Title
Rational Design, Synthesis and Evaluation of Benzothiazole Amphiphiles with Applications in Neurodegenerative Diseases

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Publication Date
2016

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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Rational Design, Synthesis and Evaluation of Benzothiazole Amphiphiles with Applications in Neurodegenerative Diseases

A Dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy

in
Chemistry

by
Jessica Lynn Cifelli

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Professor Clifford Kubiak
Professor Christina Sigurdson

2016
The Dissertation of Jessica Lynn Cifelli is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2016
Dedication

To my grandparents with love:

~Pop & Grandma~
Your love and strength guide me each and every day. Thank you for always being there.

~Grandma Nettie~
I miss you and your beautiful smile every day. You are in my heart always.
“Education is the most powerful weapon which you can use to change the world”
- Nelson Mandela
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<tr>
<td>3-AT</td>
<td>3-amino-1,2,4-triazole</td>
</tr>
<tr>
<td>Aβ</td>
<td>β-amyloid</td>
</tr>
<tr>
<td>Ach</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AchE</td>
<td>acetylcholinesterase</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>Ag₂O</td>
<td>silver oxide</td>
</tr>
<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>ARP2/3</td>
<td>actin-related protein -2/3</td>
</tr>
<tr>
<td>αS</td>
<td>α-synuclein</td>
</tr>
<tr>
<td>BAM</td>
<td>benzothiazole amphiphile</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinechoninic acid</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BTA</td>
<td>benzothiazole aniline</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DIV</td>
<td>days <em>in vitro</em></td>
</tr>
<tr>
<td>DLB</td>
<td>dementia with Lewy body</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>CJD</td>
<td>Creutzfeldt-Jakob disease</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>EG</td>
<td>ethylene glycol</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagle’s minimum essential medium</td>
</tr>
<tr>
<td>ESC</td>
<td>embryonic stem cell</td>
</tr>
<tr>
<td>EtOAc</td>
<td>ethyl acetate</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescene activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HATU</td>
<td>1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HD</td>
<td>Huntington’s disease</td>
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<tr>
<td>HFIP</td>
<td>1,1,1,3,3,3-hexafluoro-2-propanal</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
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</tbody>
</table>
IAPP  islet amyloid polypeptide
iPSC  induced pluripotent stem cell
K₂CO₃  potassium carbonate
KI  potassium iodide
log P  octanol-water partitioning coefficient
mCPBA  meta-chloroperoxybenzoic acid
MAP2  microtubule-associated protein 2
MeOH  methanol
MS  multiple sclerosis
MTT  3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium
NaOH  sodium hydroxide
Na₂SO₄  sodium sulfate
Na₂S₂O₃  sodium thiosulfate
NHS  N-hydroxysuccinimide
NDD  neurodegenerative disease
NMDA  N-methyl-D-aspartate
NMR  nuclear magnetic resonance spectroscopy
NSC  neural stem cells
PBS  phosphate buffer saline
PCC  Pearson’s correlation coefficient
PD  Parkinson’s disease
PEG  poly(ethylene glycol)
PFA  paraformaldehyde
<table>
<thead>
<tr>
<th><strong>Abbreviation</strong></th>
<th><strong>Definition</strong></th>
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<tbody>
<tr>
<td>pH</td>
<td>negative log of hydrogen ion concentration</td>
</tr>
<tr>
<td>PSD95</td>
<td>postsynaptic density protein 95</td>
</tr>
<tr>
<td>RA</td>
<td>retinoic acid</td>
</tr>
<tr>
<td>RasGRF1</td>
<td>ras protein-specific guanine nucleotide-releasing factor 1</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>rt</td>
<td>room temperature</td>
</tr>
<tr>
<td>SASA</td>
<td>solvent accessible surface area</td>
</tr>
<tr>
<td>SCA</td>
<td>spinocerebellar atrophy</td>
</tr>
<tr>
<td>SEVI</td>
<td>semen-derived enhancer of virus infection</td>
</tr>
<tr>
<td>SMA</td>
<td>spinal muscular atrophy</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>TFAA</td>
<td>trifluoracetic anhydride</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>ThT</td>
<td>thioflavin T</td>
</tr>
<tr>
<td>TsCl</td>
<td>tosyl chloride</td>
</tr>
<tr>
<td>VMAT-2</td>
<td>vesicular monoamine transporter-2</td>
</tr>
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Acknowledgements

No accomplishment can be made without the help of others. As such, I have been truly blessed to have the aid of such wonderful and truly gifted people along the way. I would first like to thank all the wonderful teachers and professors I had the privilege of being taught by on my journey. I have always had such admiration for educators but teaching in graduate school has given me a new appreciation for what a truly challenging and rewarding job it can be.

I would next like to thank my research mentors I have had during my undergraduate career. A huge thank-you to my amazing scientific mentor, Dr. Kevin Fitzpatrick, who took time out of his busy schedule to teach me synthetic chemistry in an industry setting, while driving me to be independent. Additionally, my gratitude goes out to Professor John Porco for challenging me with rigorous research and pushing me to apply for funding opportunities. The skills learned in these two labs helped to excel in both industry and during my graduate studies, for which I am very grateful.

A very special thank you goes to my boss at AMRI, Jiffry Ismail for believing in me so much as to get me hired even during a recession. I truly admire Jiffry’s skills as a boss and mentor and am forever appreciative for his understanding and genuine happiness for me leaving to pursue graduate school.

During the past five years in graduate school there have been many individuals who I am delighted to extend my gratitude. First and foremost, I would like to thank my advisor, Professor Jerry Yang. Since day one Jerry allowed me to take charge of my own research, allowing me to pursue synthetic chemistry all the way up to neuroscience, a
truly unique and gratifying experience for graduate school. He let me gain confidence in myself and become an independent and self-sufficient scientist, skills that will stay with me for life. Additionally, I would like to thank my thesis committee, Professors Nathan Gianneschi, Thomas Hermann, Clifford Kubiak and Christina Sigurdson, for all the time, helpful input and advice given to me throughout the years. I would especially like to thank Christina for being so sweet and bringing me into the world of neurodegeneration and protein misfolding journal club, from which I have learned to look at science through many different perspectives.

Research is if anything a collaborative effort, as such I would next like to thank the individuals who helped contribute to the research in this thesis. First off I thank Tim Chung for spearheading the beginning of this research by synthesizing the molecule, BAM3-EG₆. Next, I would like to thank Lara Dozier and Professor Gentry Patrick for being such wonderful collaborators on all the primary neuron work. I could not have asked for a better team to work with. Lara’s positive attitude about diving into any experiment, as well as willingness to take the time to teach me her scope and analysis methods were so admirable and for which I am truly grateful. Additionally, Gentry’s biological insight and meetings were always so helpful. I would also like to thank Professor Michael Mayer and his group members, Haiyan Liu and Panchika Prangkio for conducting all the key ion channel experiments.

Graduate school is a journey and along the way I was fortunate enough to have some wonderful colleagues in the Yang lab to work with, both past and present. A big thank you goes out to past Yang members: Dr. Alice Luong, Dr. Christina Capule, Dr. Yuchen Cao, Leibniz Hang, and Tim Chung. You all helped show me the ropes of the
new lab and definitely made my first year of graduate school enjoyable. Leibniz and Tim, thank you for being such wonderful people and making me laugh pretty much every day while you were here. To my partners in crime, Young Hun Kim and Dr. Kevin Cao, without who graduate school would have been infinitely less enjoyable. You both are truly two of the kindest people I have ever met and I am honored to call you both my friends for life. From knowing when to say “it’s fine”, to laughing hysterically, taking much needed coffee breaks, or lending a hug when needed, we’ve been through all of grad schools ups and downs and I can say I am a better person for knowing the both of you. Young, Kevin, we’ve been through this journey since day one, with you walking into lab with your mismatched laces and taped up glasses, I guess not much has changed! Thank you for all your help along the way; both in and out of lab you are truly an amazing friend. To the rest of the current Yang Lab: Dr. Geoffray Leriche, John Kim, Tak Koyanagi, Kevin Sibucao, thanks for everything and continue doing great research. John, it has been a pleasure getting to know you ever since your recruitment, you are such a sweet person.

To my wonderful boyfriend, Christian, where would I be without your calm and levelheaded voice of reason? You push me to be a better person each and every day and I am so grateful for that. I could not imagine going through graduate school without you and I am so appreciative for all the support and advice along the way. I love you and am forever thankful for your poster bringing us together.

Finally I would like to thank the people who have been with me the longest, my wonderful family: Mom, Dad, Michael, Lauren, Pop and Grandma, Aunt Marie, Aunt Sophie, Aunt Sue, Uncle John, Michelle and Justin. You guys have all been there
throughout my life journey for which I am so grateful. I love you all so much. Pop, Grandma, and Aunt Marie I am so lucky to have you in my life you each have taught me so much, from true strength, unconditional love, to never giving up. Thank you for being there for everything and anything, you are in my thoughts and heart each and every day. Mom and Dad thank you for all of the sacrifices you have each made and for allowing me to have every opportunity a child could ever hope for and more. I have been truly blessed and fortunate to have such supportive parents. Lauren, my other half, words cannot even begin to express how grateful I am to have you as a sister. Thank you so much for keeping me sane and grounded on this journey, I love you beyond anything.

Notes About the Chapters:

Chapter two, in part, is a reprint (with co-author permission) of the material as it appears in the following publications; Cifelli, J. L., Chung, T. S., Liu, H., Prangkio, P., Mayer, M., and Yang, J. (2016) “Benzothiazole Amphiphiles Ameliorate Amyloid β-Related Cell Toxicity and Oxidative Stress”, ACS Chem. Neurosci. 7(6), 682-688; and Cifelli, J. L., Dozier, L., Chung, T. S., Patrick, G. N. & Yang, J. “Benzothiazole Amphiphiles Promote the Formation of Dendritic Spines in Primary Hippocampal Neurons”, J. Biol. Chem. 291, 11981–11992 (2016). I would like to thank Tim Chung, Haiyan Liu, Panchika Prangkio, Lara Dozier, Michael Mayer, Gentry Patrick, and Jerry Yang for their invaluable contributions to this chapter: Tim Chung synthesized BAM3-EG₆, Haiyan Liu and Panchika Prangkio performed all ion channel studies, Lara Dozier and Gentry Patrick prepared and maintained all primary neuronal cultures. Additionally I
would like to thank Michael Mayer, Gentry Patrick and Jerry Yang for directing the research. The author of the dissertation is the primary author of these manuscripts. Additionally, the solubility studies, which were the author of this dissertation’s contribution were taken, in part from: Lee, N. J.; Song, J. M.; Cho, H.-J.; Sung, Y. M.; Lee, T.; Chung, A.; Hong, S.-H.; Cifelli, J. L.; Rubinshtein, M.; Habib, L. K.; Capule, C. C.; Turner, R. S.; Pak, D. T. S.; Yang, J.; Hoe, H.-S. (2016) Hexa (ethylene glycol) derivative of benzothiazole aniline promotes dendritic spine formation through the RasGRF1-Ras dependent pathway. Biochim. Biophys. Acta 1862, 284–295. Last, I thank Young Hun Kim for the preparation of the liposomes suspension used in the solvatochromism study.

Chapter three, in part, is a reprint (with co-author permission) of the material as it appears in the publication; Cifelli, J. L., Chung, T. S., Liu, H., Prangkio, P., Mayer, M., and Yang, J. (2016) “Benzothiazole Amphiphiles Ameliorate Amyloid β-Related Cell Toxicity and Oxidative Stress”, ACS Chem. Neurosci. 7(6), 682-688. I would like to thank Tim Chung and Jerry Yang for their invaluable contributions to this chapter: Tim Chung synthesized BAM3-EG₆ and Jerry Yang directed the research. The author of the dissertation is the primary author of this manuscript.

Chapter four, in part, is being prepared for publication (with co-author permission): Cifelli, J. L., Capule, C. C., & Yang, J. “Utilization of non-covalent interactions to improve amyloid-targeted binding”, Manuscript in Preparation. I would like to thank Christina Capule and Jerry Yang for their invaluable contributions to this chapter: Christina Capule synthesized and characterized all oligovalent BTA compounds and Jerry Yang directed the research and prepared the WT α-synuclein E. coli stocks.
Additionally, Dr. Eric Luth is acknowledged for the generous gift of the plasmids used for the *E. coli* expression of WT α-synuclein. The author of the dissertation is the primary author of this manuscript.

Chapter five, in part, is a reprint (with co-author permission) of the material as it appears in the publication; **Cifelli, J. L.,** Dozier, L., Chung, T. S., Patrick, G. N. & Yang, J. “Benzothiazole Amphiphiles Promote the Formation of Dendritic Spines in Primary Hippocampal Neurons”, *J. Biol. Chem.* **291**, 11981–11992 (2016). I would like to thank Tim Chung, Lara Dozier, Gentry Patrick, and Jerry Yang for their invaluable contributions to this chapter: Tim Chung synthesized BAM3-EG₆ and Lara Dozier and Gentry Patrick prepared and maintained all primary neuronal cultures. Additionally, Lara Dozier assisted with all primary neuron experiments and analysis. I would like to thank Gentry Patrick and Jerry Yang for directing the research. The author of the dissertation is the primary author of this manuscript.

Chapter six is unpublished work. I thank Jessica Young for the generous donation of NSCs and invaluable contributions to this chapter. Jessica Young reprogrammed human fibroblasts to iPSC cells, induced neural differentiation to NSCs, and FACS purified the NSCs. In addition, I would like to thank Jerry Yang for directing the research. My contribution to this chapter was the differentiation and characterization of NSCs to mature neurons. Additionally, I designed and performed all dosing studies, imaging, and western blot analysis.
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Cifelli, J. L., Capule, C. C., and Yang, J. “Utilization of non-covalent interactions to improve amyloid-targeted binding”, Manuscript in Preparation.


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Posters and Presentations


Neurodegenerative diseases (NDDs), or disorders with progressive neuronal or nervous system dysfunction, affect millions of people worldwide each year. With a steadily increasing aging population, the amount of people affected is projected to exponentially increase unless there are significant advances made in research. As such, this dissertation will explore small molecule therapeutics that can potentially alleviate common pathological features of NDDs, with a focus on Alzheimer’s disease (AD).
First, this dissertation will describe the rational design strategy towards improving the biocompatibility, of a class of amyloid-binding benzothiazoles particularly, with focus on decreasing toxicity. Here, novel benzothiazole amphiphiles or BAMs were designed, synthesized and evaluated to have a decreased toxicity over the parent compound when observed in various cell lines. In the subsequent chapters, these improved derivatives were further evaluated and modified for use in NDDs diagnostics or therapeutics. Specifically, BAMs capability to protect cells against the pathological hallmark of AD, β-amyloid (Aβ), its related toxicity and oxidative stress, was examined. Next, charged derivatives of BAM1-EG₆ were synthesized for use in improving the binding to several NDD associated amyloids, including Aβ and α-synuclein (αS). This non-covalent strategy has potential future applications to differentiate between amyloids for diagnostics, or in therapeutics if metal chelation was utilized instead of charge-charge interactions.

The last two chapters of this dissertation, analyzes the ability of BAMs to increase dendritic spine density. Since decreased synaptic density and function is associated with many NDDs, particularly as one of the initial pathologies, small molecules that could alleviate or even reverse these effects would be quite valuable. Here, the BAMs spinogenic properties were evaluated in both primary hippocampal neurons as well as human induced pluripotent stem cell (hiPSC)-derived neurons. Importantly, the ability of BAMs to counteract Aβ-induced dendritic spine loss was also examined in primary culture. Lastly, the possibility of these effects translating into humans was evaluated utilizing hiPSC-derived neurons. Collectively, this dissertation presents on small molecules with a dual-modality (i.e amyloid binding capabilities and spinogenic activity), which may find use as part of a therapeutic regiment for AD and other NDDs.
~Chapter 1~

Introduction: Neurodegenerative Diseases

1.1 Introduction to Neurodegeneration & Neurodegenerative Diseases

When defining “neurodegeneration” it may seem trivial given the ubiquity of the word today. Indeed since Patrick F. Bray used the term “neurodegenerative disease” in the title of his article in the journal of Pediatrics back in 1965, according to a PubMed search of the literature, the term “neurodegeneration” has appeared 27,908 times as of 2016. Many journals define neurodegeneration as the overarching terminology that refers to the progressive damage inflicted on nerve cells otherwise known as neurons. This devastation subsequently leads to both structural and functional damages that culminate in the eventual death of neurons. As the progressive impairments accumulate, a loss of cognitive function, including: memory loss, behavioral changes and/or motor skills also appear depending on the region of the brain affected. This fundamental definition comes from the etymology of the word where the prefix “neuro”, is defined as nerve cells or neurons, and “degeneration”, is defined as the process of progressive loss of function.
This description on its own seems relatively straightforward; however, complexity arises when exploring which disease should be categorized under the terminology of neurodegeneration.

Classically neurodegenerative diseases (NDDs) were denoted as being pathological conditions where the nervous system or neurons undergo progressive dysfunction. The medical definition redefines NDDs to encompass an etiological diverse, but related group of disorders that affect different specific subset of neurons in particular anatomofunctional regions of the nervous system. Additionally, NDDs are often progressive and largely arise for unknown reasons. Thus, historically any disease predominately impacting the nervous system, which is comprised of pathologies restricted to the nervous system were categorized as neurodegenerative. Utilizing this simplified definition, many disorders are unambiguously defined as NDDs, for example Alzheimer’s disease (AD), Parkinson’s disease (PD), Huntington’s disease (HD) and amyotrophic lateral sclerosis (ALS). Other disorders of the nervous system do not as straightforwardly fall into this definition of NDDs including multiple sclerosis (MS) and schizophrenia, where the former is primarily thought of as a demyelinating disease and the latter does not show the distinctive neuronal loss, but is a chronic disease of the nervous system with unknown causality.

1.2 Types of Neurodegenerative Diseases

There are hundreds of debilitating conditions that are placed underneath the umbrella of neurodegenerative disorders, which no doubt expanded due to the ambiguity
of categorizing what defines a neurodegenerative disease (Table 1.1). The most prevalent and studied of NDDs include: AD, PD, ALS and HD. While only a handful of diseases

<table>
<thead>
<tr>
<th>Group</th>
<th>Condition</th>
<th>Main Areas Affected</th>
<th>Pathological Markers</th>
<th>Clinical Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dementia</td>
<td>Alzheimer’s disease (AD)</td>
<td>Cerebral cortex, hippocampus</td>
<td>Amyloid plaques, neurofibrillary tangles</td>
<td>Memory loss, cognitive impairments, personality changes</td>
</tr>
<tr>
<td></td>
<td>Frontotemporal lobe dementia</td>
<td>Temporal and frontal lobes</td>
<td>Temporal and frontal atrophy</td>
<td>Behavioral changes, motor and cognitive impairment</td>
</tr>
<tr>
<td></td>
<td>Dementia with Lewy body (DLB)</td>
<td>Cerebral cortex, substantia nigra</td>
<td>Lewy bodies</td>
<td>Hallucinations, cognitive and motor impairments</td>
</tr>
<tr>
<td></td>
<td>Creutzfeldt-Jakob disease (CJD)</td>
<td>Cortex and grey matter</td>
<td>Spongiform encephalopathy</td>
<td>Personality changes, memory loss, motor impairments</td>
</tr>
<tr>
<td>Hyperkinetic disorders</td>
<td>Huntington’s disease (HD)</td>
<td>Caudate nucleus and putamen</td>
<td>Loss striatal neurons, marked caudate atrophy</td>
<td>Chorea, dementia, behavioral abnormalities</td>
</tr>
<tr>
<td></td>
<td>Dystonia</td>
<td>Brain stem and basal ganglia</td>
<td>Perinuclear inclusion bodies</td>
<td>Dystonia (focal, segmental, or generalized)</td>
</tr>
<tr>
<td>Hypokinetic disorders</td>
<td>Parkinson’s disease (PD)</td>
<td>Substantia nigra, dorsal motor nucleus of the vagus</td>
<td>Lewy bodies</td>
<td>Tremors, rigidity, chorea</td>
</tr>
<tr>
<td></td>
<td>Progressive supranuclear palsy (PSP)</td>
<td>Substantia nigra, basal ganglia, brainstem, subthalamus</td>
<td>Neurofibrillary tangles</td>
<td>Visual disturbances, bradykinesia, rigidity, lack balance, dementia</td>
</tr>
<tr>
<td></td>
<td>Multiple system atrophy (MSA)</td>
<td>Frontostriatal</td>
<td>Alpha-synuclein inclusions (glial)</td>
<td>Loss of autonomic nervous system and motor impairment</td>
</tr>
<tr>
<td>Ataxia</td>
<td>Spinocerebellar atrophies (SCA)</td>
<td>Cerebellum, various others</td>
<td>Intranuclear inclusions</td>
<td>Ataxia, muscle atrophy</td>
</tr>
<tr>
<td>Motor neuron diseases</td>
<td>Amyotrophic lateral sclerosis (ALS)</td>
<td>Upper and lower motor neurons</td>
<td>Atrophy of motor neurons</td>
<td>Progressive motor impairment and atrophy</td>
</tr>
<tr>
<td></td>
<td>Spinal muscular atrophy (SMA)</td>
<td>Mainly lower motor neurons</td>
<td>Atrophy of lower motor neurons</td>
<td>Hypotonia, weakness, and cranial nerve palsies</td>
</tr>
<tr>
<td>Other</td>
<td>Multiple sclerosis (MS)</td>
<td>White matter</td>
<td>Focal plaques of demyelination</td>
<td>Motor abnormalities, muscle weakness, coordination problems</td>
</tr>
</tbody>
</table>
receive notable attention, both in research and in the public domain, this does not down
play the importance of researching and pushing the study of all neurodegenerative
diseases forward.

Currently, there are several different methods used to classify a particular type of
neurodegenerative disease (NDD). The traditional clinicopathological practice for
categorizing NDDs is based upon their principal clinical feature and/or topography of the
lesion (Table 1.1). For example, Alzheimer’s disease (AD) can be classified as a cerebral
cortex disease with concomitant dementia, while Parkinson’s disease (PD) is a
hypokinetic basal ganglia disorder and Huntington’s disease a hyperkinetic basal ganglia
disorder (HD). ALS is a motor neuron disease characterized by upper and low motor
neuron degeneration with muscle atrophy.

As one can imagine this classification scheme lends itself to several problems
including the perceived commonality between diseases both clinically and pathologically,
as well as the characteristic of many individual NDDs affecting several different regions
of the brain. This highlights the importance for improved and perhaps a more molecular
based categorization of neurodegeneration diseases. Indeed advances in neurology,
biochemistry, genetics, and histology, specifically over the last couple decades, have
drastically aided in the nosological classification of NDDs. Importantly, this led to
studies revealing a common pathogenic mechanism of misfolded protein aggregation,
connecting seemingly diverse NDDs. Classification of many NDDs, or
proteinopathies, can now include identification of proteins with conformational changes
as well as their accumulation location (i.e. extra- or intra-cellular deposits) (Table 1.2).
### Table 1.2. Protein aggregation and deposition in neurodegenerative diseases\(^{13}\)

<table>
<thead>
<tr>
<th>Disease</th>
<th>Etiology</th>
<th>Pathological Deposition &amp; Locations</th>
<th>Disease Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alzheimer’s disease (AD)</td>
<td>Sporadic, familial (ApoE risk factor)</td>
<td>Extracellular plaques, intracellular, neurofibrillary tangles, cerebral amyloid angiopathy (CAA)</td>
<td>Aβ peptide and hyperphosphorylated tau(^5)(^{14})</td>
</tr>
<tr>
<td>Creutzfeldt-Jakob disease (CJD)</td>
<td>Sporadic, familial (PRNP mutations), transmissible</td>
<td>Synaptic, perivacuolar, perineuronal, or extracellular plaque deposits</td>
<td>Prion protein (PrP)(^5)</td>
</tr>
<tr>
<td>Huntington’s disease (HD)</td>
<td>Huntington (dominant)</td>
<td>Intracellular/nuclear inclusions</td>
<td>Huntington with polyglutamine expansion(^1)(^5)</td>
</tr>
<tr>
<td>Parkinson’s disease (PD)</td>
<td>Sporadic, familial (LRRK2, PINK1, parkin, α-synuclein)</td>
<td>Intracellular Lewy bodies, Lewy neurites</td>
<td>α-synuclein(^5)(^,)(^16)</td>
</tr>
<tr>
<td>Progressive supranuclear palsy (PSP)</td>
<td>Sporadic, rare familial (possible MAPT polymorphisms)</td>
<td>Globose neurofibrillary tangles</td>
<td>Tau protein(^5)</td>
</tr>
<tr>
<td>Multiple system atrophy (MSA)</td>
<td>Sporadic, familial</td>
<td>Glial cytoplasmic inclusions</td>
<td>α-synuclein(^5)(^,)(^17)</td>
</tr>
<tr>
<td>Spinocerebellar atrophies (SCA)</td>
<td>Familial (dominant)</td>
<td>Intranuclear inclusions</td>
<td>Polyglutamine expansion(^1)(^8)</td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis (ALS)</td>
<td>Sporadic, familial (Superoxide dismutase-1 (SOD1))</td>
<td>Intracellular inclusions</td>
<td>SOD(^1)(^9)</td>
</tr>
</tbody>
</table>

### 1.3 Protein Misfolding in Neurodegenerative Diseases

One of the hallmark features of many neurodegenerative diseases is the accumulation of intra- and extra-cellular protein aggregates in the affected degenerating region of the brain. The accumulation of misfolded proteins is caused by a combination of genetic and environmental factors in conjunction with defective protein degradation due to deficiency of the ubiquitin-proteosomal-autophagy system.\(^20\) Neuronal loss manifests by the interrelation of various pathways, which are effected by differing aggregation states (i.e. oligomers, protofibrils or fibrils), including free radical formation.
and oxidative stress\textsuperscript{21}, mitochondrial dysfunction\textsuperscript{22}, molecular chaperones\textsuperscript{23} and neuroinflammatory processes\textsuperscript{24}. Thus a central feature of NDDs is a long period of protein accumulation, followed by a cascade of symptoms with increasing severity culminating in eventual death (Figure 1.1)\textsuperscript{25}.

\textbf{Figure 1.1.} Dynamic protein aggregation leading to dysfunction and neurodegeneration. Adapted and reprinted by permission from Macmillan Publishing Ltd: \textit{Nature Reviews Drug Discovery}\textsuperscript{25}, 2015.
Examination of the underlying mechanism of protein aggregation in NDDs includes amorphous aggregation, but extracted proteinaceous deposits are typically characterized by amyloids.\textsuperscript{26} The term “amyloid” was first introduced in the medical literature in 1854 by Rudolph Virchow when describing neurological deposits that reacted with iodine and sulfuric acid, similarly to starch.\textsuperscript{27} Subsequently, light microscopy studies led to the amyloid characteristic “apple-green birefringence” observed upon binding of the diazobenzidine sulfonate dye, Congo-red\textsuperscript{28} and fluorescence microscopy studies showed increased fluorescence of the benzothiazole dye thioflavin T\textsuperscript{29} (ThT) upon binding to amyloids (Figure 1.2a) Additional progress in both science and imaging techniques led to further characterization of amyloids and their structure including an overall richness in β-pleated sheet structure (Figure 1.2b).\textsuperscript{30}

![Figure 1.2](image-url)

**Figure 1.2.** (A) Structures of the amyloid binding dyes Congo red and thioflavin T. (B) Amyloid structures exhibit a range of specific features and possess common characteristics. Representation of the 'cross-β' structure common to amyloid fibrils. (B) is adapted and reprinted by permission from Macmillan Publishing Ltd: *Nature Reviews Molecular Cell Biology*\textsuperscript{30}, 2014.
Intriguingly, it is this common β-sheet linkage between peptide strands that is energetically favorable due to stabilization by intermolecular hydrogen bonding as well as complementary sterics. The existence of an amyloid state in protein misfolding diseases may thus be the thermodynamically stable state for proteins at high concentrations. This also correlates with why a variety of sequence and functionally diverse peptides found in differing NDDs are all capable of adopting this analogous amyloid structure.

1.4 Treatment Options

Perhaps most disheartening to explore is the fact that currently there are no viable treatment options that halt or even significantly slow the progression of any neurodegenerative disease. Though the advances made to the field over the last couple decades, particularly to the fundamental understanding of the biochemistry involved in many NDDs brings new therapeutic avenues and with that, hope. Additionally, due to the plethora of different types of neurodegenerative diseases, the causes can range from familial, sporadic to even transmissible and thus treatment options in the future will likely need to be tailored per patient.

Looking at some of the drugs that are currently available for NDDs a commonality can be found, they serve mostly only to alleviate some of the behavioral and/or cognitive symptoms (Table 1.3). For example the FDA approved medications for AD, include acetylcholinesterase (AchE) inhibitors like donepezil or rivastigmine or memantine an antagonist of NMDA receptors. Acetylcholinesterase inhibitors serve to
Table 1.3. FDA approved drugs for several neurodegenerative diseases. 

<table>
<thead>
<tr>
<th>Disease</th>
<th>Drug Name</th>
<th>Mechanism of Action</th>
<th>Symptoms Relieved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alzheimer's disease (AD)</td>
<td>Donepezil</td>
<td>AchE inhibitor</td>
<td>Treatment of dementia</td>
</tr>
<tr>
<td></td>
<td>Rivastigmine</td>
<td>AchE inhibitor</td>
<td>Treatment of mild to moderate dementia</td>
</tr>
<tr>
<td></td>
<td>Galantamine</td>
<td>AchE inhibitor</td>
<td>Treatment of dementia</td>
</tr>
<tr>
<td></td>
<td>Memantine</td>
<td>NMDA antagonist</td>
<td>Treatment of moderate to severe dementia</td>
</tr>
<tr>
<td>Parkinson's disease (PD)</td>
<td>Levodopa</td>
<td>Dopamine precursor</td>
<td>Treatment of motor symptoms</td>
</tr>
<tr>
<td></td>
<td>Pramipexole</td>
<td>Dopamine receptor agonist</td>
<td>Treatment of motor symptoms</td>
</tr>
<tr>
<td></td>
<td>Ropinirole</td>
<td>Dopamine receptor agonist</td>
<td>Treatment of motor symptoms</td>
</tr>
<tr>
<td></td>
<td>Trihexyphenidyl</td>
<td>Anticholinergic</td>
<td>Tremors and dystonia</td>
</tr>
<tr>
<td></td>
<td>Pimavanserin</td>
<td>Serotonin 5-HT2A inverse agonist</td>
<td>Hallucinations and delusions</td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis (ALS)</td>
<td>Riluzole</td>
<td>Unknown, blocks glutamatergic neurotransmission</td>
<td>Delays time to tracheostomy/death</td>
</tr>
<tr>
<td>Huntington's disease (HD)</td>
<td>Tetrabenazine</td>
<td>Vesicular monoamine transporter-2 inhibitor</td>
<td>Treatment of chorea</td>
</tr>
</tbody>
</table>

inhibit breakdown of the neurotransmitter, acetylcholine (ACh), therefore relieving symptoms by counteracting the loss of cholinergic neurons and subsequent decreased levels of ACh prevalent in AD. NMDA antagonists allow for a different approach than targeting the cholinergic system by assisting in alleviation of the neuronal excitotoxicity associated in the glutamatergic system in AD.

Pharmaceutical treatments available to help mitigate symptoms associated with PD, for the most part, has been dominated by compounds that either increase dopamine
levels or mimic its action. Introduction of levodopa (L-dopa), a dopamine precursor capable of passing the blood-brain barrier (BBB), for use in PD began in 1961 by two groups, Barbeau et al. in Montreal and Birkmayer and Hornykiewicz in Vienna.\textsuperscript{37,38} They observed remarkable, albeit short-lived improvements in several symptoms including rigidity, tremors and akinesia. The short-term success of this therapeutic intervention is due to the counteraction of the diminished dopamine levels that result from the loss of dopamingenic neurons in the substantia nigra. Additionally stemming from this same intervention pathway, dopamine agonists like pramipexole\textsuperscript{39} (Mirapex®) and ropinirole\textsuperscript{40} (Requip®) are also prescribed, though not as potent as L-dopa.

For other less prevalent NDDs there are even less medications currently available for symptomatic relief. For HD there is only one FDA approved drug, tetrabenazine (Xenazine®), which serves as relief for chorea. Tetrabenazine acts by inhibiting monoamine uptake by binding to vesicular monoamine transporter-2 (VMAT-2).\textsuperscript{41} This leads to the depletion of monoamines, like dopamine, which is thought to decrease involuntary movements associated with HD. Likewise there is only one FDA approved drug, riluzole (Rilutek®) for ALS. Riluzole is thought to reduce the levels of glutamate, which is often found to be elevated in people with ALS.\textsuperscript{42}

The overall complexity of NDDs in addition to the lack of clarity in diagnosis certainly lends itself to severe difficulties in regards to treatment options. Again, as highlighted above, the current approved drugs do not address the ongoing destruction and the many interrelated pathways of neurodegeneration consequently, they fail to provide any more than minor symptomatic relief, which clearly is insufficient. In conjunction
with actual disease modifying and neuroprotective strategies a multifaceted approach to NDD therapeutics may also lend to the most optimistic therapeutics in the future.

1.5 Impact of Neurodegenerative Diseases

Neurodegenerative diseases affect millions of people throughout the world and is strongly associated with the aging process, thus with a growing aging population these numbers are only expected to grow exponentially in the next coming decades. The most prevalent NDDs are Alzheimer’s and Parkinson’s disease with over 5 million Americans diagnosed with AD and about 1 million Americans living with PD, though these numbers may in fact be larger due to the exclusion of patients that are either not diagnosed or misdiagnosed. By 2050 these numbers for AD are projected to triple or to put it into perspective, 1 person will be diagnosed with AD every 33 seconds, and the cost burden will be over $1.1 trillion annually in the US alone if no treatment or medical breakthrough is found. Additionally, AD is now among the sixth leading cause of death in the US. In regards to PD, the costs including treatment and lost income is estimated at almost $25 billion per year in the US. These numbers can be overwhelming at best but they also do not touch nor can they quantify the devastating emotional toll placed on all of the millions of families and loved ones of those affected.

Though worldwide statistics are harder to quantify, a comprehensive review of the global prevalence of dementia, estimated that 35.6 million people worldwide lived with dementia in 2010 with estimations quite staggering at 115.4 million people by 2050. With AD being the most common form of dementia (60-80%) these numbers loosely correlate with the number of people afflicted with AD worldwide. In regards to PD, the
second most prevalent NDD, the projected numbers of cases in the 10 most populous nations was around 4.1-4.6 million people in 2005, and this number is expected to double to 8.7-9.3 million people by 2030. Estimation of worldwide cases of people living with PD are as high as 10 million currently.

These statistics truly highlight the rather urgent importance of all scientific disciplines to come together through collaborations and advance neurodegeneration research to push forward the development of therapeutics for not just AD and PD but all NDDs. As such, this thesis will focus on the design, synthesis and evaluation of therapeutics for NDDs, with particular focus on AD.
Rational Design of Benzothiazole Amphiphiles with Improved Biocompatibility

2.1 Introduction

Previous work done in the Yang Lab embarked on research towards the development of potential therapeutics for neurodegenerative diseases. Due to the commonality of amyloidogenic pathology in NDDs, they sought to characterize compounds that could bind to amyloids. For example, in AD it has been shown that one of the potential pathogenic mechanism for neurotoxicity is the interaction of β-amyloid (Aβ) with cellular proteins, like catalse. Thus, Inbar and Yang developed a new surface-based therapeutic strategy for amyloidogenic NDDs where small molecules could be used to inhibit protein-amyloid interactions (Figure 2.1). This surface chemistry approach mimicked the biotechnology strategy where molecular coatings are commonly utilized to attenuate protein interactions on polymeric or metallic materials. Initially, thioflavin T (ThT) used as a proof-of-concept but its charged character makes it a poor candidate as
Figure 2.1. Illustration of the inhibition of the binding of amyloid binding proteins to aggregated amyloid fibrils by exploiting a small molecular coating.

...
Barrier (BBB), 3) protect cells from toxicity and oxidative stress induced by aggregated Aβ peptides, 4) decrease Aβ levels *in vivo,* and 5) improve memory and learning in a wild type and a mouse model for AD.

![Chemical structures of two oligo(ethylene glycol) derivatives of benzothiazole aniline (BTA), BTA-EG₄ and BTA-EG₆.](image)

**Figure 2.2.** Chemical structures of two oligo(ethylene glycol) derivatives of benzothiazole aniline (BTA), BTA-EG₄ and BTA-EG₆.

While the *in vivo* properties of BTA-EG₄ and BTA-EG₆ suggest they could provide broad therapeutic benefits for improving cognitive function in AD as well as in other neurodegenerative diseases, we also observed cytotoxicity of the compound in SH-SY5Y neuroblastoma cells that correlated with their ability to partition in membranes and induce membrane lysis. Toxicity is one of the biggest issues at every stage of drug development and the toxicity of BTA-EG₄,₆ precluded our ability to adequately evaluate the extent of its biological activity.

In order to improve the biocompatibility of this class of benzothiazoles, this chapter describes the synthesis and evaluation of the toxicity and related membrane pore-forming capability of three benzothiazole amphiphiles (BAMs) compared to the parent
compound, BTA-EG$_6$. BAMS were designed with structural alterations made to the hydrophobic core of BTA-EG$_6$. These new BAM agents showed a reduced propensity to induce ion channel-like transmembrane current fluctuations in planar lipid bilayers, a reduction in membrane lysis activity, and overall lower toxicity to SH-SY5Y neuroblastoma cells, differentiated SH-SY5Y neuroblastoma cells, and in primary neuronal culture.

**2.2. Characterization of the Solubility Properties of BTA-EG$_4$ and BTA-EG$_6$**

When deciding which oligo(ethylene glycol) derivative of benzothiazole aniline (BTA) was to be used as the parent compound for our novel structural variants, solubility was the major determining factor. Solubility is one of the fundamental parameters in drug design, playing an important role in the extent of absorption particularly in the oral administration of a drug.$^{61}$ For example, the low absorption of drugs with poor water solubility results in both inadequate as well as variable bioavailability. Unfortunately, BTA-EG$_4$ has low aqueous solubility, which can be seen as it noticeably precipitates from aqueous solutions. This low solubility suggested that, as a therapeutic BTA-EG$_4$ may not reach its target at a consistent and intended dose. In order to analyze and better quantify the difference in solubility between BTA-EG$_4$ and BTA-EG$_6$, a saturation shake-flask solubility method$^{62}$ was utilized. We found that BTA-EG$_4$ was visibly less soluble in PBS solution than BTA-EG$_6$ (Figure 2.3), and quantification revealed the solubility of BTA-EG$_6$ was increased by two orders of magnitude over BTA-EG$_4$ (Figure 2.3). Due to
this favored increase in aqueous solubility, BTA-EG$_6$ was chosen as the parent compound for our rational design of second generation Benzothiazole Amphiphiles (BAMs).

<table>
<thead>
<tr>
<th>Compound</th>
<th>MW (g/mol)</th>
<th>µg/ml</th>
<th>Solubility, S (µM)</th>
<th>logS</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTA-EG$_4$</td>
<td>416.54</td>
<td>7.0 ± 0.5</td>
<td>17 ± 1</td>
<td>1.22 ± 0.07</td>
</tr>
<tr>
<td>BTA-EG$_6$</td>
<td>504.64</td>
<td>1,247 ± 161</td>
<td>2470 ± 319</td>
<td>3.39 ± 0.11</td>
</tr>
</tbody>
</table>

Figure 2.3. Shake-flask solubility of BTA-EG$_4$ and BTA-EG$_6$ in aqueous PBS.

2.3 Rational Design of Benzothiazole Amphiphiles (BAMs) 1-3

In order to further develop benzothiazole amphiphiles as potential therapeutics for neurodegenerative diseases, we considered whether small variations in the structure of the BTA-EG$_x$ compounds could increase their therapeutic window.$^{63}$ Towards this end, we previously reported that BTA-EG$_4$ could form cation-selective pores in planar lipid bilayers.$^{59}$ The concentration required to observe ion channel-forming activity in membranes roughly correlated with the observed cytotoxicity of the compounds in human SH-SY5Y neuroblastoma cells ($IC_{50}$ ~60 µM), suggesting ion pore-mediated lysis of cells as a potentially significant factor for the observed toxicity$^{64}$ of the BTA-EG$_x$ compounds at micromolar concentrations.$^{59}$ Hence, we hypothesized that altering the hydrophobic core of these molecules would decrease their energetic driving force to partition into membranes, thereby reducing their ion channel forming capabilities and concomitant toxicity.
To test this hypothesis, we used BTA-EG₆ as the lead compound for the rational drug design of three Benzothiazole Amphiphile (BAM) derivatives (Figure 2.4). BTA-EG₆ was chosen over the tetra(ethylene glycol) BTA derivative due to its far improved aqueous solubility. For our rational design, we presumed that the benzothiazole core is required to impart efficient binding to aggregated Aβ peptides. We, therefore, examined the effects on membrane activity of the following three changes to the periphery of the benzothiazole core of BTA-EG₆: 1) removal of the 6-methyl group (as in BAM1-EG₆), 2) addition of a methyl group to the aniline nitrogen (as in BAM2-EG₆), and 3) replacement of the aniline nitrogen with a sulfur group (as in BAM3-EG₆).

**Figure 2.4.** Benzothiazole amphiphiles (BAMs) (1-3) were rationally designed to exhibit decreased hydrophobicity and hydrogen-bonding capabilities compared to the parent compound, BTA-EG₆.
Calculations (Table 2.1) suggested that these small modifications to BTA-EG₆ could affect its hydrophobic character as determined by their octanol-water partitioning coefficient (log P) and their solvent accessible surface area (SASA) without the need to modify the core benzothiazole structure, which is presumably required to impart activity.

Table 2.1. Calculated hydrophobic parameters of (BAMs) (1-3) compared to BTA-EG₆.

<table>
<thead>
<tr>
<th>Compound</th>
<th>log Pᵃ</th>
<th>SASA (Å²)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTA-EG₆</td>
<td>3.14</td>
<td>998</td>
</tr>
<tr>
<td>BAM1-EG₆</td>
<td>2.64</td>
<td>967.6</td>
</tr>
<tr>
<td>BAM2-EG₆</td>
<td>3.24</td>
<td>983</td>
</tr>
<tr>
<td>BAM3-EG₆</td>
<td>3.26</td>
<td>976.6</td>
</tr>
</tbody>
</table>

ᵃlog P values were calculated from molinspirations cheminformatics software.ᵇSASA values were calculated with PyMOL.

2.4 Synthesis of Benzothiazole Amphiphiles (BAMs) 1-3

The general synthetic procedures used to prepare benzothiazole amphiphiles (BAMs 1-3) are outlined in Scheme 2.1. For the synthesis of the benzothiazole core for BAM2, commercially available 4-hydroxy benzaldehyde (4) was alkylated with 2-chloro-N-methylacetamide (5) via an in situ Finklestein reaction.⁶⁶ The aryl ether (6) underwent a rearrangement under basic conditions to yield 4-N-(methylamino) benzaldehyde (7).⁶⁷ Microwave irradiation in ionic liquid ([pmim]Br)⁶⁸ of 2-aminothiophenol (8) with
benzaldehyde (7) afforded benzothiazole 9. An analogous microwave-assisted reaction \(^{69}\) between 8 and 12 gave 2-(4-(methylthio)phenyl)benzo[d]thiazole (13) in good yield. The methylthiol group on 13 was then oxidized to the sulfoxide via mCPBA oxidation to yield 2-(4-(methylsulfinyl)phenyl)benzo[d]thiazole (14). Pummerer rearrangement \(^{70,71}\) of compound 14 gave the \(\alpha\)-acyloxy-thioether (15), which was converted to the thiol (16). Compounds 9, 10 (commercially available), and 16 were then reacted with \(\text{EG}_6\)-Iodide (11) \(^{59}\) under standard nucleophilic substitution conditions to yield BAM1-\(\text{EG}_6\) (1), BAM2-\(\text{EG}_6\) (2), and BAM3-\(\text{EG}_6\) (3), respectively.

Scheme 2.1. Synthetic scheme of BAM1-\(\text{EG}_6\) (1), BAM2-\(\text{EG}_6\) (2) and BAM3-\(\text{EG}_6\) (3).
2.5 Hydrophobic Evaluation of Benzothiazole Amphiphiles (BAMs) 1-3

After successfully synthesizing three new BTA analogs, we first looked to examine the hydrophobic character of the new BAMs 1-3 relative to the parent compound, BTA-EG₆. In order to analyze the structural modifications and test if it was enough to alter partitioning into membranes, we took advantage of the solvatochromic nature of these compounds. In this assay, the fluorescence emission spectra were measured for each compound in octanol, water and an aqueous suspension of liposomes to mimic cell membranes. All compounds exhibited a shift of maximum fluorescence emission to shorter wavelengths in a more hydrophobic environment (i.e., in octanol compared to water) (Figure 2.5). The emission maximum in an aqueous suspension of

![Fluorescence emission spectra](image)

<table>
<thead>
<tr>
<th>Compound</th>
<th>λ_{water} (nm)</th>
<th>λ_{octanol} (nm)</th>
<th>λ_{liposomes} (nm)</th>
<th>Relative Δλ_{max} (liposomes-water)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTA-EG₆</td>
<td>429.5</td>
<td>416.5</td>
<td>423</td>
<td>50%</td>
</tr>
<tr>
<td>1</td>
<td>433</td>
<td>417.5</td>
<td>428.5</td>
<td>29%</td>
</tr>
<tr>
<td>2</td>
<td>431.5</td>
<td>415.5</td>
<td>426.5</td>
<td>31%</td>
</tr>
<tr>
<td>3</td>
<td>410</td>
<td>394</td>
<td>404.5</td>
<td>34%</td>
</tr>
</tbody>
</table>

**Figure 2.5.** Hydrophobic evaluation of BAMs 1-3 by solvatochromism. (A) Fluorescence emission properties of BAMs 1-3 and BTA-EG₆ in water, octanol, or an aqueous solution of liposomes. (B) Table of measured membrane partitioning properties.
liposomes was measured for all compounds and it was found that compounds 1-3 exhibited $\lambda_{\text{max}}$ that reflected a more polar, water-like environment compared to BTA-EG$_6$, with changes of emission max of 29-34% from water (relative to $\lambda_{\text{max}}$ in pure octanol) compared to a 50% change in $\lambda_{\text{max}}$ for BTA-EG$_6$ (Figure 2.5). These results demonstrate that the novel structural modifications in BAMs 1-3 decreased their membrane partitioning compared to BTA-EG$_6$.

2.6 BAMs 1-3 Show Decreased Membrane Lysis Over BTA-EG$_6$

Since we previously found a correlation with toxicity and the ion-channel forming properties of BTA-EG$_4$,$^{59}$ we next examined the ion channel-forming properties of compounds 1-3 compared to BTA-EG$_6$ in bilayer lipid membranes (BLMs). Similar to previous studies, we observed ion channel-like events in these cell free bilayer experiments at mid to high micromolar concentrations of the compounds (Figure 2.6). Importantly, while BTA-EG$_6$ was capable of forming ion-channel-like pores at concentrations as low as 10 µM, BAMs 1-3 required 2-10 fold higher concentrations to induce transmembrane ion fluctuations (Figure 2.6).

We also examined compounds 1-3 for their capability to induce membrane lysis (i.e., rupture of membranes) in the planar lipid bilayers. In these studies, all BAMs were found to require a higher concentration to lyse the membrane compared to BTA-EG$_6$, with BAM 1 requiring a 3-fold higher concentration to lyse the membrane and no observed membrane lysis induced by BAM 3 at any of the concentrations tested (i.e., up to 150 µM) (Figure 2.6).
2.7 BAMs 1-3 Exhibit a Decreased Toxicity Compared to BTA-EG₆

In order to evaluate if the structural modifications to BTA-EG₆ and subsequent decreased membrane lysis correlated with a decrease in toxicity, an MTT cell proliferation assay was performed to compare the toxicity of BAM 1-3 to the parent compound. We examined all toxicity comparisons in a variety of different cell lines.

First, we looked at the comparing the toxicity of BAM 1-3 to BTA-EG₆ in SH-SY5Y neuroblastoma cells. This cancer cell line was chosen as our first test model due to the previous noted toxicity of the parent compound being determined in this cell line. Additionally, the neuroblastoma cells are a good cell line for testing compounds intended for evaluation in neurological systems. Here the parent compound, BTA-EG₆ exhibited relative toxicity in SH-SY5Y cells with an IC₅₀ of 90 µM (Figure 2.7). Agreeably, our
Next generation of BAMs 1-3, which required higher concentrations to lyse PLBs, were all significantly less toxic than BTA-EG₆ with IC₅₀’s ranging from 140-171 µM (Figure 2.7).

Figure 2.7. Toxicity curves of A) BTA-EG₆, B) BAM1-EG₆, C) BAM2-EG₆, and D) BAM3-EG₆ in SH-SY5Y neuroblastoma cells.

Next, we looked at the compounds 1-3 toxicity in differentiated SH-SY5Y neuroblastoma cells. SH-SY5Y neuroblastoma cells can be stimulated with retinoic acid (RA) to differentiate into a more characteristic neuronal morphology which lends this differentiated cell line into a more relevant model for mature neurons. Upon
differentiation, cells enter exit the cell cycle entering G₀ and exhibit decreased proliferation. Furthermore, differentiated SH-SY5Y cells show increased expression of mature neuronal markers. In this cell line, BTA-EG₆ exhibited moderate toxicity to differentiated SH-SY5Y neuroblastoma cells with an IC₅₀ of 69 µM (Figure 2.8). Figure 2.8 shows that all BAMs 1-3 were once again significantly less toxic than BTA-EG₆ with IC₅₀’s ranging from 106-140 µM. Importantly, the reduced toxicity of these compounds correlated with the increased concentrations required to lyse membranes.

**Figure 2.8.** Toxicity curves of A) BTA-EG₆, B) BAM1-EG₆, C) BAM2-EG₆, and D) BAM3-EG₆ in differentiated SH-SY5Y neuroblastoma cells.
While neuroblastoma cell lines are extremely useful as model systems for the study of neurobiology, the use of primary culture is ideal. Primary cultures are not tumor-derived, and hence, are more likely to exhibit the same neuronal properties found *in vivo*. We, therefore, next tested the toxicity of compounds 1-3 compared to the parent compound, BTA-EG₆, in rat primary hippocampal neurons. In this assay, BTA-EG₆ exhibited moderate toxicity to rat primary neurons with an IC₅₀ of 9.8 µM after 24 h exposure (Figure 2.9). Satisfyingly, we found that all BAMs 1-3 were significantly less toxic than BTA-EG₆, with IC₅₀’s ranging from 44-67 µM (Figure 2.9).

**Figure 2.9.** Toxicity of A) BTA-EG₆, B) BAM1-EG₆, C) BAM2-EG₆, and D) BAM3-EG₆ in rat primary hippocampal neurons.
2.8 Concluding Remarks

We previously reported that oligo(ethylene glycol) derivatives of benzothiazole could insert into planar lipid bilayers and induce membrane lysis.\textsuperscript{59} The concentration required to observe lysis in membranes roughly correlated with the observed cytotoxicity of the compounds in human SH-SY5Y neuroblastoma cells (IC\textsubscript{50} \~60 µM), suggesting lysis of cells as the significant factor for the apparent toxicity\textsuperscript{64} of the BTA-EG\textsubscript{x} compounds at high micromolar concentrations.\textsuperscript{59} Hence, we hypothesized that altering the hydrophobic core of these molecules would decrease their energetic driving force to partition into membranes, thereby reducing their toxicity.

With a goal of generating structural analogs of BTA-EG\textsubscript{6} with reduced hydrophobic character, we designed and synthesized benzothiazole analogs 1-3 (Figures 2.4). This new set of benzothiazoles exhibited lower overall toxicity in SH-SY5Y neuroblastoma cells (Figure 2.7), differentiated SH-SY5Y neuroblastoma cells (Figure 2.8) and primary rat hippocampal neurons (Figure 2.9) when compared to the parent BTA-EG\textsubscript{6}. Interestingly, we did not find a correlation between the calculated log P values (a typical measure of hydrophobicity) and toxicity. Instead, we found a better correlation between solvent-accessible surface area (SASA) and toxicity in BTA-EG\textsubscript{6} and all of the BAM derivatives. The SASA could, therefore, represent a more useful and quantifiable alternative parameter to log P for guiding the development of additional members of this class of benzothiazole amphiphilic compounds with low toxicity. Furthermore, these results support a correlation between membrane lysis, pore-forming capabilities, and toxicity of the BAM agents, and demonstrate that the small structural changes made to
the parent BTA-EG₆ structure can lead to compounds with measurably improved biocompatibility.

In conclusion, we introduce three benzothiazole amphiphiles that exhibited improved biocompatibility compared to BTA-EG₆. The decreased toxicity of these BAM compounds correlated with their decreased capability to form ion pores in lipid bilayers and to lyse membranes.

2.9 Materials and Methods

2.9.1 Materials

Synthetic Aβ(1-42) peptide was purchased from PL Lab (Port Moody, Canada). Diphytanoylphosphatidylcholine (DiPhyPC) was purchased from Avanti Polar Lipids, Inc. SH-SY5Y human neuroblastoma cells (Product No: CRL-2266) and 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) cell proliferation assay (Product No: 30-1010K) were purchased from American Type Culture Collection (ATCC) (Manassas, VA). All other chemical reagents were purchased from either Sigma-Aldrich or Fisher and used as is unless otherwise stated.

2.9.2 Synthesis of Compounds

General:

NMR spectra were obtained on either a JEOL 500 MHz spectrometer or a Varian
400 MHz spectrometer as noted. Abbreviations for spectra splitting include: singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), broad (b). Chemical shifts are reported in ppm relative to residual solvent.

**Synthesis of 1-tosyl-3,6,9-trioxaundecanol (18):**

![Synthesis of 1-tosyl-3,6,9-trioxaundecanol (18)](image)

Tetra(ethylene glycol) (17) (2 g, 10.26 mmole, 1 equiv.) was dissolved in DCM (100 mL) and let stir for 5 minutes. After which, Ag$_2$O (3.5g, 15.44 mmole, 1.5 equiv.), KI (342 mg, 2.06 mmole, 0.2 equiv.) and tosyl chloride (2.16g, 11.33 mmole, 1.1 equiv.) was added and the reaction was let stir vigorously for 2 h. After 2 h, Ag$_2$O was filtered off through Celite and the reaction was concentrated down. Column chromatography (100% EtOAc) afforded 1-tosyl-3,6,9-trioxaundecanol (18) as a clear viscous liquid (2.00g, 56% yield).$^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 7.80 (d, 2H), 7.34 (d, 2H), 4.17 (t, 2H), 3.69-3.59 (m, 14H), 2.44 (s, 3H). ESI-MS (m/z): 371.1 [M+ Na]$^+$, 349.3[M+ H]$^+$

**Synthesis of 1-Iodo-3,6,9-trioxaundecanol (19):**

![Synthesis of 1-Iodo-3,6,9-trioxaundecanol (19)](image)
1-tosyl-3,6,9-trioxaundecanol (18) (1.00g, 2.87 mmole, 1 equiv.) and sodium iodide (860 mg, 5.74 mmole, 2 equiv.) were added to a round bottom with 10 mL of acetone and let reflux overnight under nitrogen. The reaction was then filtered to remove sodium para-tolunenesulfonate. Solvent was then removed and reaction was taken up in EtOAc and the organic phase was washed with 10% Na$_2$S$_2$O$_3$ (3x), water, and brine. The organic layer was dried over Na$_2$SO$_4$, filtered and concentrated down to yield 1-Iodo-3,6,9-trioxaundecanol (19) as a yellow oil (576 mg, 66% yield). $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 3.75-3.60 (m, 14H), 3.27 (t, 2H), 2.41 (b, OH). ESI-MS (m/z): 327.0 [M+ Na$^+$], 305.2 [M+ H$^+$]

**Synthesis of 17-hydroxy-3,6,9,12,15-pentaoxaheptadecyl 4-methylbenzene-sulfonate (20):**

Hexa(ethylene glycol) (20) (2 g, 7.08 mmole, 1 equiv.) was dissolved in DCM (100 mL) and let stir for 5 minutes. After which, Ag$_2$O (2.46g, 10.63 mmole, 1.5 equiv.), KI (235 mg, 1.42 mmole, 0.2 equiv.) and tosyl chloride (1.49 g, 7.79 mmole, 1.1 equiv.) was added and the reaction was let stir vigorously for 2 h. After 2 h, Ag$_2$O was filtered off through Celite and the reaction was concentrated down. Column chromatography (10% MeOH/EtOAc) afforded 17-hydroxy-3,6,9,12,15-pentaoxaheptadecyl 4-methylbenzene-sulfonate (21) as a clear viscous liquid (2.80 g, 91% yield). $^1$H NMR (500 MHz, CDCl$_3$):
δ7.80 (d, 2H), 7.34 (d, 2H), 4.16 (t, 2H), 3.67-3.58 (m, 22H), 2.44 (s, 3H). ESI-MS (m/z): 459.2 [M+ Na]^+

Synthesis of 17-iodo-3,6,9,12,15-pentaoxaheptadecan-1-ol (11):

1-tosyl-3,6,9,12,15-pentaoxaheptadecanol (21) (1g, 2.29 mmole, 1 equiv.) and sodium iodide (687 mg, 4.58 mmole, 2 equiv.) were added to a round bottom with 20 mL of acetone and let reflux overnight. The reaction was then diluted with EtOAc (10 mL) and washed with 10% Na$_2$S$_2$O$_3$ (3x). The organic layers were washed with brine, dried over Na$_2$SO$_4$, filtered and concentrated down to yield 17-iodo-3,6,9,12,15-pentaoxaheptadecan-1-ol (11) as a yellow oil (938 mg, quantitative yield). $^1$H NMR (500 MHz, CDCl$_3$): δ 3.75-3.60 (m, 22H), 3.27 (t, 2H), 2.36 (b, OH). ESI-MS (m/z): 415.1 [M+ Na]^+

General synthesis of BTA-EG$_x^{51}$: 

BTA-EG$_4$: n = 3 (47%)
BTA-EG$_6$: n = 5 (46%)
A microwave vial was charged with 17-iodo-3,6,9,12,15-pentaoxaheptadecan-1-ol (EG6-I) (11) or 1-Iodo-3,6,9-trioxaundecanol (EG4-I) (19) (109 mg, 0.277 mmole, 1 equiv.), benzo[b]thiazole aniline (BTA) (100 mg, 0.416 mmole, 1.5 equiv.), potassium carbonate (115 mg, 0.832 mmole, 3 equiv.) and tetrahydrofuran (THF) (2 mL). The mixture was irradiated under MW (125 °C, 2 h). The mixture was filtered, concentrated and normal phase column chromatography (4% MeOH/DCM) followed by reverse phase column chromatography (3:1 MeOH/H2O) yielded: **BTA-EG6** (67 mg, 46% yield). \(^1\)H NMR (500 MHz, CDCl3): \(\delta\) 7.89 (2H), 7.86 (1H), 7.62 (1H), 7.24 (1H), 6.70 (2H), 3.74-3.58 (m, 22H), 3.38 (t, 2H), 2.46 (s, 3H). ESI-MS \(m/z\): 506.3 [M+ H]+, 527.3 [M+ Na]+ or **BTA-EG4** (188 mg, 47% yield). \(^1\)H NMR (400 MHz, CDCl3): \(\delta\) 7.87 (d, 2H), 7.83 (d, 1H), 7.63 (s, 1H), 7.23 (d, 1H), 6.68 (d, 2H), 3.76-3.64 (m, 14H), 3.37 (t, 2H), 2.47 (s, 3H). ESI-MS \(m/z\): 417.2 [M+ H]+

**Alkylation of 4-hydroxy benzaldehyde (6):**

4-Hydroxy benzaldehyde 4 (2g, 16.5 mmole, 1.1 equiv.) and anhydrous potassium carbonate (K₂CO₃) (4.14g, 29.9 mmole, 2 equiv.) were dissolved in acetone (30 mL) and let stir under nitrogen (N₂) for 30 min. Then 2-chloro-N-methylacetamide 5 (1.61g, 15 mmole, 1 equiv.) and potassium iodide (KI) (249 mg, 1.5 mmole, 0.1 equiv.) were added
and let reflux for 24 h. After cooling to room temperature (RT), solids were filtered off and the solvent was removed and replaced with dichloromethane (DCM). Extraction was done with 10% sodium hydroxide (NaOH) followed by column chromatography purification (95% DCM/ methanol (MeOH)) to yield compound 6 as a white solid (2.4 g, 83% yield). 

$^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 9.91 (s, 1H), 7.88 (d, 2H), 7.04 (d, 2H), 6.50 (b, 1H), 4.57 (s, 2H), 2.93 (s, 3 H). ESI-MS ($m/z$): 194.12 [M+ H]$^+$

**Synthesis of 4-N-(methylamino) benzaldehyde (7):**

To a round bottom with dry toluene, compound 6 (300 mg, 1.55 mmole, 1 equiv.) and potassium hydroxide (KOH) pellets (174 mg, 3.10 mmole, 2 equiv.) were added and let reflux for 24 h. After cooling to RT, the reaction was put on ice and water was added. The organic layer was washed 3x with water, dried, and concentrated. Column chromatography (50% ethyl acetate (EtOAc)/Hexanes) yielded compound 7 as a red solid (64 mg, 30% yield). 

$^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 9.72 (s, 1H), 7.71 (d, 2H), 6.61 (d, 2H), 4.41 (b, 1H), 2.91 (s, 3 H). ESI-MS ($m/z$): 136.19 [M+ H]$^+$
Synthesis of 1-pentyl-3-methylimidazolium bromide ([pmIm]Br):  

Commercially available 1-methylimidazole (22) (0.871 g, 0.010 moles, 1 equiv.) and 1-bromopentane (23) (1.66 g, 0.011 moles, 1.1equiv.) were placed under sonication for 2 h. The clear two layers turn into a viscous, opaque single layer upon completion. For purification, excess 1-bromopentane was removed by washing with ethyl acetate and ether followed by drying yielded 1-pentyl-3-methylimidazolium bromide ([pmIm]Br) as a clear viscous liquid (2.09g, 88%). \(^1\)H NMR (500 MHz, CDCl\(_3\)): \(\delta\) 10.55 (s, 1H), 7.45 (d, 1H), 7.43 (d, 1H), 4.32 (t, 2H), 4.11 (s, 3H), 1.92 (m, 2H), 1.33(m, 4H), 0.88(t, 3H). ESI-MS (m/z): 153.1 [M- Br]^+

Synthesis of benzothiazole (9):  

A microwave vial was charged with 2-aminothiophenol 7 (45 mg, 0.36 mmole. 1 equiv.), followed by 1-pentyl-3-methylimidazolium bromide ([pmIm]Br) (29 mg, 0.18 mmole, 0.5 equiv.) and then 4-(methylamino)benzaldehyde 8 (49mg, 0.36 mmole, 1
equiv.). The mixture was irradiated under MW conditions (150 °C, 4 min). The reaction mixture was extracted with ether/H₂O (4x). The ether was evaporated and the compound was purified by column chromatography (25%DCM/70%Hexanes/5%EtOAc), affording compound 9 as a light orange solid (55 mg, 64% yield). ¹H NMR (500 MHz, CDCl₃): δ 8.02 (d, 1H), 7.96 (d, 2H), 7.84 (d, 1H), 7.44 (t, 1 H), 7.32 (t, 1 H), 6.66 (d, 2 H), 2.92 (s, 3 H). ¹³C NMR (400 MHz, CDCl₃): δ 169.05, 154.53, 151.82, 134.73, 129.32 (2C), 126.25, 124.50, 122.68, 122.53, 121.60, 112.24 (2C), 30.54. ESI-MS (m/z): 241.0 [M+H]⁺

**General protocol for hexa(ethylene glycol) addition:**

![Scheme](image)

Synthesis of 17-iodo-3,6,9,12,15-pentaoxaheptadecan-1-ol (EG₆-I) (11) was prepared as previously described⁵⁹. A microwave vial was charged with EG₆-I (1 equiv.), benzothiazole aniline 9 or 10 (2 equiv.), potassium carbonate (3 equiv.) and tetrahydrofuran (THF). The mixture was irradiated under MW (125 °C, 2 h). The mixture was filtered, concentrated and normal phase column chromatography (4% MeOH/EtOAc) followed by reverse phase column chromatography (3:1 MeOH/H₂O) yielded compound 1 (285 mg, 48% yield) or compound 2 (13 mg, 30% yield). **BAM1-EG₆** (1): ¹H NMR (500 MHz, CDCl₃): δ 7.99 (d, 1H), 7.92 (d, 2H), 7.84 (d, 1H), 7.43 (t, 1 H), 7.30 (t, 1
H), 6.76 (d, 2 H), 4.97 (b, 1 H), 3.73-3.58 (m, 22 H), 3.39 (t, 2 H). $^{13}$C NMR (500 MHz, CDCl$_3$): $\delta$ 168.92, 154.51, 151.38, 134.74, 129.13 (2 C), 126.24, 124.54, 123.20, 122.55, 121.60, 113.28 (2 C), 71.68, 69.81-69.03 (69.81, 69.59, 69.45, 69.30, 69.24, 69.23, 69.03), 68.74, 60.44, 43.86. HR/MS (ESI +): Calcd for [C$_{25}$H$_{34}$N$_2$O$_6$S + Na] 513.2030 found 513.2029 [M+Na]$^+$ or BAM2-EG$_6$ (2): $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 7.96 (d, 1 H), 7.93 (d, 2 H), 7.84 (d, 1 H), 7.42 (t, 1 H), 7.29 (t, 1 H), 7.29 (d, 2 H), 6.76 (d, 2 H), 3.72-3.28 (m, 24 H), 3.07 (s, 3 H). $^{13}$C NMR (500 MHz, CDCl$_3$): $\delta$ 168.94, 154.64, 151.39, 134.74, 129.17 (2 C), 126.19, 124.40, 122.49, 121.57, 121.55, 111.80 (2 C), 72.76, 71.0-70.50 (71.00, 70.88, 70.85, 70.80, 70.76, 70.75, 70.71, 70.50), 68.73, 61.93, 52.29, 39.26. HR/MS (ESI-TOFMS +): Calcd for [C$_{26}$H$_{36}$N$_2$O$_6$S + Na] 527.2191 found 527.2187 [M+Na]$^+$

2-((4-(methylthio)phenyl)benzo[d]thiazole (13):

2-amino thiophenol 8 (376 mg, 3 mmol, 1 equiv.), [pmlm]Br (400 mg, 0.5 equiv), 4-(methylthio)benzaldehyde 12 (457 mg, 3 mmol, 1 equiv.) were added respectively, into a 5 mL microwave tube with a stir bar. The reaction tube was microwaved for 4 min at 130°C. The reaction mixture was dissolved in diethyl ether and extracted with water to remove the ionic liquid solution. The diethyl ether was removed under reduced pressure
and the crude solid 13 was purified by recrystallization in a 3:1 mixture of hexanes:EtOAc (547 mg, 71% yield). Spectra matched previously reported sample.\textsuperscript{73} \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}): \(\delta\): 8.05 (d, 1H), 8.01 (d, 2H), 7.90 (d, 1H), 7.49 (t, 1H), 7.38 (t, 1H), 7.33 (d, 2H), 2.55 (s, 3H). ESI-MS (m/z): 258.25 [M+H]\textsuperscript{+}

2-(4-(methylsulfinyl)phenyl)benzo[d]thiazole (14):

\[
\begin{array}{c}
\text{13} \\
\text{mCPBA} \\
\text{DCM} \\
\text{80\%} \\
\text{14}
\end{array}
\]

2-(4-(methylthio)phenyl)benzo[d]thiazole 13 (300 mg, 1.1 mmol) was dissolved in 6 mL of DCM. \textit{meta}-chloroperoxybenzoic acid (\textit{m}-CPBA) (242 mg, 1.4 mmol) was dissolved in 4 mL of DCM and added dropwise at 0 °C to the methyl sulfide 13 solution over a period of 20 min. NaHCO\textsubscript{3} (80 mg) was added and the solution was let stir. The reaction mixture was monitored by TLC analysis (100% EtOAc) until completion. The white precipitate was filtered away and the DCM was removed under reduced pressure to afford a white solid. The solid was purified by recrystallization in 100% EtOAc to give product 14 (254 mg, 80% yield). \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}): \(\delta\): 8.26 (d, 2H), 8.11 (d, 1H), 7.94 (d, 1H), 7.78 (d, 2H), 7.53 (t, 1H), 7.44 (t, 1H), 2.79 (s, 3H). ESI-MS (m/z): 274.17 [M+H]\textsuperscript{+}, 296.10 [M+Na]\textsuperscript{+}
2-(4-(methylsulfinyl)phenyl)benzo[d]thiazole (14) (50 mg, 0.18 mmol) was dissolved in 2 mL of freshly distilled DCM in an oven dried 50 mL round bottom. Trifluoroacetic anhydride (TFAA) (0.15 mL) was added to the reaction flask and the reaction was gently refluxed at 40 °C for 2 h under N₂. The solvent was removed under reduced pressure to afford the crude product 15 (72 mg, approximately quantitative conversion). The product was taken on to the next step without further purification. 

1H NMR (500 MHz, CDCl₃): δ 8.07 (m, 3H), 7.92 (d, 1H), 7.58 (d, 8Hz, 2H), 7.53 (t, 1H), 7.43 (t, 1H), 5.70 (s, 2H)

4-(benzo[d]thiazol-2-yl)benzenethiol (16):

((4-(benzo[d]thiazol-2-yl)phenyl)thio)methyl 2,2,2-trifluoroacetate (15) (72mg, 0.19 mmol) was dissolved in 3 mL of MeOH and 0.6 mL of 1M NaOH was added to the reaction flask and refluxed under N₂ for 1 h. The reaction mixture was cooled and the solvent was removed under reduced pressure. 0.6mL of 1M HCl was then added to the
crude mixture and the product was extracted into EtOAc by washing the aqueous layer with 3 x 2mL of EtOAc. The organic layer was washed with a saturated NaCl solution and dried over Na$_2$SO$_4$. The EtOAc was removed under reduced pressure to afford the crude product 16 (44 mg, 93% crude yield). $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 8.08 (d, 1H), 7.95 (d, 2H), 7.90 (d, 1H), 7.51 (t, 1H), 7.39 (m, 3H), 3.68 (s, 1H). ESI-MS (m/z): 244.28 [M+H]$^+$

17-((4-(benzo[d]thiazol-2-yl)phenyl)thio)-3,6,9,12,15-pentaoxaheptadecan-1-ol (3):

In an oven dried 50 mL round bottom, solid sodium hydride (NaH) (2 mg, 0.074 mmol) was added and the round bottom was tightly capped with a rubber septum. The round bottom was purged with N$_2$. The crude 4-(benzo[d]thiazol-2-yl)benzenethiol (16) (12mg, 0.05 mmol, 1 equiv.) was dissolved in 1mL of freshly distilled dimethylformamide (DMF) and added dropwise to the round bottom flask containing NaH. The reaction mixture was stirred for 30 min. 17-iodo-3,6,9,12,15-pentaoxaheptadecan-1-ol (EG$_6$-I, 20 mg, 0.05 mmol, 1 equiv.) was dissolved in 1 mL of freshly distilled DMF in a separate vial and added dropwise into reaction mixture. The reaction was then refluxed under N$_2$ for 12 h. The reaction mixture was cooled to RT and the solvent was removed under reduced pressure. The product was purified via silica gel
flash chromatography (using a gradient of EtOAc:MeOH 0-4%) to afford the desiredproduct 3 as a yellow oil (Rf=0.24, 100% EtOAc). The yellow oil product was purified once more using a reverse-phase preparatory plate (using a 3:1 mixture of MeOH: H2O as eluent) to give the final product 3 (11mg, 44% yield). **BAM3-EG6 (3):** 

\[ ^1H \text{NMR (500 MHz, CDCl}_3) : \delta \ 8.04 \text{ (d, 1H)}, 7.99 \text{ (d, 2H)}, 7.89 \text{ (d, 1H)}, 7.48 \text{ (t, 1H)}, 7.41 \text{ (d, 2H)}, 7.37 \text{ (t, 1H)}, 3.74-3.70 \text{ (m, 4H)}, 3.64 \text{ (m, 16H)}, 3.60-3.58 \text{ (m, 2H)}, 3.20, \text{ (t, 2H).} \]

\[ ^13 \text{C NMR (500 MHz, CDCl}_3) : \delta 167.42, 154.09, 140.57, 134.88, 130.83, 128.01, 127.86, 126.38, 125.18, 123.08, 121.62, 72.50, 70.64-70.30 \text{ (70.64, 70.59, 70.55, 70.53, 70.50, 70.30)}, 69.68, 61.74, 32.08. \]


**2.9.3 Saturation Shake-Flask Solubility Method**

To determine the difference in solubility between BTA-EG4 and BTA-EG6, a standard saturation shake-flask solubility method was performed with noted changes. Briefly, ~5–10 mg of each compound was added into 5 mL of PBS (pH 7.4) and was shaken vigorously for 24 h at room temperature. After equilibrium was reached, the remaining undissolved compound was removed by centrifugation. The concentration of dissolved compound was then determined by measuring the UV–Vis absorbance at 355 nm (i.e., \( \lambda_{\text{max}} \) for both compounds). Dilutions in PBS were necessary for BTA-EG6 in order to obtain absorbance values under 1 absorbance unit (AU), which facilitates precision in the measurement. No dilutions were necessary for BTA-EG4 due to its poor solubility in PBS. Concentrations were then determined by comparison to calibration
curves for each respective compound. Data were presented as mean ± SD, n=3.

2.9.4 Estimation of Log P and Solvent Accessible Surface Area (SASA)

Log P values were calculated using molinspiration cheminformatics software and solvent accessible surface area (SASA) values were calculated with PyMOL.

2.9.5 Measurement of Fluorescence Emission Spectra

The emission spectra of benzothiazoles in different environments was evaluated as previously described. Briefly, BAMs 1-3 and BTA-EG₆ were diluted to a final concentration of 50 µM in deionized H₂O, pure octanol, and a liposome suspension. The liposomes were prepared from a total lipid concentration of 10 mM of diphytanoylphosphatidylcholine (DiPhyPC) in water by the gentle dehydration rehydration method followed by tip sonication. 200 µL of each sample was transferred to a cuvette (Helma Analytics, Quartz SUPRASIL (QS), 10 mm) and the fluorescence emission spectrum was measured in a PTI spectrofluorimeter (0.5 nm step size) in water, octanol and an aqueous liposome suspension for BAMs 1-3 and BTA-EG₆. Maximal excitation and emission values (λ_max) for all compounds in PBS were as follows: BTA-EG₆ (Ex/Em 355/420 nm), BAM1-EG₆ (Ex/Em 355/420 nm), BAM2-EG₆, (Ex/Em 365/428 nm), and BAM3-EG₆ (Ex/Em 335/398 nm). Each experiment was repeated at least three separate times and error bars denote standard deviation from the mean. Data were processed using Origin 7.0 (MicroCal Software, Inc., Northampton, MA).
2.9.6 Planar Lipid Bilayer Studies

The procedure for PLB recordings was performed as previously described. Briefly, planar lipid bilayers were prepared from a solution of 20 mg/mL 1,2-diphytanoyl-sn-glycero-3-phosphatidylcholine (DiPhyPC) in decane by the painting method. Both compartments of the recording chamber were filled with an electrolyte solution containing 1 M CsCl and 10 mM HEPES buffer (pH = 7.4). Currents were recorded at 5 kHz bandwidth with a sampling frequency of 25 kHz using a Geneclamp-500 amplifier (Axon instruments), and further filtered at 200 Hz using a Gaussian low-pass filter for analysis. For this experiment, we defined the minimum concentration of pore formation to be the concentration where the frequency of observed channel events was greater than 0.05 per minute.

2.9.7 SH-SY5Y Neuroblastoma Cultures

SH-SY5Y human neuroblastoma cells were grown in 1:1 Eagle’s Minimum Essential Medium (EMEM) and Ham’s F12 supplemented with 10% Fetal Bovine Serum (FBS). Cells were maintained in a humidified incubator at 37 °C in 95% air and 5% carbon dioxide (CO₂). Complete media was exchange 2 times a week.

2.9.8 MTT Cell Proliferation Assay in SH-SY5Y Neuroblastoma

An MTT cell proliferation assay was performed as previously described. Briefly cells were plated at a density of 50,000 cells/well in 100 µL of 1:1 EMEM and Ham’s F12, supplemented with 10% FBS and without phenol red. After adhering overnight, the medium was replaced with 100 µL of fresh medium containing various concentrations of
either BTA-EG₆ or BAMs-EG₆ with final concentrations (0-500 µM). Cells were exposed to these solutions for 24 hours at 37 °C. MTT cell viability assay was then used to determine cell viability. Briefly, the MTT reagent (10 µL of the solution from the commercial kit) was then added and the cells were incubated for 3 additional hours. The cells were subsequently solubilized with detergent reagent (100 µL of the solution from the commercial kit) and incubated at room temperature overnight. The cell viability was determined by measuring the absorbance at 570 nm using a Spectramax 190 microplate reader (Molecular Devices). All results were expressed as percent reduction of MTT relative to untreated controls (defined as 100% viability) and the average absorbance value for each treatment was blanked with the absorbance reading of wells containing only media, MTT reagent, and detergent reagent.

2.9.9 Differentiated SH-SY5Y Neuroblastoma Cultures

SH-SY5Y neuroblastoma cells were differentiated as previously described. Briefly, SH-SY5Y human neuroblastoma cells were grown in complete medium [1:1 Eagle’s Minimum Essential Medium (EMEM) and Ham’s F12 supplemented with 10% Fetal Bovine Serum (FBS)]. After adhering overnight, cells were differentiated by addition of 10 µM all-trans-retinoic acid (RA) to the complete medium. Medium with RA was replaced every 2 days for 8 days total. Cells were maintained in a humidified incubator at 37 °C in 95% air and 5% carbon dioxide (CO₂).

2.9.10 MTT Cell Proliferation Assay in Differentiated SH-SY5Y Neuroblastoma

SH-SY5Y neuroblastoma cells were differentiated as noted above. For the MTT
assay, cells were plated at a density of 50,000 cells/well in 100 µL of 1:1 EMEM and Ham’s F12, supplemented with 10% FBS and without phenol red. After adhering overnight, medium was replaced with differentiating medium by addition of 10 µM all-trans-retinoic acid (RA). Medium with RA was replaced every 2 days for 8 days total. After 8 days, the media was removed and 100 µL of new, RA-free medium containing various concentrations of either BTA-EG₆ or BAMs-EG₆ with final concentrations (0-500 µM). Cells were exposed to these solutions for 24 hours at 37 °C. MTT cell viability assay as mentioned above was then used to determine cell viability. All results are presented as percent reduction of MTT relative to untreated cells (100% viability).

2.9.11 Neuronal Cultures

Rat dissociated hippocampal neurons from postnatal day 1 Sprague Dawley® outbred rats of both sexes were plated at a density of 45,000 cells/cm² onto poly-D-lysine-coated 96-well plates. Neurons were maintained in B27 supplemented Neurobasal media (Invitrogen) until days in vitro (DIV) 18-23 as previously described.⁷⁵,⁷⁶

2.9.12 Measurement of Cell Viability in Primary Culture

An MTT cell viability assay was performed. Briefly, 21 DIV rat dissociated hippocampal neurons in 96-well plates were dosed with 100 µL of various samples solutions of either BTA-EG₆ or BAMs 1-3 with final concentrations (0-250 µM). Cells were exposed to these solutions for 24 h at 37 °C, 5% CO₂. An MTT cell viability kit (ATCC, Product No: 30-1010K) was then used to determine cell viability. Briefly, 20 µL of the provided MTT reagent was added per well and cells were placed in the incubator
for 3 h. The insoluble intracellular purple formazan was then dissolved by the addition of 100 µL of detergent reagent provided and let solubilize overnight at room temperature. The cell viability was determined by measuring the absorbance at 570 nm using a Spectramax 190 microplate reader (Molecular Devices). All results are presented as percent reduction of MTT relative to untreated cells (100% viability), and all wells were blanked with absorbance values from the wells containing medium, MTT reagent and detergent only.

**Notes About the Chapter:**

Chapter two, in part, is a reprint (with co-author permission) of the material as it appears in the following publications; Cifelli, J. L., Chung, T. S., Liu, H., Prangkio, P., Mayer, M., and Yang, J. (2016) “Benzothiazole Amphiphiles Ameliorate Amyloid β-Related Cell Toxicity and Oxidative Stress”, *ACS Chem. Neurosci.* 7(6), 682-688; and Cifelli, J. L., Dozier, L., Chung, T. S., Patrick, G. N. & Yang, J. “Benzothiazole Amphiphiles Promote the Formation of Dendritic Spines in Primary Hippocampal Neurons”, *J. Biol. Chem.* 291, 11981–11992 (2016). I would like to thank Tim Chung, Haiyan Liu, Panchika Prangkio, Lara Dozier, Michael Mayer, Gentry Patrick, and Jerry Yang for their invaluable contributions to this chapter: Tim Chung synthesized BAM3-EG₆, Haiyan Liu and Panchika Prangkio performed all ion channel studies, Lara Dozier and Gentry Patrick prepared and maintained all primary neuronal cultures. Additionally I would like to thank Michael Mayer, Gentry Patrick and Jerry Yang for directing the research. The author of the dissertation is the primary author of these manuscripts. Additionally, the solubility studies, which were the author of this dissertation’s
Benzothiazole Amphiphiles Ameliorate β-Amyloid Related Cell Toxicity and Oxidative Stress

3.1 Introduction

Oxidative damage due to an imbalance of production and degradation of reactive oxygen species (ROS) in neurons is a normal part of the aging process, and is accelerated in Alzheimer’s disease (AD). In particular, elevated levels of ROS are found associated with regions of high accumulation of β-amyloid (Aβ) in the brains of patients with AD. The brain is highly susceptible to oxidative stresses due to slow regeneration, high oxygen consumption and low levels of antioxidants, making it important to develop methods to combat oxidative stress in AD.

Recent evidence supports a detrimental interaction between aggregated forms of Aβ and the antioxidant enzyme catalase. Catalase plays an important role in maintaining the normal levels of ROS by catalyzing the degradation of hydrogen peroxide (H₂O₂). The interaction between Aβ and catalase causes deactivation of
catalase and subsequently an increase in cellular levels of $H_2O_2$\textsuperscript{52,84}. Compounds that can inhibit Aβ-catalase interactions in cells may, therefore, represent a new therapeutic strategy for treatment of AD.

We previously reported the design, synthesis, and evaluation of two oligo(ethylene glycol) derivatives of benzothiazole aniline (BTA), BTA-EG\textsubscript{6} and BTA-EG\textsubscript{4}, which exhibited a variety of advantageous properties for the potential treatment of neurodegenerative diseases such as AD\textsuperscript{51–53}. The \textit{in vivo} properties of these BTA-EG\textsubscript{x} compounds suggest that they may be capable of slowing down cognitive decline associated with AD. While the therapeutic potential of this class of compounds is attractive, these BTA-EG\textsubscript{x} compounds exhibited some toxicity in neuroblastoma cells at concentrations below 100 μM, which we showed correlated with their capability to form pores in membranes and induce membrane lysis\textsuperscript{59}. To further advance this class of compounds as AD therapeutics, BAMs 1-3 (described in Chapter 2) were rationally designed, synthesized and exhibited reduced toxicity in a variety of cell lines.

In this chapter we evaluate the capability of all new BAM derivatives’ capability to protect cells against Aβ-induced toxicity and oxidative stress. These BAMs were found to retain the parent compound’s ability to protect against Aβ-induced toxicity and oxidative stress. Additionally, BAMs were found to decrease co-localization and binding of catalase to aggregated Aβ peptides in cells. Thus, the cytoprotective effects of these compounds support a previously proposed mechanism of formation of bioresistive coatings on aggregated Aβ peptides, leading to inhibition of catalase-amyloid interactions that would otherwise promote cellular increases in $H_2O_2$. These BAM compounds, thus,
represent a new family of potential anti-Aβ therapeutics that may have utility as part of a combined therapeutic regiment for AD.

### 3.2 Binding of BAMs to Aggregated Aβ

In order to evaluate whether 1-3 could bind to aggregated Aβ peptides with similar affinity as BTA-EG₆ after hydrophobic structural modifications, we used a preparation of Aβ that mimicked the heterogeneous population of aggregated Aβ species present in the brains of AD patients. This preparation contained ~12% small Aβ oligomers (MW <15kDa corresponding to monomers-trimers), ~16% medium-sized oligomers (MW 20-65 kDa corresponding to 5-15 mers), and ~72% soluble protofibrils (MW >150 kDa corresponding to >30 mers) (Figure 3.1). We then used a previously

![Figure 3.1](image)

**Figure 3.1.** (A) Amino acid sequence of Aβ(1-42) (B) Western blot detection of the composition of Aβ(1-42) aggregation states.
reported fluorescence-based binding assay to estimate $K_d$ values for the association of the BAMs to this preparation of aggregated Aβ (1-42) peptides. All compounds were found to bind Aβ in the mid nanomolar range (Figure 3.2). These binding affinities were comparable to previously reported $K_d$ values for the binding of BTA-EG$_6$ to aggregated Aβ (1-42).

![Figure 3.2](image)

**Figure 3.2.** Plot of the fluorescence intensity binding curve of (A) BTA-EG$_6$ ($\lambda = 420$ nm), (B) BAM1-EG$_6$ ($\lambda = 420$ nm), (C) BAM2-EG$_6$ ($\lambda = 428$ nm) and (D) BAM3-EG$_6$ ($\lambda = 398$ nm) with aggregated Aβ(1-42).
3.3 BAMs Readily Internalize into Differentiated SH-SY5Y Cells

Next, we looked to assess if the hydrophobic modifications made to BAMs 1-3 affected their ability to internalize into cells. Here, the intrinsic fluorescence of the compounds was utilized to detect cellular internalization in differentiated SH-SY5Y neuroblastoma cells. Live-cell fluorescent microscopy showed that BAMs 1-3 all readily internalized into cells with no apparent subcellular localization, similar to BTA-EG₆ (Figure 3.3).

Figure 3.3. Representative z-slices from the middle of the cells showing cellular internalization of (A) BAM1-EG₆, (B) BAM2-EG₆, (C) BAM3-EG₆ and (D) BTA-EG₆ in differentiated SH-SY5Y cells. Scale bar, 25 µm.
3.4 Cytoprotection Against Aβ Induced Toxicity

Since BTA-EG\textsubscript{X} compounds were previously shown to reduce the toxicity of Aβ (1-42) in cells through the formation of protein-resistive coatings on amyloid aggregates,\textsuperscript{51,52} we next examined whether BAMs 1-3 also exhibited such cytoprotective properties. Exposure of differentiated SH-SY5Y cells to 25 µM concentrations of aggregated Aβ (1-42) peptides resulted in a 35% decrease in cell viability (Figure 3.4), as determined by an MTT cell proliferation assay. As expected, BAMs 1-3 were capable of reducing the toxicity of Aβ, leading to a dose-dependent trend of increasing cell viability to ~80% at concentrations of 20 µM of compounds in the presence of 25 µM Aβ (Figure 3.4).

![Figure 3.4. Cytoprotective effects of BTA-EG\textsubscript{6} and BAMs 1-3 against aggregated Aβ toxicity (25 µM) in differentiated SH-SY5Y neuroblastoma cells. UT= untreated cells. Data expressed as mean values ± SD, n = 3 or more for each concentration. *p < 0.05 or **p < 0.01 compared to cells incubated with 25 µM Aβ alone (i.e., in the absence of small molecules).]
3.5 Cytoprotection Against Aβ Induced Oxidative Stress

In order to evaluate the BAMs for their ability to inhibit Aβ-induced oxidative stress, we analyzed the cellular H$_2$O$_2$ levels in the presence of aggregated Aβ with or without the addition of compounds 1-3. Differentiated SH-SY5Y cells were first incubated in the presence of 25 µM aggregated Aβ. After 24 h, we observed a 4-fold increase in cellular H$_2$O$_2$ levels compared to untreated cells (Figure 3.5). As a positive control, addition of 3-AT, a known catalase inhibitor, to cells also showed about a 4-fold increase in H$_2$O$_2$ levels over the control cells (Figure 3.5). When cells were treated

![Graph](image)

**Figure 3.5.** Reduction of the Aβ-induced increase of H$_2$O$_2$ release by BTA-EG$_6$ or BAMs 1-3 in SH-SY5Y cells. Cells treated with Aβ (25 µM) or 3AT, a catalase inhibitor show ~4 fold increase in H$_2$O$_2$ compared to untreated cells. Cells that were treated with aggregated Aβ (25 µM) that was pre-incubated with 1-40 µM concentrations of BTA-EG$_6$ or BAMs 1-3 show a dose-dependent decrease in H$_2$O$_2$ release. Cells treated with compounds alone showed no statistical change in H$_2$O$_2$ release relative to untreated cells. (*p < 0.05 or **p < 0.01 compared to cells treated with 25 µM Aβ alone). Data expressed as mean values ± SD, n ≥ 3 for each concentration.
with aggregated Aβ in the presence of various concentrations (0-40 µM) of BTA-EG₆ or BAMs 1-3, a dose dependent reduction in H₂O₂ levels was observed for all compounds. Furthermore, the levels of cellular H₂O₂ when cells were incubated with 25 µM Aβ in the presence of 40 µM concentrations of any of the compounds tested were statistically indistinguishable from control cells (Figure 3.5). Interestingly, BAM 3 showed significantly improved reductions of Aβ-induced increases in cellular H₂O₂, with statistically significant activity observed at concentrations as low as 1 µM.

We hypothesized that this inhibition of Aβ-induced increases in cellular H₂O₂ levels was due to the capability of the BAM agents to inhibit catalase-Aβ interactions. In order to rule out the possibility that the BAM agents exhibited inherent anti-oxidant properties that were independent from their capability to bind to aggregated Aβ, we incubated BAMs 1-3 over 24 h (i.e., same time course as cellular oxidative stress assay) in the presence of 3 mM H₂O₂ and monitored these reactions for any products from oxidation by mass spectrometry (Table 3.1). We did not observe any oxidation products

<table>
<thead>
<tr>
<th>Compound</th>
<th>Exact Mass (g/mol)</th>
<th>In Water: ESI-MS (m/z)</th>
<th>In H₂O₂: ESI-MS (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAM1-EG₆</td>
<td>490.21</td>
<td>491.27 [M+H]⁺</td>
<td>491.27 [M+H]⁺</td>
</tr>
<tr>
<td></td>
<td></td>
<td>513.22 [M+Na]⁺</td>
<td>513.20 [M+Na]⁺</td>
</tr>
<tr>
<td>BAM2-EG₆</td>
<td>504.23</td>
<td>505.25 [M+H]⁺</td>
<td>505.25 [M+H]⁺</td>
</tr>
<tr>
<td></td>
<td></td>
<td>527.22 [M+Na]⁺</td>
<td>527.24 [M+Na]⁺</td>
</tr>
<tr>
<td>BAM3-EG₆</td>
<td>507.17</td>
<td>508.20 [M+H]⁺</td>
<td>508.16 [M+H]⁺</td>
</tr>
<tr>
<td></td>
<td></td>
<td>530.23 [M+Na]⁺</td>
<td>530.24 [M+Na]⁺</td>
</tr>
</tbody>
</table>

**Table 3.1.** Mass spectrometry analysis of BAMs 1-3 incubated in water or 3 mM hydrogen peroxide (H₂O₂) for 24 hours.
for any of the BAM agents under these conditions, supporting that the observed decrease in cellular H₂O₂ levels by compounds 1-3 in the presence of Aβ was likely due to their capability to target aggregated Aβ.

### 3.6 BAMs as Inhibitors of Catalase-Aβ Interactions

BTA-EG₆ was previously shown to inhibit catalase-Aβ interactions both in cells and in cell-free assays. In order to confirm that the BAM agents could also inhibit catalase-Aβ interactions, we dosed Aβ with or without the inclusion of compounds 1-3 and examined the extent of intracellular co-localization of Aβ and catalase compared to cells that were treated with Aβ alone (Figure 3.6). This co-localization was determined by fixing the cells after incubation with Aβ and compounds, fluorescent immunolabeling of Aβ and catalase, and quantifying co-localization using the Pearson’s correlation coefficient (PCC) within the entire three-dimensional volume of the cells. When cells were treated with Aβ, the PCC for colocalization of Aβ and catalase was found to be 0.57, indicating a substantial degree of colocalization. However, when treated with BTA-EG₆ or BAMs 1-3, the PCC for Aβ and catalase reduced to 0.19-0.09 (Figure 3.6). These results suggest that the tested BAM compounds were indeed capable of reducing the interaction of Aβ with catalase, with both BAM1 and BAM3 showing the highest reduction in co-localization of catalase and Aβ among the compounds tested.

Additionally, while these results do not rule out any affects the BAM compounds may have on cellular uptake of Aβ, we have previously shown that BTA-EG₄ and BTA-EG₆ did not cause any statistically changes in the uptake of aggregated Aβ peptides in
cells. We, therefore, presume that the effects of the BAM agents reduce Aβ-induced increases in H₂O₂ through inhibiting intracellular catalase-Aβ interactions, and not merely affecting cellular uptake of Aβ.

**Figure 3.6.** Co-localization of aggregated Aβ (1-42) with catalase in SH-SY5Y cells. Fluorescence micrographs of representative z-slices within a cell illustrate the reduced co-localization of catalase (red) and Aβ peptides (green) in the presence of 40 µM concentrations of the BTA-EG₆ or BAMs 1-3. The nuclei of imaged cells are depicted in blue and merged images are shown on the right. Scale bar = 20 µm.
To further confirm that BAMs 1-3 were able to inhibit catalase-Aβ interactions, we used a previously described semi-quantitative ELISA-based assay\textsuperscript{51} to evaluate the relative interaction of human catalase to aggregated Aβ in the presence of BAMs 1-3 or BTA-EG\textsubscript{6}. In this assay, aggregated Aβ was deposited into wells of a 96-well plate and then incubated with catalase in the presence of increasing concentrations of compound. Results from this assay then reveal the concentrations of small molecule required to observe inhibition of aggregated Aβ as well as the maximal extent of the inhibition. We found that all compounds effectively decrease the interaction of catalase and Aβ at similar concentrations and to a similar extent (Figure 3.7). These results further support that BAMs 1-3 are capable of forming protein-resistive coatings on aggregated Aβ.

Figure 3.7. Inhibition of catalase-Aβ interactions by BTA-EG\textsubscript{6} and BAMs 1-3. The percentage of bound catalase to Aβ was decreased in the presence of increasing concentrations of A) BTA-EG\textsubscript{6}, B) BAM1-EG\textsubscript{6}, C) BAM2-EG\textsubscript{6} and D) BAM3-EG\textsubscript{6}. 100% bound was defined as the amount of catalase bound to Aβ in the vehicle controls (1% BSA/PBS). Data expressed as mean values ± SD, n = 3 for each concentration.
3.7 Concluding Remarks

While the experiments described here focus on the effects of the BAM agents on the interaction of aggregated Aβ with catalase, Aβ has been shown to interact with a variety of other cellular proteins.\textsuperscript{51,87,88} We have shown previously that molecules such as BTA-\textsc{eg}\textsubscript{6} are capable of forming protein-resistive coatings on a multiple amyloid surfaces,\textsuperscript{51,85} suggesting that the new BAM agents may find more general utility by simultaneously inhibiting a variety of protein-amyloid interactions that can cause damage to cells.

In conclusion, we introduce three benzothiazole amphiphiles (BAMs) that were able to 1) bind to aggregated Aβ, 2) decrease the toxicity of Aβ in differentiated neuroblastoma cells, 3) decrease Aβ-induced increases in cellular \textsc{H}_{2}\textsc{O}_{2} levels, 4) reduce the co-localization of Aβ and catalase in cells, and 5) inhibit the interaction of catalase and aggregated Aβ in solution. These results are consistent with the capability of these benzothiazoles to form protein-resistive coatings on aggregated Aβ and to diminish deleterious interactions between catalase and Aβ in cells (Figure 3.8). The capability of

![Inhibit Protein-Amyloid Interactions](image)

**Figure 3.8.** Illustration of the inhibition of Aβ-proteins, like catalase, to Aβ aggregates by binding of BAMs.
the BAM agents to neutralize Aβ activity makes it possible for catalase to maintain normal cellular levels of H₂O₂ in an Aβ-rich environment, leading to reduced toxicity of Aβ to cells. These new BAM agents, thus, represent an exciting new family of compounds that have potential therapeutic value for treating AD and possibly other amyloid-related diseases. In particular, the new compound BAM3-EG₆ (3) exhibited the greatest biocompatibility and anti-Aβ activity of the compounds tested, and can serve as a lead candidate for further development of inhibitors of protein-amyloid interactions as potential therapeutics for AD.

3.8 Materials and Methods

3.8.1 Materials

Synthetic Aβ(1-42) peptide was purchased from PL Lab (Port Moody, Canada). SH-SY5Y human neuroblastoma cells (Product No: CRL-2266) and 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) cell proliferation assay (Product No: 30-1010K) were purchased from American Type Culture Collection (ATCC) (Manassas, VA). The Amplex red hydrogen peroxide kit (# A22188) was from Molecular Probes of Invitrogen Co. and 3-amino-1,2,4-triazole (3AT) (# A8056) was from Sigma-Aldrich. All other chemical reagents were purchased from either Sigma-Aldrich or Fisher and used as is unless otherwise stated.
3.8.2 Preparation and Characterization of Aggregated Aβ(1-42)

Aggregated Aβ(1-42) was prepared as previously described. Briefly, Aβ(1-42) was initially solubilized in 100% 1,1,1,3,3,3,3-hexafluoro-2-propanal (HFIP) to 1 mM concentration at RT for 21 h with shaking. The solution was sonicated and vortexed before it was diluted in cold nanopure water (2:1 H₂O:HFIP). Aliquotted fractions were lyophilized for 2 days, followed by storage at -80 °C until use. Solutions of Aβ were obtained by dissolving Aβ in sterile PBS to a concentration of 100 µM and incubated at 37 °C for 3 days before use. Western blot analysis of the three day incubated Aβ was carried out to determine composition. We estimated the relative abundance of monomers, oligomers, and protofibrils of Aβ using ImageJ and percentage of each composition was calculated by dividing the intensity of each aggregation state over the total intensity for Aβ in the lane. This preparation of Aβ lead to a composition of ~12% monomers, ~16% low MW oligomers, and ~72% mixture of soluble protofibrils/fibrils. Aggregated Aβ was also characterized by EM, MALDI-TOF, and binding by thioflavin T (data not shown).

3.8.3 Measurement of the Binding Affinity of BTA-EG₆ and BAMs to Aggregated Aβ (1-42) Peptides

Binding of compounds to aggregated Aβ (1-42) was measured according to a previously described assay. Briefly, 200 µL of various concentrations of BTA/BAM compounds in PBS were incubated in the absence or presence of 10 µg of pre-aggregated Aβ (total volume 220 µL). Solutions were allowed to equilibrate overnight at room temperature. Samples were then centrifuged at 16,000 x g for 20 min at 4°C. The supernatants were removed and the pellet was re-suspended in 220 µL of fresh PBS.
Fluorescence of the bound molecule was determined using a spectrofluorometer (Photon Technology International, Inc., Birmingham, NJ). Each experiment was repeated at least three times and error bars denote standard deviation from the mean. Graphs shown in Figure 3.2 were fit using the following one-site specific binding algorithm to determine $K_d$: $Y = B_{\text{max}} \times X / (K_d + X)$, where $X$ is the concentration of small molecule, $Y$ is the specific binding intensity, and $B_{\text{max}}$ is the apparent maximal observable fluorescence upon binding to Aβ. Data was processed using Origin 7.0 (MicroCal Software, Inc., Northampton, MA).

3.8.4 Evaluation of the Cellular Internalization of BAMs 1-3 and BTA-EG$_6$

Differentiated SH-SY5Y neuroblastoma cells were plated in DMEM without phenol red (supplemented with 10% FBS and 4 mM L-glutamine) on 35 mm glass bottom dishes (MatTek) and incubated overnight. The growth media was removed and solutions of compounds in media were added to cells and allowed to incubate for 12 h before imaging. The cells were washed with Hanks’ balanced saline solution (HBSS) (3x) immediately before imaging. All Images were acquired on a Yokagawa spinning disk system (Yokagawa, Japan) built around an Axio Observer Z1 motorized inverted microscope (Carl Zeiss Microscopy GmbH, Germany) with a 40x, 1.40 NA oil immersion objective. An Evolve 512x512 EMCCD camera (Photometrics, Canada) was used with ZEN imaging software (Carl Zeiss Microscopy GmbH, Germany). Environmental conditions were maintained at 37 ºC, 5% CO$_2$ with a heated enclosure and CO$_2$ controller (Pecon, Germany). The intrinsic fluorescence of BTA-EG$_6$ (Ex/Em 355/420 nm), BAM1-EG$_6$(Ex/Em 355/420 nm), BAM2-EG$_6$, (Ex/Em 365/428 nm), and
BAM3-EG\textsubscript{6} (Ex/Em 335/398 nm) were imaged with excitation by a 405 nm, 50 mw DPSS laser and emission monitored at (Em/bp: 450/50 nm).

3.8.5 Cytoprotection of Differentiated SH-SY5Y Neuroblastoma Cells

Differentiated SH-SY5 cells were exposed to solutions containing a final concentration of 25 µM Aβ and various concentrations of the BTA-E\textsubscript{6} and BAMs (0-20 µM), in RA-free medium for 24 hours at 37°C. Please refer to the supporting information with regard to pretreatment of all Aβ samples and note that the concentration of aggregated Aβ reported here refers to the Aβ concentration if all Aβ would be present in monomeric, dissolved form. The MTT reagent (10 µL of the solution from the commercial kit) was then added and the cells were incubated for 3 additional hours. The cells were subsequently solubilized with detergent reagent (100 µL of the solution from the commercial kit) and incubated at room temperature overnight. The cell viability was determined by measuring the absorbance at 570 nm using a Spectramax 190 microplate reader (Molecular Devices). All results were expressed as percent reduction of MTT relative to untreated controls (defined as 100% viability) and the average absorbance value for each treatment was blanked with the absorbance reading of wells containing only media, MTT reagent, and detergent reagent.

3.8.6 Assay for Hydrogen Peroxide Release from Differentiated SH-SY5Y Neuroblastoma Cells

Cells were differentiated as previously mentioned in DMEM without phenol red and supplemented with 10% FBS, 4 mM L-glutamine, and 10 µM RA for 8 days.
Solutions of Aβ aggregates with and without compounds in RA-free medium were incubated with the cells for 24 h. Hydrogen peroxide release was determined by adding 20 µL/well of a solution containing 250 µM Amplex red reagent and 0.5 U/mL Horseradish Peroxidase (HRP) dissolved in complete medium. After 30 minutes the absorbance at 560 nm was measured. For controls, cells were incubated with 40 µM of each compound alone; a positive control of 20 mM 3AT (a catalase inhibitor) was also added in each experiment. All experiments were done at least in triplicate and control wells of medium alone were used to blank all samples.

3.8.7 Confocal Microscopy of the Cellular Co-localization of Aβ and Catalase

SH-SY5Y cells were cultured on 35 mm dishes (MatTek) and incubated overnight in a 1:1 mixture of EMEM and Ham’s F12 supplemented with 10% FBS. The growth medium was removed and solutions containing aggregated Aβ (5µM) with or without compounds (40 µM), were added in fresh medium. The cells were incubated for 12 hours (37 °C, 5% CO₂). To visualize the co-localization of aggregated Aβ with catalase, the cells were rinsed (3x, PBS), fixed with 4% paraformaldehyde in PBS (pH 7.4) and permeabilized with 0.25% Triton-X in PBS. Cells were then blocked with 10% goat serum/PBS (1h, RT) and then incubated with primary antibodies: mouse anti-Aβ (6E10, Covance) and rabbit anti-catalase (Abcam) antibody on a shaker at 4°C overnight. To detect the primary antibodies the following fluorescently-labeled secondary antibodies were used: TRITC-conjugated goat anti-mouse (Jackson ImmunoResearch) and an Alexa Fluor® 488-conjugated goat anti-rabbit (Jackson ImmunoResearch) and incubated in the dark, (1h, RT). Matteks were then rinsed (3x, PBS with the last rinse
containing NucBlue® fixed cell stain, Molecular Probes) and then mounted using Dako fluorescent mounting medium (Product # S3023) and let dry before imaging with a Olympus FV1000 spectral deconvolution confocal system equipped with an Olympus IX81 inverted microscope. The co-localization was visualized and Pearson’s correlation coefficient (PCC) of the entire three-dimensional volume of the cell was determined using ImageJ. The images shown are fluorescence micrographs of representative z-slices within cells.

3.8.8 Decrease in Catalase-Aβ Binding Using Small Molecules

The inhibition of catalase-Aβ interactions by small molecules was determined by a previously described protocol. Briefly, the wells of a 96-well plate were coated with a solution of aggregated Aβ in PBS (2h, 1.3 µM). After removal of solutions containing excess Aβ, all wells were blocked with 1 % BSA/PBS (1 h), washed with PBS and then incubated for 2 h with a human catalase solution (0.20 µM, in 1 % BSA/PBS buffer). After removal of solutions containing excess catalase, solutions of various concentrations of small molecules in 1 % BSA / PBS buffer were incubated in the wells for 12 h. Wells were then washed with 1% BSA/PBS and incubated for 1h with monoclonal mouse anti-catalase IgG (clone 1A1, 2.2 nM in 1 % BSA/PBS). Excess solution was removed; wells were washed with PBS and then incubated for 45 min with an alkaline phosphatase conjugated polyclonal secondary rabbit IgG (anti-mouse IgG, 6.8 nM in 1 % BSA/PBS). The relative amount of secondary IgG bound was quantified by adding a solution containing p-nitrophenyl phosphate (NPP, 2.7 mM, in 0.1 M diethanol amine/ 0.5 mM magnesium chloride, pH 9.8) to each well. The enzymatic hydrolysis reaction of NPP by
alkaline phosphatase was stopped after 45 min. by addition of 0.25 N sodium hydroxide. The concentration of p-nitrophenoxide was then quantified at A$_{405}$ using a UV-Vis microplate reader (Molecular Devices). Each. Each data point from this assay represents the average of at least three independent experiments. Error bars represent standard deviations. Graphs were normalized, plotted and fitted with the sigmoidal curve fitting option in Prism 6.0 (GraphPad Software Inc.).

**Notes About the Chapter**

Chapter three, in part, is a reprint (with co-author permission) of the material as it appears in the publication; Cifelli, J. L., Chung, T. S., Liu, H., Prangkio, P., Mayer, M., and Yang, J. (2016) “Benzothiazole Amphiphiles Ameliorate Amyloid β-Related Cell Toxicity and Oxidative Stress”, *ACS Chem. Neurosci.* 7(6), 682-688. I would like to thank Tim Chung and Jerry Yang for their invaluable contributions to this chapter: Tim Chung synthesized BAM3-EG$_6$ and Jerry Yang directed the research. The author of the dissertation is the primary author of this manuscript.
Utilization of Non-Covalent Electrostatic Interactions to Improve Amyloid-Targeted Binding

4.1 Introduction

The conversion of normally soluble proteins into amyloids, or proteinaceous aggregates with high cross-beta sheet structure, continues to be found associated with a broad range of diseases.\textsuperscript{30,90} Ranging from neurodegenerative disorders like Alzheimer’s disease\textsuperscript{91} (AD) or Parkinson’s disease\textsuperscript{92} (PD), to nonneuropathic localized diseases like type II diabetes\textsuperscript{93}, and even to sexual transmitted diseases like HIV\textsuperscript{94}, with the associated aggregating protein or peptide for each disease being: \(\beta\)-amyloid peptide (A\(\beta\)), \(\alpha\)-synuclein (\(\alpha\)S), islet amyloid polypeptide (IAPP) and semen-derived enhancer of virus infection (SEVI), respectively (Table 4.1). Not surprisingly, the presence of amyloids and their diverse links to pathology has made the study and detection of amyloids an intensely pursued area of study, spanning a variety of scientific disciplines.
Table 4.1. List of amyloids and their associated diseases and precursor proteins.\textsuperscript{95,96}

<table>
<thead>
<tr>
<th>Amyloid</th>
<th>Precursor Protein</th>
<th>Disease/Syndrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Amyloid (Aβ)</td>
<td>Amyloid Precursor Protein (APP)</td>
<td>AD</td>
</tr>
<tr>
<td>α-Synuclein (αS)</td>
<td>α-Synuclein (αS)</td>
<td>PD</td>
</tr>
<tr>
<td>Huntingtin (poly Q expansion)</td>
<td>Huntingtin</td>
<td>HD</td>
</tr>
<tr>
<td>PrPsc (scrapie isoform)</td>
<td>Prion Protein (PrPc)</td>
<td>Spongiform encephalopathies</td>
</tr>
<tr>
<td>ATTR</td>
<td>Wild-type transthyretin (TTR)</td>
<td>Senile systemic amyloidosis</td>
</tr>
<tr>
<td>Tau (hyperphosphorylation)</td>
<td>Tau</td>
<td>Fronto-temporal dementias</td>
</tr>
<tr>
<td>Superoxide dismutase (SOD)</td>
<td>Superoxide dismutase (SOD)</td>
<td>ALS</td>
</tr>
<tr>
<td>Immunoglobulin light chain (or fragments)</td>
<td>Immunoglobulin light chain</td>
<td>Primary systemic amyloidosis</td>
</tr>
<tr>
<td>Fibrinogen α-chain variants</td>
<td>Fibrinogen</td>
<td>Fibrinogen amyloidosis</td>
</tr>
<tr>
<td>γ-Crystallin</td>
<td>Crystallin</td>
<td>Cataract</td>
</tr>
<tr>
<td>Amylin or Islet amyloid polypeptide (IAPP)</td>
<td>Precursor protein proIAPP</td>
<td>Type II diabetes</td>
</tr>
<tr>
<td>Semen-derived enhancer of viral infection (SEVI)</td>
<td>Prostatic acid phosphatase (PAP)</td>
<td>HIV</td>
</tr>
</tbody>
</table>
The detection of amyloids has a long history utilizing Congo red (CR)\textsuperscript{28}, which labels amyloids an “apple-green” birefringence under polarized light, or thioflavin T (ThT)\textsuperscript{29}, which has enhanced fluorescence upon binding to amyloids. While these probes are still most commonly used as detection agents for clinical/pathological analysis, advancements in amyloid-binding agents are still necessary. In considering the improvements for binding to amyloids, the structure and formation of fibrils can be analyzed. Though the overall structure of many amyloids remains unknown, amyloid fibrils are made by the assembly of monomeric units and display many binding sites along the surface (Figure 4.1). For example, previous work by Wu et al.\textsuperscript{97,98} and Lockhart et al.\textsuperscript{99,100} have shown that several ThT and benzothiazole aniline (BTA) analogs bind to Aβ as well as αS at several different binding sites. Important to note is that some of these adjacent binding sites are even close enough for interactions between the two, including FRET (~ 2-10 nm)\textsuperscript{99}.

**Figure 4.1.** Model of multiple binding sites (BS) along an amyloid fibril with the potential for interactions between several different binding sites.
The ability to have multiple close interacting binding sites on amyloids led the Yang lab to synthesize several oligovalent derivatives of the known amyloid binder, BTA in order to test the hypothesis that multivalency could be used to improve binding to amyloids. A multivalent design strategy can transform compounds with low affinity to high avidity, resulting in great improvements in the $K_d$. Indeed, these oligovalent BTA derivatives did show a trend of increasing binding to aggregated amyloids as a function of increasing numbers of oligomers. These oligomeric compounds have potential applications where their large size is beneficial. For example, they have been shown to coat SEVI with improved efficacy than the parent monomer to prevent the interaction of HIV and the cell membrane leading to a decrease in HIV infectivity.\textsuperscript{101}

While a multivalent approach did indeed improve binding to amyloids, this approach also comes with some drawbacks including: 1) the large size of amyloid-binding oligomers, which is not ideal for crossing the BBB, 2) the potential for decreased bioavailability, and 3) the compounds were challenging to make synthetically. In order to combat these potential issues, we devised a new strategy to improve the binding of amyloid-targeting molecules, by utilizing non-covalent interactions, such as, charge-charge secondary interactions to improve binding.

In this chapter, we show that this electrostatic approach improved binding to two different types of amyloids, Aβ as well as αS. A 1:1 mixture of negatively (-) and positively (+) charged BAM1-EG\textsubscript{6} derivatives showed a 10-fold enhancement of binding to aggregated Aβ peptides over the neutral parent compound, BAM1-EG\textsubscript{6}, and a 3-fold enhancement for αS. This enhancement, as expected, was diminished under high salt conditions as electrostatic interactions become screened. Furthermore, this strategy had
the ability to improve binding to aggregated Aβ to the same degree as a covalently attached dimer of BTA, which exhibited an enhancement of binding by 8-fold to aggregated Aβ peptides, compared to the analogous monomeric BTA compound. Additionally, these results demonstrate that functionalizing the end of the hexa(ethylene) glycol tail is in close enough proximity to promote interactions between molecules bound to different binding pockets along an amyloid surface. This proof-of-concept opens up future opportunities to use other interactions, such as, metal chelation between amyloid-binders to improve the binding of small molecules to amyloids, with applications ranging from AD and beyond.

4.2 Multivalent Approach of Binding to β-Amyloid

Work done previously in the Yang lab focused on initially using the concept of multivalency in order to improve binding to amyloids. We hypothesized that oligovalent-BTA derivatives connected by flexible linkers had the potential to hit multiple binding sites along the fibril surface of amyloids, thereby improving binding (Figure 4.2). It is known that multivalent interactions mostly exhibit negative cooperativity due to various factors like strain, if the geometry of the binding site and oligovalent ligand does not match, or loss of entropy by the constraint of the linker. In order to examine if these oligovalent-BTA derivatives did exhibit negative cooperativity we fit the saturation binding curves of the monomer (compound 24) and oligomers (compounds 25-28) to Aβ with a one-site specific binding curve with Hill slope. A Hill coefficient less than 1 signifies negative cooperativity, while a positive Hill slope denotes positive cooperativity. Negative cooperativity was indeed observed for all oligomeric
derivatives with the exception of the tetramer, which was within error of exhibiting no cooperativity (h=1) (Figure 4.3).

**Figure 4.2.** Model of a multivalent strategy utilizing various oligovalent BTA compounds 25-28 to improve binding to amyloid fibrils over the monomeric BTA analog 24.
Figure 4.3. Multivalent interactions show negative cooperativity towards binding to aggregated β-amyloid (1-42) peptides. Binding saturation curves of (A) Compound 24, (B) Compound 25, (C) Compound 26, (D) Compound 27 and (E) Compound 28 to Aβ peptides. (F) Hill coefficients derived from fitting the saturation binding plots with a one-site specific binding curve with Hill slope \( Y = B_{\text{max}} \cdot X^h/(K_d^h + X^h) \).

While negative cooperativity is observed in the case of most multivalent systems, in order to better demonstrate the benefit of multivalent interactions we thus utilized the following enhancement factor, \( \beta \), proposed by Whitesides and co-workers:\(^{102}\)

\[
\beta = \frac{K_{\text{multi}}}{K_{\text{mono}}}
\]

This enhancement factor allows for a clear way to demonstrate the benefit of binding, especially when there is an unknown number of total binding sites (N), as is the case in a heterogeneous mixture of amyloids. Utilizing this enhancement factor, with respect to
increasing multivalency in reference to the monomer, compound 24, we observed an enhancement of 8-115 going from the dimer (25) to pentamer (28) (Table 4.2).

**Table 4.2.** Table comprised of the dissociation constants ($K_d$) upon binding Aβ and enhancement factor ($\beta$) of the oligovalent compounds 25-28 compared to the monomeric compound 24. NA = not applicable

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_d$ to Aggregated Aβ Peptides (nM)</th>
<th>Enhancement Factor ($\beta$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer (24)</td>
<td>235 ± 75</td>
<td>NA</td>
</tr>
<tr>
<td>Dimer (25)</td>
<td>29 ± 4</td>
<td>8</td>
</tr>
<tr>
<td>Trimer (26)</td>
<td>26 ± 6</td>
<td>9</td>
</tr>
<tr>
<td>Tetramer (27)</td>
<td>20 ± 5</td>
<td>12</td>
</tr>
<tr>
<td>Pentamer (28)</td>
<td>2.0 ± 0.4</td>
<td>118</td>
</tr>
</tbody>
</table>

### 4.3 Synthesis of Charged-BAM1-EG₆ Derivatives

While our multivalent approach was successful in improving the binding to aggregated Aβ, this methodology also comes with some disadvantages. Some of these include, the large molecular weight of the oligomers, decreased bioavailability, as well as non-facile synthesis. For application where these characteristics would not be beneficial, we devised a new strategy where we could utilize non-covalent interactions like charge-
charge secondary interactions to improve binding. Here, we synthesized two oppositely charged derivatives of BAM1-EG\textsubscript{6} (Scheme 4.1).\textsuperscript{104} First, a tert-butyl ester functional group was added to BAM1-EG\textsubscript{6} through a \textit{S}N\textsubscript{2} reaction with tert-butyl bromoacetate (29). Deprotection of compound 30 with trifluoroacetic acid (TFA) led to the free carboxylic acid derivative of BAM1-EG\textsubscript{6}, compound 31. Finally, HATU coupling was utilized between taurine (32) or (2-aminoethyl)trimethylammonium chloride (33) to carboxylic acid derivative 31, yielded (-)BAM1-EG\textsubscript{6} and (+)BAM1-EG\textsubscript{6}, respectively (Scheme 4.1).

\begin{center}
\includegraphics[width=\textwidth]{Scheme_4.1.png}
\end{center}

\textbf{Scheme 4.1.} Synthetic scheme of (+)BAM1-EG\textsubscript{6} and (-)BAM1-EG\textsubscript{6} from the neutral parent compound, BAM1-EG\textsubscript{6}.
4.4 Electrostatic Approach: Binding to β-Amyloid

Having a pair of charged BAM1-EG₆ derivatives, which could theoretically interact, helping to promote binding to amyloid (Figure 4.4) we next wanted to test the binding of a 1:1 mixture of the charged derivatives compared to the parent compound.

Figure 4.4. Model of an electrostatic strategy utilizing positively and negatively charged BAM1-EG₆ derivatives to improve binding to amyloid fibrils.
To achieve this objective, we first examined the binding of a 1:1 mixture of the positively and negatively charged BAM1-EG₆ derivatives to aggregated Aβ (1-42) using a fluorescence based binding assay. Here, an improved binding constant (Kₐ) of 20 nM was observed for the 1:1 charged mixture to Aβ compared to 170 nM for the uncharged parent compound, BAM1-EG₆ (Table 4.3). Utilizing the same enhancement factor, β, this translates into a notable enhancement of 10 for the binding of the charged mixture to Aβ (Table 4.3). Impressively, this was an even further improved enhancement for binding to aggregated Aβ than what was observed for the covalently linked BTA-dimer 25 (i.e. an enhancement of 8). In order to test if the improvement in binding was indeed due to the charge-charge interactions between the two charged BAM1-EG₆ derivatives, we next looked at the binding under high salt conditions, which would

**Table 4.3.** Secondary interactions improve binding to aggregated β-amyloid (1-42) peptides. Table comprised of the dissociation constants (Kₐ) upon binding Aβ peptides and enhancement factor (β) of the 1:1 mixture of (+/-) BAM1-EG₆ with or without 500 mM NaCl compared to the neutral compound, BAM1-EG₆. NA = not applicable

<table>
<thead>
<tr>
<th>Compound</th>
<th>Kₐ to Aggregated Aβ Peptides (nM)</th>
<th>Enhancement Factor (β)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAM1-EG₆</td>
<td>170 ± 30</td>
<td>NA</td>
</tr>
<tr>
<td>1:1(+/-) BAM1-EG₆</td>
<td>20 ± 2</td>
<td>10</td>
</tr>
<tr>
<td>High Salt 1:1(+/-) BAM1-EG₆</td>
<td>80 ± 15</td>
<td>2</td>
</tr>
</tbody>
</table>
theoretically screen out any such interactions. Expectantly, under high salt conditions (500 mM NaCl) the observed enhancement of binding significantly diminished, showing that charge-charge interactions could account for most of the improvement of binding of (+/-)BAM1-EG₆ over BAM1-EG₆ observed under low salt conditions (Table 4.3).

Additionally, the cooperativity between the binding of the two charged BAM1 derivatives was estimated by the Hill coefficient, which was derived by fitting the saturation binding plots (Figure 4.5) with a one-site specific binding curve with Hill slope \[ Y = B_{\text{max}} \cdot \frac{X^h}{(K_d^h + X^h)}. \] Here, we found positive cooperativity for the 1:1 (+) BAM1-EG₆:(-) BAM1-EG₆ mixture to Aβ (h = 3.3). This cooperativity was reduced within error from the Hill coefficient of the neutral parent compound under high salt conditions (Figure 4.5).

**Figure 4.5.** Secondary interactions show positive cooperativity towards binding to aggregated β-amyloid (1-42) peptides. Binding saturation curves of (A) BAM1-EG₆, (B) (+/-) BAM1-EG₆, and (C) (+/-) BAM1-EG₆ in high salt conditions (500mM NaCl) to Aβ peptides. Normalized semi-log plots of (D) BAM1-EG₆, (E) (+/-) BAM1-EG₆, and (F) (+/-) BAM1-EG₆ in high salt to Aβ peptides. (G) Representation of graphs (E), (F), and (G) on the same plot to visualize change in K_d and Hill coefficient. (H) Hill coefficients derived from fitting the saturation binding plots with a one-site specific binding curve with Hill slope \[ Y = B_{\text{max}} \cdot \frac{X^h}{(K_d^h + X^h)}. \]
It should be noted that while no improvement to binding was observed for (+) BAM1-EG₆ compared to the parent compound, a slightly improved binding for (-) BAM1-EG₆ alone to Aβ was observed over BAM1-EG₆ (Table 4.4). We hypothesized that this was an outcome of the binding studies being conducted in DI water (pH = 5.5), which was chosen due to proximity to the isoelectric point (pI) (i.e. pH where the peptide has an overall neutral charge) for Aβ (pI~5.3). Indeed, this improvement observed for (-) BAM1-EG₆ was lost when the pH was raised from 5.5 to 7.4.

Table 4.4. pH dependence of (+) and (-) BAM1-EG₆ binding to aggregated β-amyloid (1-42) peptides.

<table>
<thead>
<tr>
<th></th>
<th>pH 5.5</th>
<th>pH 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_d$ to Aggregated Aβ Peptides (nM)</td>
<td>Enhancement Factor ($\beta$)</td>
</tr>
<tr>
<td>BAM1-EG₆</td>
<td>170 ± 30</td>
<td>NA</td>
</tr>
<tr>
<td>(+) BAM1-EG₆</td>
<td>290 ± 50</td>
<td>0.6</td>
</tr>
<tr>
<td>(-) BAM1-EG₆</td>
<td>40 ± 7</td>
<td>4</td>
</tr>
</tbody>
</table>

4.5 Electrostatic Approach: Binding to α-Synuclein

In order to see if this approach could be applied more generically to other amyloids other than aggregated Aβ, we next tested the binding of the charged derivatives to aggregated αS proteins. A significant improvement to binding was once again
observed for the 1:1 mixture of charged derivatives compared to the uncharged parent compound (Table 4.5). Here, a $K_d$ of 400 nM was observed for the mixture compared to 1,100 nM for the uncharged parent compound, BAM1-EG$_6$, with an enhancement factor of 3. Additionally, under high salt conditions this enhancement was completely abolished (Table 4.5).

**Table 4.5.** Secondary interactions improve binding to aggregated $\alpha$-synuclein. Table comprising the dissociation constants ($K_d$) upon binding $\alpha$-synuclein peptides and enhancement factor ($\beta$) of the 1:1 mixture of (+/-) BAM1-EG$_6$ with or without 500 mM NaCl compared to the neutral compound, BAM1-EG$_6$. NA = not applicable

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_d$ to Aggregated $\alpha$S Peptides (nM)</th>
<th>Enhancement Factor ($\beta$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAM1-EG$_6$</td>
<td>1,100 ± 200</td>
<td>NA</td>
</tr>
<tr>
<td>1:1(+/-) BAM1-EG$_6$</td>
<td>400 ± 70</td>
<td>3</td>
</tr>
<tr>
<td>High Salt 1:1(+/-) BAM1-EG$_6$</td>
<td>1,400 ± 60</td>
<td>1</td>
</tr>
</tbody>
</table>

Furthermore, a Hill coefficient of 3.8 showed once again positive cooperativity for the binding event of the charged mixture, which was decreased under high salt conditions to
a similar extent as the neutral BAM1-EG₆ (Figure 4.6). This further reinforces that this electrostatic approach is capable of improving the binding to various amyloids.

**Figure 4.6.** Secondary interactions show positive cooperativity towards binding to aggregated α-synuclein peptides. Binding saturation curves of (A) BAM1-EG₆, (B) (+/-) BAM1-EG₆, and (C) (+/-) BAM1-EG₆ in high salt conditions (500 mM NaCl) to α-synuclein peptides. Normalized semi-log plots of (D) BAM1-EG₆, (E) (+/-) BAM1-EG₆, and (F) (+/-) BAM1-EG₆ in high salt to α-synuclein peptides. (G) Representation of graphs (D), (E), and (F) on the same plot to visualize change in $K_d$ and Hill coefficient. (H) Hill coefficients derived from fitting the saturation binding plots with a one-site specific binding curve with Hill slope $[Y = B_{max} \times \frac{X^h}{(K_d^h + X^h)}]$. 

### 4.6 Difference of Binding Site Density Between Aβ and αS

The difference of the enhancement to binding gained by the 1:1 mixture of charged BAM1 derivatives to either aggregated Aβ (1-42) or αS peptides brought to light an interesting phenomenon. We hypothesized that the observed difference of enhancement (i.e. 10-fold enhancement to aggregated Aβ and only 3-fold enhancement to aggregated αS) was most likely do the difference of amino acid residues (Figure 4.7),
which could led to a difference in binding site density for the BAM1 compounds to bind to along each respective fibril. Thus, if there were less potential binding pockets for BAM1 to bind to, this could lower the probability of two charged species to bind cooperatively, and thus lower the overall enhancement to binding, as was observed for αS compared to Aβ.

Figure 4.7. Amino acid sequences of monomeric human (A) Aβ (1-42) and (B) αS peptides. Acidic residues are depicted in red and basic residues are depicted in blue.

BAM1 to bind to this would lower the probability of two charged species to bind cooperatively and thus, lower the overall enhancement to binding, which was observed for αS compared to Aβ. In order to estimate the binding site density for aggregated Aβ and αS, the same methodology as utilized by Lockhart et al. was employed. Here, the ratio of $K_{d1}$ and $K_{d2}$ was taken to estimate binding site density where: 1) $K_{d1}$, is the
dissociation constant determined when the ligand concentration was fixed and the protein concentration was varied and 2) $K_{d2}$, is the dissociation constant determined when the protein concentration was fixed and the ligand concentration was varied. Using BAM1-EG$_6$ as a representative for BAM/BTA compounds, additional binding studies were performed where the protein concentration was now varied while holding the concentration of the ligand, BAM1-EG$_6$ constant. Here, we determined the $K_{d1}$ of BAM1-EG$_6$ to be 6.3 µM to aggregated Aβ peptides and 50 µM to αS peptides making the binding site densities of 37 and 45 monomers per ligand binding site, respectively (Figure 4.8). The lower average available binding pockets observed for BAM1 to αS over Aβ could, thus, be one possible explanation for the difference in observed enhancement to binding for the charged derivatives to αS compared to Aβ.

Figure 4.8. Difference of binding site density for BAM1-EG$_6$ in aggregated β-amyloid (1-42) peptides and α-synuclein. Binding saturation curves and normalized semi-log plots of BAM1-EG$_6$ to varying concentrations of either Aβ or αS peptides. $K_d$ derived from fitting the saturation binding plots with a one-site specific binding curve with Hill slope $[Y = B_{max} * X^h / (K_d^h + X^h)]$. $K_{d1}$ = varying protein concentration, $K_{d2}$ = varying ligand concentration, $K_{d1}/K_{d2}$ = approximation of binding site density.
4.7 Concluding Remarks

In conclusion, we illustrated that our electrostatic approach improved binding to two different types of amyloids, Aβ as well as αS. A 1:1 mixture of negatively (-) and positively (+) charged BAM1-EG₆ derivatives showed a 10-fold enhancement of binding to Aβ peptides over the neutral parent compound, BAM1, and a 3-fold enhancement for αS. Interestingly, this difference in enhancement of the two amyloids may also be useful for differentiating between amyloids and we hypothesized that this is due to the varying binding site densities and arrangements of the two amyloids. This enhancement of binding was lost under high salt conditions as electrostatic interactions are screened out. Furthermore, this strategy of mixing two charged BAM1 derivatives together had the ability to improve their binding to aggregated Aβ at the same degree as the covalently attached dimer of BTA (25). We additionally demonstrated that functionalizing the end of the hexa(ethylene glycol) tail of BAM/BTA derivatives is close enough for interactions to occur between different binding pockets along amyloid fibrils. This opens up future opportunities to use other interactions, like metal chelation between amyloid-binders to improve binding to amyloids, with applications ranging from AD and PD, to the growing field of amyloid-diseases.
4.8 Materials and Methods

4.8.1 Materials

Synthetic Aβ(1-42) peptide was purchased from PL Lab (Port Moody, Canada). All other chemical reagents were purchased from either Sigma-Aldrich or Fisher and used as is unless otherwise stated.

4.8.2 Compounds

General:

BAM1-EG₆ was synthesized as previously mentioned in Chapter 2.¹⁰⁴ Oligovalent BTA molecules (24-28) were synthesized as previously reported.¹⁰¹ NMR spectra were obtained on either a JEOL 500 MHz spectrometer or a Varian 400 MHz spectrometer as noted. Abbreviations for spectra splitting include: singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), broad (b). Chemical shifts are reported in ppm relative to residual solvent.

Synthesis of tert-butyl protected BAM1-EG₆ (30):

\[
\text{BAM1-EG₆} + \text{Br-}O\text{O} \rightarrow \text{NalH} \rightarrow \text{THF, Overnight} 61\% \rightarrow \text{BAM1-EG₆} \]

\[
\text{29} \quad \text{30}
\]
BAM1-EG$_6$ (1 equiv.) was dissolved in dry THF under N$_2$. To this solution sodium hydride (29) (5 equiv.) was added under N$_2$ and let stir for 1 h. Next, tert-butyl bromoacetate (4 equiv.) was slowly added and the solution was let stir overnight at room temperature. The NaBr salts were then filtered off, rinsed with excess THF and column chromatography (100% EtOAc) afforded the protected carboxylic acid BAM1-EG$_6$ derivative 30 as a yellow oil with 61% yield. ESI-MS: (positive ion mode) [M + H]$^+$ = 605.16, [M + Na]$^+$ = 627.16. $^1$H NMR (500 MHz, CDCl$_3$): 7.92 (1H), 7.85 (2H), 7.83(1H), 7.40 (1H), 7.29 (1H), 6.70 (2H), 4.14 (2H), 3.75-3.63 (22 H), 3.38 (2H), 1.46 (9H)

**Synthesis of carboxylic acid derivative of BAM1-EG$_6$ (31):**

![Synthesis of carboxylic acid derivative of BAM1-EG$_6$ (31)](image)

The deprotection of compound 30 was performed with TFA/DCM (30:70). The mixture was let stir for 1 h. For a workup, the solution was diluted in DCM and then extracted with 0.1 M HCl (2x), water, followed by a brine wash. The organic layer was dried over Na$_2$SO$_4$, filtered and concentrated. Column chromatography (10-20% MeOH/DCM) afforded the carboxylic acid derivative (31) of BAM1-EG$_6$ as a yellow-red oil (93% yield). ESI-MS: (positive ion mode): [M + H]$^+$ = 549.21. ESI-MS: (negative ion mode): [M-H] = 547.30. $^1$H NMR (500 MHz, CDCl$_3$): 7.95 (1H, d), 7.87 (2H,d), 7.83(1H,d), 7.40 (1H,t), 7.29 (1H,t), 6.67 (2H,d), 4.02 (2H, s), 3.75-3.62 (22 H, m), 3.34
(2H). $^{13}$C NMR (300 MHz, CDCl$_3$): 172.4, 170.0, 151.0, 150.9, 132.8, 129.9(2C), 125.4, 123.6, 121.8, 121.4, 121.0, 113.9 (2C), 71.6-68.9, 43.9, 29.9

**General HATU coupling reactions:**

Compound 31 (1 equiv.) dissolved in dry DMF under N$_2$. HATU (1.1 equiv.) was then added to the solution and let stir for 10 min. The coupling agent (2-aminoethanesulfonic acid (32) or (2-aminoethyl)trimethylammonium chloride) (33) was then added (2 equiv.) followed by DIPEA (3 equiv.) and let stir overnight, at RT. DMF was then removed and purification by normal phase and reverse phase column chromatography (75% MeOH/H$_2$O) afforded [(-) BAM1-EG$_6$] as a yellow oil (20 % yield) or [(+) BAM1-EG$_6$] as a yellow solid (33 % yield).

[Diagram of the reaction]

**(-) BAM1-EG$_6$ diisopropylethylamine salt:** $^1$H NMR (500 MHz, MeOD): 7.86-7.82 (m, 4H), 7.43 (t, 1H), 7.31 (t, 1H), 6.74 (d, 2H) 3.95 (2H), 3.62-3.35 (m, 24H), 3.29(2H), 3.22 (2H), 2.98 (2H), 2.87 (4H, DIPEA), 1.15(18H, DIPEA). $^{13}$C NMR (500 MHz, D$_2$O): 172.1, 169.7, 152.9, 151.1, 133.4, 128.7 (2C), 126.4, 124.5, 121.4, 121.2, 120.4, 112.3 (2C), 71.9, 70.13, 69.6-69.2,
68.7, 62.4, 54.2, 49.4, 42.4, 34.5 17.6, 16.1, 12.0. HR/MS: Calculated for [C\textsubscript{29}H\textsubscript{40}N\textsubscript{3}O\textsubscript{10}S\textsubscript{2}] Theoretical: 654.2155, Mass Found: 654.2163

\[ (+) \text{BAM1-EG}_6 \]: \textsuperscript{1}H NMR (500 MHz, D\textsubscript{2}O):
7.91-7.83 (m, 4H), 7.43 (t, 1H), 7.32 (t 1H), 6.77 (d, 2H) 4.02 (2H), 3.71-3.38 (m, 24H), 3.51(2H), 3.38 (2H), 3.18 (9H). \textsuperscript{13}C NMR (500 MHz, D\textsubscript{2}O): 171.9, 169.7, 153.6, 151.9, 133.8, 128.7, 128.6, 126.1, 124.3, 121.3, 121.1, 120.8, 112.0 (2C), 70.4, 69.8-69.6, 64.3, 52.7(3C), 42.5, 33.0. HR/MS: Calculated for [C\textsubscript{32}H\textsubscript{49}N\textsubscript{4}O\textsubscript{7}S\textsuperscript{+}] Theoretical: 633.3322, Mass Found: 633.3321

\textbf{4.8.3 Preparation of Aggregated A\textbeta(1-42)}

Synthetic A\textbeta(1-42) peptide was purchased from PL Lab (Port Moody, Canada). Aggregated A\textbeta(1-42) was prepared as previously described.\textsuperscript{89} Briefly, A\textbeta(1-42) was initially solubilized in 100% 1,1,1,3,3,3-hexafluoro-2-propanal(HFIP) to 1 mM concentration at RT for 24 h with shaking. The solution was sonicated and vortexed before it was diluted in cold nanopure water (2:1 H\textsubscript{2}O:HFIP). Aliquoted fractions were lyophilized for 2 days, followed by storage at -80 °C until use. Solutions of aggregated A\textbeta were obtained by dissolving A\textbeta in sterile PBS or DI water to a concentration of 100 \mu M and incubated at 37 °C for 3 days before use.
4.8.4 Purification and Preparation of Aggregated α-Synuclein Peptides.

Recombinant α-synuclein and frozen glycerol stock (pET5a αSynWT)\textsuperscript{106} were a generous gift from Dr. Eric Luth (Selkoe Lab, Harvard Institutes of Medicine) and Professor Jerry Yang (UCSD). Purification from BL21 E. coli is described briefly below. A starter culture of transformed bacteria (pET5a αSynWT) was set up (LB broth with ampicillin [100 ug/mL final]) and let grown overnight. The next day, 25 mL of the started culture was added to 500 mL of LB + Amp. Protein expression was initiated by addition of IPTG [0.119 g/500 mL] when OD\textsubscript{600} was between 0.5-1. After 4 h flasks were pelleted (5,000 x g for 20 min) and supernatant was removed. Pellets could be stored at -80 °C at this point before continuing with purification. Pellets were then re-suspended in 20 mM Tris, 25 mM NaCl, 1 mM EDTA, pH 8.0 transferred into a flask, boiled for 1-2 minutes in a microwave to deactivate proteases quickly, followed by boiling in a water bath (90 °C) for 30 minutes. Samples were then centrifuged (20,000 x g for 30 min) and the supernatant was collected and filtered through a 0.2 μm filter. α-synuclein was then further purified by running through 2x 5ml HiTrapQ HP anion exchange columns followed by SEC (Superdex 200). Pure α-synuclein fractions, as determined by Coomassie and western blot, were lyophilized and stored at -80 °C until use. Solutions of aggregated α-synuclein were obtained by dissolving α-synuclein in sterile PBS or water to a concentration of 100 μM and incubated at 37 °C for 7 days before use.
4.8.5 Measurement of the Binding Affinity of BAMs to Aggregated Aβ (1-42) and α-Synuclein Peptides

Binding of compounds to aggregated Aβ (1-42) was measured according to a previously described assay. Briefly, 200 µL of various concentrations of BTA/BAM compounds in PBS, DI H₂O or 500 mM NaCl/ DI H₂O (high salt conditions) were incubated in the absence or presence of 10 µg of pre-aggregated amyloid (total volume 220 µL). Solutions were allowed to equilibrate overnight at room temperature. Samples were then centrifuged at 16,000 x g for 20 min at 4°C. The supernatants were removed and the pellet was re-suspended in 220 µL of fresh PBS, DI H₂O or 500 mM NaCl respectively. Fluorescence of the bound molecule was determined using a spectrofluorometer (Photon Technology International, Inc., Birmingham, NJ). Each experiment was repeated at least three times and error bars denote standard deviation from the mean. Graphs were fit using the following one-site specific binding algorithm with Hill slope to determine $K_d$: $Y = B_{max} \times X^h / (K_d^h + X^h)$, where X is the concentration of small molecule, Y is the specific binding intensity, $B_{max}$ is the apparent maximal observable fluorescence upon binding to Aβ and h is the Hill slope. Data was processed using Origin 7.0 (MicroCal Software, Inc., Northampton, MA).

4.8.6 Measurement of the Binding Site Density of Aggregated Aβ (1-42) and α-Synuclein Peptides

Binding site density for Aβ or αS was approximated by $K_{d1}/K_{d2}$, where $K_{d1} = $ dissociation constant when varying the protein concentration and $K_{d2} = $ dissociation constant when varying the ligand concentration. Both followed the same general protocol as mentioned above (i.e. for $K_{d2}$). The only difference in determining $K_{d1}$ was
that the ligand was kept constant (500 nM), and the protein concentration was varied from 0-100 µM. $K_d$ in each case was derived from fitting the saturation binding plots with a one-site specific binding curve with Hill slope $[Y = B_{max} \cdot \frac{X^h}{(K_d^h + X^h)}]$.

**Notes About the Chapter:**

Chapter four, in part, is being prepared for publication (with co-author permission): **Cifelli, J. L., Capule, C. C., & Yang, J.** “Utilization of non-covalent interactions to improve amyloid-targeted binding”, *Manuscript in Preparation*. I would like to thank Christina Capule and Jerry Yang for their invaluable contributions to this chapter: Christina Capule synthesized and characterized all oligovalent BTA compounds and Jerry Yang directed the research and prepared the WT α-synuclein *E. coli* stocks. Additionally, Dr. Eric Luth is acknowledged for the generous gift of the plasmids used for the *E. coli* expression of WT α-synuclein. The author of the dissertation is the primary author of this manuscript.
5.1 Introduction

Dendritic spines are specialized protrusions responsible for receiving excitatory synaptic inputs, providing an important function in communication between neurons. The morphology of dendritic spines and their overall density correlates with synaptic function and are strongly implicated in memory and learning. Consequently, alteration or misregulation of dendritic spines can influence synaptic function playing a major role in various neurological and psychiatric disorders such as autism, fragile X syndrome, Parkinson’s disease (PD) and Alzheimer’s disease (AD). For example, in AD there is mounting evidence suggesting deficits begin with alterations of hippocampal synaptic function caused by β-amyloid (Aβ) peptide prior to neuronal loss. Therefore treatment strategies that target the initial synaptic loss, rather than
late stage disease intervention, may provide a better prognosis for the treatment of AD. Furthermore, since most cognitive disorders elicit abnormalities in the form and function of dendritic spines, it would be desirable to target them directly using a small molecule to alter or alleviate these spine changes.

We previously reported the design, synthesis, and evaluation of two oligo(ethylene glycol) derivatives of benzothiazole aniline (BTA), BTA-EG\(_6\) and BTA-EG\(_4\), which interestingly, showed the capability to improve memory and learning in cognitive performance tests in both wild-type mice and in a mouse model for AD.\(^{53,55}\) This \emph{in vivo} activity of BTA-EG\(_{4,6}\) was also accompanied by a phenotypic increase in dendritic spine density.\(^{53,55}\) Due to the scarcity of small molecules known to increase dendritic spine density, this rare feature of benzothiazole amphiphiles is of particular interest and could be utilized as a tool to help study the relationship between spines and cognitive function.

Benzothiazole amphiphiles (BAMs) 1-3 have been shown to exhibit substantially less toxicity compared to the parent compound, BTA-EG\(_6\) (Chapter 2). They could therefore be utilized to further study the spinogenic properties of this class of compounds without fear of toxic effects precluding the analysis. In this chapter we show that these new BAM agents are capable of promoting an increase in dendritic spine density and can serve as either a pre- or co-treatment to negate the overall net spine loss induced by the presence of aggregated A\(\beta\) peptides. Additionally, these compounds are capable of directly inhibiting aggregated A\(\beta\) from inducing spine loss mediated by a Cofilin-dependent pathway. This spinogenic activity was dose-dependent in primary neurons, and, using BAM1-EG\(_6\) (1) as a representative example, we demonstrate that the increase
in spine density is reversible by removal of the compound from the cellular environment. Time-dependent imaging studies of primary neurons treated with BAM1-EG6 reveal that these benzothiazoles can increase spine density through promoting the formation of new spines. Signal transduction studies support that these molecules promote spine formation by involving the activation of the Ras-ERK1/2 pathway and do not directly affect overall F/G actin ratios or ARP2 expression levels in primary neurons. Taken together, these results demonstrate that these BAM agents represent new potential tools to study the relationship between dendritic spines and cognitive behavior and may open up a new avenue to explore the use of spinogenic agents for the treatment of neurodegenerative and other spine-related cognitive disorders.

**5.2 Effects of BAMs 1-3 on Dendritic Spine Density**

BTA-EG6 was first used to assess increases in spine density in primary hippocampal neurons as a control due to its previously published ability to increase spine density.\(^{65}\) In order to visualize all spines, we used a virally transfected membrane-targeting palGFP that is known to reliably fluorescently label dendritic spines.\(^{122-124}\) Moreover, expression was limited to less than 18 h to minimize any artifacts from viral transduction. After confirming an observed increase in dendritic spine density in BTA-EG6-treated neurons over treatment with vehicle control (Figure 5.1), neurons were next treated with 1 or 5 \(\mu\)M concentrations of benzothiazoles 1-3. All new compounds 1-3 showed a dose-dependent increase in spine density after a 24 h exposure (Figure 5.1). In
Figure 5.1. Spinogenic properties of BTA-EG₆ and BAMs 1–3 observed in rat primary hippocampal neurons. (A) Representative spine segments (23 microns) for BTA-EG₆ and BAMs 1–3 compared with control (0.1% DMSO). (B) Quantitative representation of spine number per micron for all compounds compared with control. Data are expressed as mean values ± SEM, n ≥ 54, *p ≤ 0.001; **p ≤ 0.0001 as determined by unpaired t test compared with control.
addition, compounds 1-3 were able to produce a statistically significant increase in net spine density at a lower concentration compared to BTA-EG₆, suggesting the structural differences (and possibly the decreased hydrophobic character) of 1-3 compared to BTA-EG₆ results in overall increased spinogenic activity. There was no observed change in spine density when the cells were treated with the vehicle control (0.1% DMSO).¹²⁵

Using BAM1-EG₆ as a representative compound for this class of benzothiazoles, we next examined the effects of BAM agents on the density of neuronal puncta containing pre- and post-synaptic markers. We dosed neurons for 24 h with and without BAM1-EG₆ (5 µM) and then examined the colocalization of PSD95 (post-synaptic marker) and Synapsin (pre-synaptic marker). In addition to the increase in dendritic spine density (Figure 5.1), we also observed an increase in the density of colocalized PSD95-Synapsin puncta in neurons dosed with BAM1-EG₆ over the control (Figure 5.2).

The cumulative distribution of spine length and width was also measured for neurons dosed with BAM1-EG₆. No observable difference in average spine length or width was found compared to cells treated with a vehicle control (Figure 5.3a,b).

To evaluate the maximum effect of the BAM agents on spine density increase, we dosed primary neurons for 24 h in the presence of 1-25 µM BAM1-EG₆. The maximum observed increase in spine density was ~20%, occurring with a dose of 5 µM with no further increase at higher concentrations (Figure 5.3c).

A time course of spinogenic activity was also examined in three separate experiments: In the first experiment, BAM1-EG₆ was exposed to primary neurons at a constant concentration (5 µM) in the culture medium for up to 72 h. At various time points, we fixed cells and measured spine density (as estimated by spine number per µm).
**Figure 5.2.** Analysis of the localization of synaptic proteins of neurons exposed to BAM1-EG₆. (A) Representative images of dendrites (GFP), PSD95 (post-synaptic marker) and Synapsin (pre-synaptic marker) from neurons dosed with control (0.1% DMSO) or BAM1-EG₆. Scale bar = 20 microns (B) Representative images of spine segments (20 microns) for cells treated with BAM1-EG₆ compared to control (0.1% DMSO) labeled with pal-GFP (green), PSD95 (red) and Synapsin (blue). (C) Quantitative representation of spine number per micron for BAM1-EG₆ compared to control. (D) Quantitative representation of amount of colocalized PSD95 and Synapsin puncta per micron for neurons treated with BAM1-EG₆ compared to control. Data are expressed as mean values ± SEM, n ≥ 48, **p < 0.0001 as determined by unpaired t-test compared to control.

This experiment revealed a trend of increasing average spine density between 2 and 24 hours, with statistically significant spine density increase reached after a 12 h exposure of the neurons to BAM1-EG₆ (Figure 5.3d). The spinogenic activity of the benzothiazoles reached equilibrium within 24 h, and this maximal increase of ~20% in spine density levels (compared to treatment with vehicle) persisted for up to 3 days upon exposure to a constant concentration of the BAM1-EG₆.
Figure 5.3. Examination of the spinogenic properties of BAM1-EG6 observed in rat primary hippocampal neurons. Cumulative distribution of spine length (A) or width (B) of control cells versus cells treated with compound BAM1-EG6 (1 µM). (C) Concentration-dependent effects of neurons dosed for 24 h with 1-25 µM of BAM1-EG6 on spine density. (D) Kinetics of spine density increase in cells exposed to BAM1-EG6 compared to vehicle control (0.1% DMSO). Neurons were dosed and then fixed at 2, 4, 12, 24, 48 and 72 h. (E) Effects of removal of BAM1-EG6 on dendritic spine number after treatment of cells for 24 h. After 24 h, BAM1-EG6 was washed out (w.o.) and spine changes were monitored for an additional 24 and 48 h (48 and 72 h total time). The dendritic spine density 24 h after removal of BAM1-EG6 is indistinguishable from control cells. (F) Effect on spine density increases of adding additional doses of BAM1-EG6 every 24 h over a total incubation time of 72 hours. Neurons were dosed at 24 h (1x), 48 h (2x) and 72 h (3x) with no observable additional increase of dendritic spine density compared to the 1x dose. Data are expressed as mean values ± SEM, n ≥ 54. *p ≤ 0.01, **p ≤ 0.0001, n.s. = not significant, as determined by unpaired t-test compared to control. Arrows denote time points when aliquots of cells were fixed and analyzed.

In a second experiment, we evaluated whether the spine density increases induced by the benzothiazoles persisted after the compounds have been removed from the culture
medium. Primary hippocampal neurons were dosed for 24 h with 5 µM BAM1-EG₆, resulting in the expected ~20% increase in spine density levels compared to treatment with vehicle alone (0.1% DMSO). The cells were then rinsed and the culture medium was replaced with compound-free medium, and we monitored the average spine density on the cells over an additional 48 h. The initial spine increase after 24 h exposure to BAM1-EG₆ did not persist once we removed the compound, with the density of spines returning back to normal levels (i.e., to the spine density observed in control cells) within 24 h of removal of BAM1-EG₆ (Figure 5.3e).

In a third experiment, we monitored the effect on spine density in primary neurons by adding fresh aliquots of BAM1-EG₆ every 24 h to the culture medium over a 72 h period. We incubated primary neurons initially in culture medium containing a final concentration of 5 µM BAM1-EG₆ (1x dose). At 24 hours (2x dose) and 48 hours (3x dose) of incubation, an additional 2 µL of a 5 mM BAM1-EG₆ DMSO stock (final concentration 5 µM, 0.1% DMSO) was added to the culture medium. We found that further addition of BAM1-EG₆ every 24 h (which putatively increased the final concentration of compound after every addition) did not result in further increases in dendritic spine density above the original observed increase of ~20% after 24 h exposure of 5 mM BAM1-EG₆ (Figure 5.3f).

5.3 BAMs 1-3 Promote the Formation of New Dendritic Spines

An observed increase in dendritic spine density by benzothiazoles 1-3 could arise either by promoting the formation of new spines or by increasing the stability of previously formed dendritic spines. In order to help elucidate which mechanistic pathway
BAMs promotes dendritic spine density alterations, we monitored the changes in spine number in real time by periodically capturing live confocal images of primary neurons over a 4 h time period. To account for baseline changes in spine dynamics, neurons were monitored 1 h prior to dosing. Live imaging then continued up to 3 h after dosing with either compound 1 (5 µM) or the vehicle control to gain insight into the spine changes induced by compound 1. We observed that dosing with compound 1 led to a statistical increase in new spines compared to the control 60 min after dosing, while no significant change in spine loss was observed over the same time period (Figure 5.4).

**Figure 5.4.** Live cell imaging showing the increase in formation of new spines upon dosing with BAM1-EG₆ (compound 1) (A) Representative segments (20 microns) of live cells before (-time) and after dosing (+time) with BAM1-EG₆ (5 µM) or vehicle control (0.1% DMSO). * denotes new spines. (B) Quantitative representation of the total dendritic spines gained or lost per 20 micron segments for either BAM1-EG₆ or vehicle control. N= 6, *p < 0.01 compared to control at same time point by unpaired t-test.
5.4 BAMs 1-3 Promote Ras Signaling

Ras and RasGRF1, a guanine nucleotide exchange factor involved in Ras signaling, are important intermediates in the regulation of spine density.\textsuperscript{126} Previous work has reported that BTA-EG\textsubscript{4} could promote spine density increases \textit{in vitro} in murine primary hippocampal neurons and \textit{in vivo} in the hippocampus of wt mice and a 3x tg mouse model for AD.\textsuperscript{53,55} The increase in spine density in neurons by BTA-EG\textsubscript{4} correlated with an increase in expression of RasGRF1 compared to control cells. In order

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure5.5.png}
\caption{Analysis of protein expression and activation of Ras signaling in primary neurons exposed to benzothiazole agents. Relative expression levels of A) RasGRF1 and B) active Ras in primary neurons upon dosing with 5 µM of BTA-EG\textsubscript{6} and BAMs 1-3. Data are expressed as mean values ± SEM, n= 3 or more for each concentration. n.s. = not significant compared to control *p ≤ 0.05 compared to control **p ≤ 0.01 compared to control as determined by unpaired t test.}
\end{figure}
to test whether the spinogenic activity induced by compounds 1-3 and BTA-EG₆ operated along a similar mechanistic path as BTA-EG₄, we analyzed the effects of these compounds on the expression level of both RasGRF1 and active Ras in rat dissociated primary neurons. When we exposed primary neurons to 5 µM concentrations of compounds 1-3 or BTA-EG₆, we observed about a 2-fold increase in RasGRF1 expression levels (Figure 5.5a). Additionally, about a ~1.5 fold increase in active Ras/total Ras was also observed for compounds 1-3 or BTA-EG₆ compared to control cells (Figure 5.5b).

5.5 BAMs 1-3 Do Not Directly Affect Overall Cellular Cytoskeletal Actin Dynamics or Actin-Related Protein-2 (ARP2)

Due to the rapid changes in dendritic spine dynamics as seen in both live-imaging and fixed time points we next sought to examine the effects of BAMs on cytoskeletal dynamics. The actin-related protein -2/3 (ARP2/3) complex plays a central role in the regulation of actin organization. Therefore we first inspected whether our BAM agents affected ARP2 expression levels in primary neurons. We did not find a statistical difference in ARP2 expression between the control cells and cells treated with 5 µM of BAMs 1-3 or BTA-EG₆ (Figure 5.6a).

We next examined whether the BAM agents had an effect on the overall actin dynamics within neurons. We utilized a G-actin/F-actin assay kit, which enabled us to probe both F- and G-actin levels from primary lysates that had been dosed with 5 µM of BAMs 1-3 and BTA-EG₆ for 24 h. We did not observe a significant difference in the ratio
of F-actin over total actin levels in neurons treated with any of the compounds compared to control neurons (Figure 5.6b).

![Diagram of cytoskeletal proteins analysis](image)

**Figure 5.6.** Analysis of cytoskeletal proteins in primary neurons exposed to benzothiazole agents. (A) Relative expression levels of ARP2 in primary neurons upon dosing with 5 µM of BTA-EG6 and BAMs 1-3. (B) Ratio of F-actin (filamentous actin) over total actin (F- plus G-actin (globular actin)) in primary neurons treated with compound (5 µM) or vehicle control. Data are expressed as mean values ± SEM, n= 3 or more for each concentration. n.s. = not significant compared to control.

### 5.6 BAMs 1-3 Inhibit Aβ-induced Cofilin Activation and Negate Aβ-Induced Net Spine Loss

Since BAMs 1-3 were able to increase dendritic spine density in primary hippocampal neurons (Figure 5.1), we examined whether co- or pre-treatment of neurons with these compounds could negate net spine loss in neurons exposed to aggregated β-
amyloid (Aβ 1-42), the toxic peptide cleavage product of the amyloid precursor protein (APP) associated with AD.

For co-treatment, we treated primary neurons for 3 days with medium containing aggregated Aβ (1-42) with and without the presence of BAMs 1-3 or BTA-EG₆. We observed around a 20% decrease in spine density in primary neurons that were incubated in the presence of 1 µM aggregated Aβ (1-42) alone for 3 days (Figure 5.7). In contrast, when we treated primary neurons with 1 µM Aβ (1-42) and 1 or 5 µM concentrations of BAMs 1-3 or BTA-EG₆, we observed a net increase in dendritic spine density by ~ 20% compared to control (Figure 5.7). Furthermore, the observed net increase in spine density in cells treated simultaneously with Aβ (1-42) and BAMs 1-3 or BTA-EG₆ were ~ 50% higher than in cells treated with Aβ (1-42) alone.

For the pre-treatment regimen, we pre-treated primary neurons for 1 day in medium containing BAMs 1-3 or BTA-EG₆ (or vehicle control). This pre-treatment presumably increased the dendritic spine density in cells exposed to compounds (Figure 5.1). Next, we replaced the medium and added in 1 µM Aβ (1-42) for 3 additional days. When we pre-treated primary neurons with 5 µM concentrations of BAMs 1-3 or BTA-EG₆, we observed no statistically difference in overall spine number compared to neurons that were not exposed to compounds or Aβ peptide (Figure 5.8). These results demonstrate that pre-treatment with BTA-EG₆ and benzothiazoles 1-3 effectively protects cells from an overall net decrease in dendritic spine density induced by aggregated Aβ (1-42) peptides.
Figure 5.7. Co-treatment with compounds counteracts Aβ associated net spine loss in rat primary hippocampal neurons. Representative spine segments (23 microns) of primary neurons dosed with Aβ, or Aβ plus BTA-EG₆ or BAMs 1-3 compared to control (0.1% DMSO) and quantitative representation of spine number per micron for all dosing experiments compared to control. Data are expressed as mean values ± SEM, n = 42. ##p ≤ 0.01, as determined by unpaired t-test compared to control. *p ≤ 0.001, **p ≤ 0.0001 as determined by unpaired t-test compared to cells treated with Aβ alone.
Figure 5.8. Pre-treatment with compounds counteract Aβ associated net spine loss in rat primary hippocampal neurons. Representative spine segments (23 microns) and quantitative representation of spine number per micron for dosing of primary neurons pre-treated with 0 (control) or 5 µM BTA-EG₆ or BAMs 1-3 for 24 h followed by addition of aggregated Aβ. Data are expressed as mean values ± SEM, n = 42. n.s. = not significant, **p ≤ 0.0001 as determined by unpaired t-test compared to control.
We next wanted to examine whether the BAM agents could specifically inhibit a mechanistic pathway for Aβ-induced spine loss. Cofilin promotes actin depolymerization and its activation is promoted through dephosphorylation of p-Cofilin. Shatz and coworkers recently reported that Aβ aggregates induce spine loss through a pathway that involves activation of Cofilin-dependent depolymerization of actin upon binding of Aβ.

![Figure 5.9](image)

**Figure 5.9.** Compounds counteract Aβ associated net spine loss in rat primary hippocampal neurons by blocking Aβ-induced activation of Cofilin. Relative expression levels of p-Cofilin/total Cofilin in primary neurons treated with Aβ alone or Aβ plus BTA-EG₆ or BAMs 1-3 compared to control expression levels. Data are expressed as mean values ± SEM, n = 3. #p ≤ 0.01, n.s. = not significant, *p ≤ 0.001, as determined by unpaired t-test compared to control.
aggregates to the PirB receptor. We observed a statistical decrease in p-Cofilin/total Cofilin levels in primary neurons that had been dosed with aggregated Aβ alone (Figure 9), which agrees well with the previous report by Shatz. However, when neurons had been dosed with BAMs 1-3 or BTA-EG₆ together with aggregated Aβ, the ratio of p-Cofilin/total Cofilin was the same as cells that were not exposed to aggregated Aβ peptides (Figure 5.9). This results supports that the BAM agents are able to block the Aβ-induced activation of Cofilin, thereby blocking a specific mechanism for Aβ-induced spine loss.

### 5.7 Discussion

Many cognitive disorders are accompanied with loss of dendritic spines, yet there are few examples of molecules that promote the formation of new dendritic spines. The capability to promote spine density through the administration of a pharmacological drug could lead to a better understanding of the underlying circuitry affecting cognitive behavior, and ultimately to novel approaches for treatment of cognitive disorders.

While reducing toxicity of benzothiazole agents is an important step towards improving their biocompatibility (Chapter 2), it is also important to assess whether the new BAMs 1-3 retain the potential beneficial biological activity of the parent compound. The capability of BTA-EG₄ to promote an increase in dendritic spine density is a distinctive and extremely rare property for any small molecule reported to date. The results demonstrate that the new benzothiazoles 1-3 are indeed capable of promoting dose-dependent increases in dendritic spine density in primary hippocampal neurons.
(Figure 5.1), with maximal spine density increases of ~20% after 24 hours of exposure to cells.

The activity of BAM1-EG₆ was further examined as a representative for this class of compounds to further evaluate the spinogenic effects of this class of benzothiazoles. Analysis of the colocalization of both pre- and post-synaptic markers, Synapsin and PSD95, of neurons exposed to BAM1-EG₆ showed both an increase in the spines density and an increase in the density of the number of colocalized PSD95/Synapsin puncta (Figure 5.2). This result supports that the promotion of new dendritic spines may correlate with an increase in the number of synapses formed in neurons exposed to the BAM agents. Furthermore, the analysis of the cumulative distribution of spine width and length of cells exposed to BAM1-EG₆ showed no difference compared to control cells (Figure 5.3a,b), demonstrating that the increase in spine density by BAM agents does not affect the overall distribution of spine morphology in the cells.¹²⁹

Temporal studies showed that the spine density increased within 12 h in neurons and stably persisted for 72 h in the presence of BAM1-EG₆. However, the spine density increase in cells exposed to BAM1-EG₆ returned to basal levels (i.e., levels comparable to control neurons) within 24 h of removal of this compound from the medium (Figure 5.3d,e). This capability of BAM agents to reversibly control the magnitude of spine density changes in primary neurons may be very attractive as a tool for further studies on the relationship between dendritic spines and other parameters related to neural circuitry.

Live cell imaging and biochemical studies support that these benzothiazoles promote the formation of new dendritic spines in neurons (Figure 5.4) through a mechanism that is accompanied by an increase in both RasGRF1 expression and active
Ras levels (Figure 5.5a,b). Previous studies showed that shRNA knockdown of RasGRF1 in primary neurons completely blocked the effect of BTA-EG4 on spine density increases\textsuperscript{55}, further supporting the involvement of Ras signaling in the spinogenic activity of the BAM agents.

The kinetic data gained from the live cell imaging and from imaging of fixed cells after various short exposures to the BAM agents indicate that changes in spine dynamics begin within the first couple hours, with significant net increases in dendritic spine density observed by 12 h (Figure 5.4 and 5.3d). The surprising speed at which we observe changes in spine dynamics suggests that cytoskeletal reorganization\textsuperscript{130,131} plays an important role in the mechanism of action of the BAM agents. While we did not find a statistical difference in either the global F-/G-actin ratios or ARP2 expression levels in neurons exposed to the BAM agents (Figure 5.6a,b), these results do not rule out the possibility that the BAM agents act on more localized cytoskeletal machinery that affects dendritic spine dynamics.

Finally, we showed that co- and pre-treatment of neurons with BAMs 1-3 and BTA-EG\textsubscript{6} were able negate the net dendritic spine loss observed in the presence of aggregated A\textsubscript{β} (Figures 5.7 and 5.8). Importantly, we found that the BAM agents could block A\textsubscript{β}-induced activation of Cofilin (Figure 5.9), which has been implicated as a specific mechanism for A\textsubscript{β}-induced synaptic dysfunction in AD through the binding of aggregated A\textsubscript{β} to the PirB receptor.\textsuperscript{88} Since BTA-EG\textsubscript{6} was previously shown to bind to aggregated A\textsubscript{β} and act as a general inhibitor of protein-amyloid interactions\textsuperscript{51,52}, we hypothesize that the BAM agents inhibit A\textsubscript{β}-induced spine loss by binding to A\textsubscript{β} and inhibiting its interaction with the PirB receptor\textsuperscript{88} (and, thus, leading to the downstream
inhibition of Cofilin activation). Collectively, these results support a dual mode of action of BTA-EG₆ and the BAM agents on the spine density of primary neurons exposed to aggregated Aβ: 1) these compounds are capable of directly inhibiting Aβ from inducing spine loss through a Cofilin-dependent pathway, and 2) these compounds can act on an Aβ-independent pathway to promote the formation of dendritic spines. These results, therefore, demonstrate that BAMs 1-3 have potential to counteract one of the earliest observed pathological events associated with AD.¹⁴,¹²¹

5.8 Concluding Remarks

In conclusion, we used a novel set of benzothiazole amphiphiles 1-3 with improved biocompatibility compared to the previously reported BTA-EGₓ compounds⁵²,⁵³,⁵⁵ to analyze the details of spinogenic properties of this class of compounds. These new compounds were capable of 1) promoting dose-dependent increases in dendritic spine density, 2) temporally and reversibly controlling elevated spine levels, and 3) protecting against Aβ-induced dendritic spine loss. Current efforts are focused on identification of the cellular target for the BAM agents and additional mechanistic details leading to the spinogenic activity of these compounds. These novel benzothiazoles represent a significant step towards the development of new tools to study and treat spine related disorders, and may also lead to a new class of general cognitive enhancers.
5.9 Materials and Methods

5.9.1 Materials

Pierce™ BCA Assay kit (#23225), Active Ras Pull-down and Detection Kit (#16117), and Pierce™ phosphatase inhibitor tablets (#88667) were purchased from Thermo Scientific. G-actin/F-actin In Vivo Assay Kit (#BK037) was purchased from Cytoskeleton, Inc. Protease inhibitor tablets (# 05892791001) were purchased from Roche. Primary antibodies used were: rabbit anti-Synapsin (EMD Millipore AB1543), mouse anti-PSD95 (EMD Millipore CP35), mouse anti-RasGRF1 (BD 610149), mouse anti-Ras (Thermo #1862335), mouse anti-GADPH (Sigma G8795), rabbit anti-Actin (Cytoskeleton #AAN01), rabbit anti-Arp2 (Santa Cruz sc-15389), mouse anti-Aβ (6E10) (Biolegend® #803001), rabbit anti-p-Cofilin (Santa Cruz sc-12912-R) and rabbit anti-Cofilin (Santa Cruz sc-33779). Secondary antibodies used were as follows: goat anti-mouse AlexaFluor 568 (Invitrogen A110040), goat anti-rabbit AlexaFluor 647 (Invitrogen A21244), ECL™ Horseradish Peroxidase (HRP) linked anti-mouse (GE #NA931) and anti-rabbit (GE #NA934). Amersham™ ECL™ Prime Western Blotting Detection Reagent (RPN2232) was purchased from GE Healthcare. Synthetic Aβ(1-42) peptide was purchased from PL Lab. All chemical reagents were purchased and used as is from Sigma Aldrich or Fisher unless otherwise stated.
5.9.2 Compounds

The synthesis of BTA-EG₆, BAM1-EG₆ (1), BAM2-EG₆ (2) and BAM3-EG₆ (3) can be found in Chapter 2.

5.9.3 Neuronal Cultures

Rat dissociated hippocampal neurons from postnatal day 1 Sprague Dawley® outbred rats of both sexes were plated at a density of 45,000 cells/cm² onto poly-D-lysine-coated coverslips in 24-well plates. Neurons were maintained in B27 supplemented Neurobasal media (Invitrogen) until days in vitro (DIV) 18-23 as previously described. Overall neuronal health was monitored visually weekly and throughout the experimental process.

5.9.4 Neuronal Treatments

For all neuronal treatments, the following general protocol was followed: Briefly, 18-23 DIV rat dissociated hippocampal neurons were dosed with various concentrations (0-50 µM final concentration) of BAMs 1-3 or BTA-EG₆ (with 0.1% final DMSO concentration) for various incubation times (24-72 h depending on the experiment). After dosing and at the desired time point, the medium was removed and cells were rinsed with PBS-MC (phosphate-buffered saline, 1 mM MgCl₂ and 0.1 mM CaCl₂). Following rinsing, cells were fixed with 4% paraformaldehyde (PFA)/sucrose in PBS for 10 min at RT. After fixation, coverslips were carefully rinsed (3 x PBS-MC) and then mounted onto slides (Polysciences Inc., 18606) for imaging.
**5.9.5 Colocalization of PSD95 and Synapsin**

After following the general procedure for neuronal treatment, fixed cells were then permeabilized, blocked in 5% BSA/PBS-MC, and incubated overnight at 4°C with anti-Synapsin (1:1000) and anti-PSD95 (1:1000). After incubation with Alexafluor conjugated secondary antibodies, the coverslips were mounted for imaging.

**5.9.6 Sindbis Production**

PalGFP SinRep5 DNA was obtained as a generous gift from Takahiro Furuta (Kyoto University, Kyoto, Japan). Recombinant Sindbis virion production was accomplished through RNA transcription using the SP6 mMMessage mMachine kit (Ambion, Austin, TX). Electroporation of RNA into baby hamster kidney cells (BHK) was completed using a BTX ECM 600 electroporator (BTX, Holliston, MA) at 220V, 129Ω, and 1050µF. After 24 h, virion was collected and concentrated by centrifugation at 20,000 rpm for 90 min using a Beckman Coulter Optima MAX Ultracentrifuge (Beckman Coulter, Indianapolis, IN). The treated neurons were infected with palGFP expressing sindbis 18 h prior to fixation. The palGFP (membrane targeting) signal was imaged directly in fixed neurons.

**5.9.7 Confocal Microscopy and Dendritic Spine Analysis**

For all imaging of neurons, we used a Leica DMI6000 inverted microscope outfitted with a Yokogawa Spinning disk confocal head, an Orca ER High Resolution CCD camera (6.45 µm/pixel at 1×) (Hamamatsu), Plan Apochromat 63×/1.4 na objective,
and PerkinElmer solid-state laser with 488nm excitation. Confocal z-stacks were acquired in all experiments and all imaging was acquired in the dynamic range of 8-bit acquisition (0–255 pixel intensity units, respectively) with Volocity (PerkinElmer) imaging software. Imaged dendrites from one secondary dendrite per cell (after 1 branch) at a distance of 40-80 µm from the soma were straightened using ImageJ. Spine density was estimated by the number of manually counted spines divided by dendrite segment length. The analyzer was blind to treatment and statistical significance was determined between experimental conditions by either unpaired t tests (two groups) or by ANOVA and indicated post hoc multiple-comparison test (>2 experimental conditions).

5.9.8 Western Blot Analysis

Rat dissociated neurons were lysed with RIPA buffer containing both protease and phosphatase inhibitors. Protein concentration was determined by BCA assay and proteins were separated by SDS-PAGE followed by transfer onto nitrocellulose membranes. Membranes were blocked with either 5% BSA or milk in Tris-Buffered Saline with Tween 20 (TBST), followed by incubation with primary antibodies overnight at 4 °C with shaking. Proteins were visualized using the appropriate HRP-labeled secondary by ECL and detection was carried out on film (Freedom Imaging, SRX-101A). The density of each band was then quantified using ImageJ software.

5.9.9 Measurement of Cellular Active Ras Levels

To analyze the levels of active Ras in the rat primary neuronal lysates treated with BTA-EG₆, BAMs 1-3 or control, we used an Active-Ras Pulldown and Detection Kit
(Thermo) according to the manufacturers guidelines. Briefly, fresh lysates were used for each assay using the provided lysis buffer with protease and phosphatase inhibitors added. Cells were then scraped and transferred to microcentrifuge tubes, vortexed briefly, and incubate on ice for 5 minutes. The microcentrifuge tubes were then centrifuged at 16,000 x g at 4 °C for 15 minutes and the supernatant (total lysate samples) was transferred to a new tube. Aliquots of the total lysate were saved for the Pierce BCA assay for protein concentration determination as well as for the total Ras load for each sample. The rest of the supernatant was transferred to a spin cup containing 100 µL of a 50% glutathione resin slurry and 80 μg of GST-Raf1-RBD, vortexed and then incubated for 1 h at 4 °C with gentle shaking. Following incubation, the spin cups with collection tube were centrifuged 6,000 x g for 30 seconds. The resin was the washed with lysis buffer (400 µL x 3) with centrifugation (6,000 x g for 30 seconds) after each rinse. The spin cups were then transferred to a new collection tube and 50 µL of 2X reducing buffer (1 part β-mercaptoethanol: 20 parts 2x SDS Sample Buffer) was added to each cup and let incubate for 2 minutes. Each tube was then centrifuged (6,000 x g for 2 minutes) and the flow through was taken on for western blot analysis (active Ras samples).

### 5.9.10 Measurement of Cellular F-Actin/G-Actin Ratio

To analyze the levels of filamentous (F-actin) and globular (G-actin) in rat primary neuronal lysates treated with BTA-EG₆, BAMs 1-3 or control, we used a G-actin/F-actin In Vivo Assay Kit (Cytoskeleton) according to the manufacturers guidelines. Briefly, primary neurons were scraped with the LAS2 buffer provided, homogenized with a 200 µL pipette tip, and incubated at 37 °C for 10 minutes. The
lysates were then centrifuged at 350 x g for 5 minutes at RT to pellet cellular debris. 100 µL of each supernatant was then transferred to ultracentrifuge tubes (Beckman Coulter, Ref # 343778) and centrifuged at 100,000 x g at 37 °C for 1 h to separate the insoluble F-actin (pellet) from the soluble G-actin (supernatant). The pellet was depolymerized with the provided F-actin depolymerization buffer (100 µL, 1 h, 4 °C). Samples were then analyzed by western blot and ImageJ was used to quantify the amount of G- and F-actin in each sample.

5.9.11 Real Time Imaging of Spine Changes in Rat Primary Hippocampal Neurons

For this study, 21 DIV neurons plated in 35 mm dishes (MatTek) were rinsed 3x with an excess of HBSS and then left in HBSS for the duration of imaging. For live imaging we kept cells at 37 °C and used a Leica DMI6000 inverted microscope outfitted with a Yokogawa Spinning disk confocal head, an Orca ER High Resolution CCD camera (6.45 µm/pixel at 1×) (Hamamatsu), Plan Apochromat 63×/1.4 na objective, and PerkinElmer solid-state laser with 488nm excitation. The spine changes on the same neuron were monitored 1 h before dosing (-60 min) and up to 3 h after dosing (+180 min). Dosing occurred at t=0 and consisted of either 0 (for control) or 5 µM BAM1-EG₆. For each condition, neurons from three different neuronal preparations (prep) were used and two neurons per prep were monitored. Confocal z-stacks were acquired in all experiments and all imaging was acquired in the dynamic range of 8-bit acquisition (0–255 pixel intensity units, respectively) with Volocity (PerkinElmer) imaging software. For analysis, imaged dendrites were straightened using ImageJ and the same
dendrite length was analyzed for each condition. Spines gained were counted as any new spines found at each respective time point and spines lost were counted as spines that disappeared from the analyzed segment. The analyzer was blind to treatment.

5.9.12 Preparation and Characterization of Aggregated Aβ(1-42)

Aggregated Aβ(1-42) was prepared as previously described.\textsuperscript{89} Briefly, Aβ(1-42) was initially solubilized in 100% 1,1,1,3,3,3-hexafluoro-2-propanal (HFIP) to 1 mM concentration at RT for 21 h with shaking. The solution was sonicated and vortexed before it was diluted in cold nanopure water (2:1 H\textsubscript{2}O:HFIP). Aliquotted fractions were lyophilized for 2 days, followed by storage at -80 °C until use. Solutions of Aβ were obtained by dissolving Aβ in sterile PBS to a concentration of 100 µM and incubated at 37 °C for 3 days before use. Western blot analysis of the three day incubated Aβ was carried out to determine composition. We estimated the relative abundance of monomers, oligomers, and protofibrils of Aβ using ImageJ and percentage of each composition was calculated by dividing the intensity of each aggregation state over the total intensity for Aβ in the lane. This preparation of Aβ lead to a composition of ~12% monomers, ~16% low MW oligomers, and ~72% mixture of soluble protofibrils/fibrils. Aggregated Aβ was also characterized by EM, MALDI-TOF, and binding by thioflavin T.

5.9.13 Rescue of Net Aβ-Induced Spine Loss in Rat Primary Hippocampal Neurons

We followed the same general dosing procedure as described for neuronal treatment, except with the following noted changes. For Aβ co-treatment, 18 DIV rat
dissociated hippocampal neurons were dosed with a final concentration of 1 µM of aggregated Aβ(1-42) with or without the presence of 1 or 5 µM of BAMs 1-3 or BTA-EG₆ for 3 days. Control cells were treated with vehicle control only (0.1% DMSO) for the three-day period. For Aβ pre-treatment, rat dissociated hippocampal neurons were dosed with 5 µM of BAMs 1-3 or BTA-EG₆ for 24 h. After a wash out of compounds, neurons were dosed with a final concentration of 1 µM of aggregated Aβ(1-42) for 3 days. For both treatments, after dosing medium was removed, cells were rinsed with PBS-MC, fixed with 4% paraformaldehyde (PFA)/sucrose in PBS for 10 min at RT, and mounted onto slides (Polysciences Inc., 18606) for imaging. All analysis was done blinded and each experiment was repeated at least three separate times from neurons from three different preparations.

Notes About the Chapter:

Chapter five, in part, is a reprint (with co-author permission) of the material as it appears in the publication; Cifelli, J. L., Dozier, L., Chung, T. S., Patrick, G. N. & Yang, J. “Benzothiazole Amphiphiles Promote the Formation of Dendritic Spines in Primary Hippocampal Neurons”, J. Biol. Chem. 291, 11981–11992 (2016). I would like to thank Tim Chung, Lara Dozier, Gentry Patrick, and Jerry Yang for their invaluable contributions to this chapter: Tim Chung synthesized BAM3-EG₆ and Lara Dozier and Gentry Patrick prepared and maintained all primary neuronal cultures. Additionally, Lara Dozier assisted with all primary neuron experiments and analysis. I would like to thank Gentry Patrick and Jerry Yang for directing the research. The author of the dissertation is the primary author of this manuscript.
6.1 Introduction

The translation of basic science research into human studies is an intricate and precarious process. To put this into perspective, on average it takes over 13 years for a new drug to proceed from the discovery phase to being approved with a failure rate exceeding 95%. This translates into each new successful drug currently costing upwards of $1.8 billion. It is therefore of great interest to minimize the failures in clinical trials. This entails accurately predicting the efficacy of the drug in humans before entering the clinic. The use of animal models have been used for therapeutic development and target validation; however, this practice is costly, time consuming, and in many cases, fails to accurately predict efficacy in humans.
Human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) represent exciting and potentially more reliable efficacy models for human therapeutics.\textsuperscript{137,138} While the use of ESCs in culture has been around since the 1980s\textsuperscript{139}, ethical and religious controversy hindered funding and public support of the technology. Takahashi’s reprogramming adult mice\textsuperscript{140} and human\textsuperscript{141} fibroblasts into pluripotent stem cells by transcription factors was truly revolutionary for the field. Human iPSC technology provides several attractive attributes for drug and disease research including: 1) the ability to provide a practically inexhaustible supply of normal human cells\textsuperscript{141}, 2) the capability to differentiate these cells to generate specific cell types\textsuperscript{142,143}, and 3) the unique capacity to generate either disease- or patient-derived cells\textsuperscript{144–146}.

iPSCs have been shown to successfully differentiate to neural stem cells (NSCs) and subsequently into neurons.\textsuperscript{147,148} In this chapter, we sought to examine the spinogenic activity of BAMs in neurons derived from human iPSCs. Here, we found that upon overnight treatment with BAMs 1-3, these neurons displayed an increase in PSD95 puncta over the control. Additionally, RasGRF1 expression was found to be increased in the BAM treated neurons compared to the control neurons. Together, these results excitingly indicate that the same machinery found to be activated in rat primary culture by BAMs is also targeted in human neurons derived from iPSCs.

### 6.2 Differentiating Human iPSC-Derived Neural Stem Cells (NSCs)

Human neural stem cells (NSCs) were derived from human induced pluripotent stem cell (hiPSC) and subsequently purified by fluorescence activated cell sorting (FACS) as previously mentioned by Yuan and coworkers (Figure 6.1).\textsuperscript{149} This methodology
employs the use of the Yamanaka factors\textsuperscript{140}, (Oct3/4, Sox2, klf4, and c-Myc) to induce pluripotency from adult human fibroblasts. Subsequently, neural induction is carried out utilizing serum-free embryoid body (SFEB)\textsuperscript{150} and dual inhibition\textsuperscript{151} of SMAD signaling, with Noggin and SB-431542\textsuperscript{152}. Purification of NSCs then employed the utilization of

**Figure 6.1.** Flowchart depicting the differentiation and isolation of NSCs from human reprogrammed fibroblast cells.
FACS isolation with a variety of cell surface markers (CD184⁺, CD271⁻, CD44⁻ and CD24⁺).

The isolated NSCs were subsequently characterized by the undifferentiated precursor markers Nestin, an intermediate filament protein; and SOX2, a transcription factor essential for pluripotency (Figure 6.2).

**Figure 6.2.** Immunohistochemistry characterization of NSCs. Undifferentiated precursor specific marker staining of (A) Nestin and (B) SOX2. Additional images of the (C) staining of nuclei with DAPI, (D) composite image of A-B, (E) bright field image of NSCs, and (F) composite of A, B, C, and E. Scale bar = 25 µm.
After characterizing the NSCs, we next sought to differentiate them into mature neurons. In order to accomplish this goal, we followed a previously described differentiation method utilizing fibroblast growth factor (FGF) removal and addition of brain-derived neurotrophic factor (BDNF) and dibutyryl cyclic adenosine monophosphate (dbcAMP).\textsuperscript{145,148,149} The differentiation process was monitored weekly by live cell microscopy (Figure 6.3).

\textbf{Figure 6.3.} Morphology changes associated with the differentiation of NSCs over time. Live cell images of A) undifferentiated NSCs, B) 1 week, C) 2 weeks, and D) 4 weeks differentiated NSC. Scale bar = 100 µm.
Additionally, mature neurons from several different preparations were then stained with the neuronal specific marker, microtubule-associated protein 2 (MAP2) (Figure 6.4).

![Image of immunohistochemistry](image)

**Figure 6.4.** Immunohistochemistry of the neuronal specific marker MAP2 from several different preparations of neurons differentiated from NSCs. MAP2 staining from preparations 1 (A), 2 (B), and 3 (C-E). DAPI staining (D) and composite (E) of image C. Top panel scale bar = 100 µm, bottom panel scale bar = 50 µm.

### 6.3 BAMs Increase PSD95 Puncta in Human iPSC-Derived Neurons

Having successfully differentiating human NSCs into mature neurons, we next sought to examine if BAMs 1-3 could elicit the same increase in PSD95 puncta as
previously observed in rat primary hippocampal culture (Chapter 5). For this experiment, we dosed 3-month-old neurons derived from NSCs with 0 or 5 µM BAMs 1-3 for 24 hours. Neurons were then fixed and immunochemistry was performed to examine PSD95 puncta located off of MAP2 positive neuron dendrites (Figure 6.5).

![Figure 6.5](image)

**Figure 6.5.** Analysis of the localization of synaptic proteins of neurons exposed to BAM1-EG₆. (A) Representative images of dendrite segments (MAP2) and PSD95 (postsynaptic marker) from iPSC derived neurons dosed with control (PBS) or BAMs 1-3. Scale bar = 25 microns (B) Quantitative representation of amount of normalized PSD95 puncta for MAP2 positive neurons treated with BAM1-EG₆ compared to control. Data are expressed as mean values ± SEM, n ≥ 9, *p < 0.01 as determined by unpaired t-test compared to control.
fold increase in PSD95 puncta was observed for neurons treated with BAMs 1-3 compared to the control neurons (PBS). This data suggests that BAMs 1-3 are targeting the same synaptic machinery in humans as they had previously been observed to do in rat primary hippocampal culture (Chapter 5).

6.4 BAMs Activate RasGRF1 Expression in Human iPSC-Derived Neurons

Having observed a phenotypic increase in PSD95 puncta in human iPSC- derived neurons, we next sought to examine if the same RasGRF1 dependent pathway was being activated as previously observed in rat primary culture (Chapter 5). For this experiment, 3-month old mature iPSC-derived neurons were exposed to solutions of BAMs 1-3 in PBS (0 or 5 µM) for 24 hours. Subsequently, cells were lysed and western blot analysis was performed in order to quantify any changes in RasGRF1 expression. Here, once again a significant increase in RasGRF1 expression was observed in neurons that were dosed with 5 µM BAMs 1-3 over the control (PBS) (Figure 6.6). This data further suggests that the RasGRF1 dependent pathway of spinogenesis is also being activated in human derived neurons.
Figure 6.6. Analysis of protein expression and activation of Ras signaling in iPSC derived neurons exposed to benzothiazole agents. Relative expression levels of RasGRF1 upon dosing with 5 μM of BTA-EG₆ and BAMs 1-3. Data are expressed as mean values ± SEM, n= 3 or more for each concentration. n.s. = not significant compared to control *p < 0.05 compared to control as determined by unpaired t test.

6.5 Concluding Remarks

The ability to properly assess a drug’s potential human efficacy is of great importance. The use of human induced pluripotent stem cells (iPSCs) allows for a new avenue for drug discovery and development. Here, FACS-purified human neural stem cells (NSCs) derived from human induced pluripotent stem cell (hiPSC) were further
differentiated into mature neurons. These mature human neurons expressed MAP2 and PSD95. Upon dosing with BAMs 1-3 the density of PSD95 puncta along dendrites was found to be higher than control neurons. Additionally, the expression of RasGRF1 was increased over control neurons as well. Taken together, this data suggests that the same RasGRF1 spinogenic pathway is being activated in humans to a similar extent as what was observed in rats (Chapter 5). Excitingly, this new evidence seems to validate BAMs pharmacology effects in humans, as well as minimizing the perceived risk in their possible role as therapeutic agents for diseases with associated decreases in spine density, like NDDs.

6.6 Future Directions

Current work in Yang lab is utilizing photoaffinity labeling (PAL) to covalently link BAM chemical probes to its potential binding partner, with hopes of determining the exact binding partner of BAMs. Once a potential binding partner is deduced from pull-down studies with a variety of lysates (i.e. neuroblastoma, human brain, mice brain), further knockout/overexpression studies will be employed in primary neurons as supplementary confirmation. If the binding partner has a previously unknown function in dendritic spine regulation these studies will be of fundamentally importance to the field of neuroscience. Additionally, lysates from the iPSC-derived neurons can be analyzed to further confirm any newly discovered pathways in humans. Future work will employ the use of animal models to further characterize and better correlate spine density changes with memory and learning in mice or rats over time. Furthermore, donated human brain tissue may be utilized to further probe the translational efficacy of BAMs into humans,
with hopes of providing alleviation of spine density loss associated with a large number of NDDs.

6.7 Materials and Methods

6.7.1 Materials

Human iPSC-derived FACS purified NSCs were a generous gift from Jessica Young in Goldstein Lab (Department of Cellular and Molecular Medicine, UCSD). DMEM/F12 + Glutamax (#10565-018), N-2 supplement (#17502-048), and B27 supplement (#170504-044) were purchased from Gibco. Human basic fibroblast growth factor (bFGF) (#100-18B) and BDNF (#450-02B) were purchased from PeproTech. Penicillin-Streptomycin (Pen/Strep) (#17-602E) was purchased from Lonza. Accutase (AT-104) was purchased from Innovative Cell Technologies, Inc. Sterile 0.4 µm cell strainers (352340) were purchased from BD Falcon. Laminin (#L2020) and poly-l-ornithine (#P3655) were purchased from Sigma. Coverslips, 25 mm #1 German Glass (#CLS-1760-025) were purchased from Chemglass. 6-well culture plates (#353046) were purchased from Falcon. Human Neural Stem Cell Immunocytochemistry Kit (#A24354) was purchased from Molecular Probes by Life Technologies. Primary antibodies used were: mouse anti-NESTIN (Life Technologies #A24345), rabbit anti-SOX2 (Life Technologies #A24339), mouse anti-MAP2 (Sigma #M1406), rabbit anti-PSD95 (Invitrogen #51-6900), mouse anti-RasGRF1 (BD 610149), and mouse anti-GADPH (Sigma G8795). Secondary antibodies used were: Alexa Fluor® 488 donkey anti-mouse
(Life Technologies #A24350), Alexa Fluor® 555 donkey anti-rabbit (Life Technologies #A24342), TRITC-conjugated goat anti-mouse (Jackson ImmunoResearch), Alexa Fluor® 488-conjugated goat anti-rabbit (Jackson ImmunoResearch), ECL™ Horseradish Peroxidase (HRP) linked anti-mouse (GE #NA931). Amersham™ ECL™ Prime Western Blotting Detection Reagent (RPN2232) was purchased from GE Healthcare. Protease inhibitor tablets (# 05892791001) were purchased from Roche. Pierce™ BCA Assay kit (#23225) was purchased from Thermo Scientific.

6.7.2 hIPSC-Derived Neural Stem Cell (NSC) Culture

Human iPSC-derived, FACS purified NSCs were maintained in base medium composed of: DMEM/F12 medium with Glutamax supplemented with N-2 (0.5x), B-27 (0.5x), bFGF (20 ng/mL), and Pen/Strep (1x). bFGF was added fresh every week or as needed. Cells were plated on 6-well plates that were coated with laminin (5 µg/mL) and poly-l-ornithine (20 µg/mL) and incubated at 37 °C, 5% CO₂ and 95% humidity. Base medium was changed every 2-3 days and cells were split at a ratio of 1:3 at 80-90% confluency with Accutase.

6.7.3 hIPSC-Derived Neural Stem Cell (NSC) Differentiation

For the differentiation of hiPSC-derived, NSCs into neurons the same base medium was used except for the removal of bFGF and addition of BDNF (20 ng/mL) and dbcAMP (0.5 mM). This differentiation medium was changed every 3-4 days and cells were maintained at 37 °C, 5% CO₂ and 95% humidity. Cells were plated on either 6-well
dishes, MatTeks, or 8-well chamber slides coated with laminin (5 µg/mL) and poly-l-ornithine (20 µg/mL) and let differentiate for up to 3 months.

### 6.7.4 Immunochemistry of hIPSC-Derived Neural Stem Cells (NSCs)

A human neural stem cell immunocytochemistry kit (A24354) was utilized for characterization of human NSCs. The kit was used to confirm expression of the common undifferentiated precursor markers Nestin as well as SOX2. Briefly, medium was removed and cells were rinsed with PBS. Cells were fixed with the provided fixative solution (4% PFA I DPBS) for 15 min at room temperature (RT), followed by permeabilization with the provided permeabilization solution (0.5% Triton X-100 in DPBS) for 15 min at RT. After permeabilization, cells were blocked with the blocking solution (3% BSA/DPBS) for 1 hour at RT. After removal of blocking solution, primary antibodies diluted to 1X in blocking solution were added and let incubate at 4 °C overnight. After washing (3x), the appropriate secondaries were added (diluted to 1X in blocking solution) and incubated for 1 hour at RT. Cells were washed 3x with 2 drops/mL of the provided NucBlue® fixed cell stain (DAPI) added in the last wash step (incubate 5 minutes). Slides or coverslips were mounted with Vectashield® and imaged with an FV1000 confocal microscope (Olympus).

### 6.7.5 Immunochemistry of hIPSC-Derived Neurons

Human iPSC-derived neurons were stained for microtubule-associated protein 2 (MAP2), a neuron specific marker, as well as postsynaptic density protein 95 (PSD-95), a
post-synaptic marker. Briefly, cells were dosed for 24 hours with or without BAMs 1-3 [5 μM in PBS]. After dosing, cells were rinsed (3 x PBS), fixed with 4% PFA (15 minutes, RT), permeabilized with 0.3% Triton X-100/DPBS (10 minutes RT), blocked with 3% BSA/DPBS (1 h, RT), and incubated overnight at 4 °C with primaries: MAP2 (1:500) and PSD95(1:1000) in blocking solution. Secondaries were diluted 1:250 in blocking solution and let incubate for 1 hour at RT. Slides or coverslips were mounted with Vectashield® and imaged with an FV1000 confocal microscope (Olympus). PSD-95 puncta were quantified per micron along MAP2 positive neurons.

6.7.6 Western Blots for hIPSC-Derived Neurons

From protein express analysis of either dosed or control hiPSC-derived neurons, western blotting was performed. Briefly, cells were dosed for 24 hours with or without BAMs 1-3 [0 or 5 μM in PBS]. After dosing, cells were rinsed on ice (3x with ice cold PBS), lysed with NP40 buffer with protease inhibitors. Lysates were agitated for 30 minutes at 4 °C, followed by centrifugation at 4 °C (20 minutes, 12,000 rpm). Supernatants were aspirated into a new ice-cold tube and a BCA assay was performed to determined protein concentration. Lysates were frozen and stored -80 °C until use for western blot analysis. Briefly, proteins were separated by SDS-PAGE followed by transfer onto nitrocellulose membranes. Membranes were blocked with either 5% BSA or milk in Tris-Buffered Saline with Tween 20 (TBST), followed by incubation with primary antibodies overnight at 4 °C with shaking. Proteins were visualized using the appropriate HRP-labeled secondary by ECL and detection was carried out on film
(Freedom Imaging, SRX-101A). The density of each band was then quantified using ImageJ software.

**Notes About the Chapter:**

Chapter six is unpublished work. I thank Jessica Young for the generous donation of human NSCs and invaluable contributions to this chapter. Jessica Young reprogrammed human fibroblasts to iPSC cells, induced neural differentiation to NSCs, and FACS purified the NSCs. In addition, I would like to thank Jerry Yang for directing the research. My contribution to this chapter was the differentiation and characterization of NSCs to mature neurons. Additionally, I designed and performed all dosing studies, imaging, and western blot analysis.
~References~


125. DMSO was used for all compounds due to its necessity to solubilize BTA-EG4.


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