SMALL MOLECULE UBIQUITIN-PROTEASOME SYSTEM INHIBITOR LIBRARY SCREEN REVEALS BOTULINUM NEUROTOXIN TYPE A INHIBITORS

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

EDANUR ŞEN

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOLOGY

FEBRUARY 2021

Approval of the thesis:

SMALL MOLECULE UBIQUITIN-PROTEASOME SYSTEM INHIBITOR LIBRARY SCREEN REVEALS BOTULINUM NEUROTOXIN TYPE A INHIBITORS

submitted by **EDANUR ŞEN** in partial fulfillment of the requirements for the degree of **Master of Science** i**n Biology, Middle East Technical University** by,

Prof. Dr. Halil Kalıpçılar
Dean, Graduate School of **Natural and Applied Sciences** _____

Prof. Dr. Ayşe Gül Gözen
Head of the Department, **Biology** _____

Assoc. Prof. Dr. Erkan Kiriş
Supervisor, **Biology, METU** _____

Examining Committee Members:

Prof. Dr. Mesut Muyan
Biology, METU _____

Assoc. Prof. Dr. Erkan Kiriş
Biology, METU _____

Asst. Prof. Onur Çizmecioğlu
Molecular Biology and Genetics, Bilkent University _____

Date: 15.02.2021

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name : Edanur Şen

Signature :

ABSTRACT

SMALL MOLECULE UBIQUITIN-PROTEASOME SYSTEM INHIBITOR LIBRARY SCREEN REVEALS BOTULINUM NEUROTOXIN TYPE A INHIBITORS

Şen, Edanur
Master of Science, Biology
Supervisor: Assoc. Prof. Dr. Erkan Kiriş

February 2021, 67 pages

Botulinum neurotoxins (BoNTs), known as the most potent bacterial toxins, cause potentially lethal disease botulism. BoNTs are classified as category A bioterror agents by many countries because they are easy to produce and have extreme toxicities. On the other hand, BoNTs are considered a therapeutic marvel in medicine because they have been extensively utilized not only for cosmetic purposes to get rid of wrinkles but also to treat various conditions in clinics, including movement disorders. These toxins specifically target cholinergic nerve terminals and block acetylcholine release by cleaving Soluble N-ethylmaleimide-sensitive Factor Attachment Protein Receptor (SNARE) complex proteins, which are crucially important for neuroexocytosis. There are eight serologically distinct serotypes of BoNTs (BoNT/A–G, BoNT/X); however, only serotypes A, B, E, and F lead to human botulism. Among these, BoNT/A is the most widely used serotype in clinics and also the most studied serotype as more than half of botulism cases are due to BoNT/A poisoning. BoNT/A has the longest-lasting effect as compared to other serotypes. For example, both BoNT/A and BoNT/E serotypes cleave the

same protein, SNAP-25, and the half-life of BoNT/E is limited to days in cells while BoNT/A can be active for up to 6 months in the neuronal cytosol. The underlying mechanisms for the half-life differences between different serotypes have not been well understood. However, there has been heightened research interest in understanding how BoNT/A manages to escape destructive protein degradation machinery in neurons. Recent studies have identified the E3 ligases and deubiquitinases (DUBs) critical for the destruction of BoNT/A. More specifically, one specific E3 ligase, HECTD2, leads to ubiquitination of the enzymatic component of BoNT/A Light Chain (LC) in cells, but the dominant DUB activity of VCIP135 inhibits the proteolytic degradation of the LC. In addition to this, another DUB, USP9X, indirectly affects the stability of BoNT/A in cells. Therefore, the persistence of BoNT/A in the cell can be potentially modified by affecting these factors using small molecule modulators. Modulation of BoNT half-life in cells by small molecules can be important for research purposes to understand intoxication/recovery mechanisms, as well as for the generation of effective countermeasures against botulism. In this study, the main goal was to screen a focused ubiquitin-proteasome system (UPS) inhibitor library to reveal compounds modulating BoNT/A activity and half-life in cells. Our screen utilizing mouse embryonic stem cell-derived motor neurons identified 10 potential lead compounds affecting BoNT/A mediated SNAP-25 in neurons. Then, tested dose-dependent effects of the selected compounds and further tested their potential toxic effects in the cell. Following, we explored the effects of the lead compounds on the stability of LC in cells. Small molecules WP1130, b-AP15, NSC632839, PR-619, Celastrol, MDBN, PYR-41, and SL-01 exhibited efficacy against BoNT/A LC action and half-life in cells. Among these, PR-619 appears to be highly crucial as it is an inhibitor of VCIP135 that has been identified as a crucial DUB affecting the half-life of BoNT/A LC. To the best of my knowledge, this is the first study in the literature showing that UPS targeting small molecules can modulate BoNT/A LC action and half-life in cells.

Keywords: Botulism, Botulinum neurotoxin (BoNT), Synaptosomal-associated protein 25 (SNAP25), Motor Neuron, Synaptic Transmission

ÖZ

BOTULİNUM NÖROTOKSİN SEROTİP A İNHİBİTÖRLERİNİN UBİKİTİN-PROTEAZOM SİSTEM İNHİBİTÖR KÜTÜPHANESİNİN TARANMASIYLA BELİRLENMESİ

Şen, Edanur
Yüksek Lisans, Biyoloji
Tez Yöneticisi: Doç. Dr. Erkan Kiriş

Şubat 2021, 67 sayfa

Botulinum nörotoksini (BoNT) ölümcül olan botulizm hastalığına sebep olan, bilinen en güçlü bakteriyel toksindir. Yüksek toksisiteye sahip olması ve kolayca üretilebiliyor olması sebebi ile bu toksin birçok ülke tarafından kategori A biyolojik ajanlar sınıfında değerlendirilmektedir. Aynı zamanda, kozmetik amaçlı olarak kırışıklıklardan kurtulmak için kullanılmasının yanı sıra, hareket bozukluklarının da içinde olduğu birçok hastalığın tedavisinde klinik alanda uygun doz ve uygulama ile yaygın olarak kullanılmaktadır. BoNT spesifik olarak kolinerjik sinir terminallerini hedef alır ve nöroeksositoz için kritik öneme sahip olan SNARE kompleks proteinleri keserek asetilkolin salınımını bloke eder. Botulinum nörotoksininin serolojik olarak farklı 8 farklı serotipi vardır (BoNT/A–G, BoNT/X) ancak yalnızca A, B, E ve F serotipleri insanda botulizme neden olur. Bu serotipler arasında, BoNT/A klinik alanda en yaygın kullanılan serotiptir, ayrıca botulizm vakalarının yarısından fazlası BoNT/A zehirlenmesine bağlı olduğu için üzerinde en fazla çalışma yapılan serotiptir. Serotip A, diğer serotiplere kıyasla en uzun ömüre sahiptir. Örnek olarak, Serotip A ve E, aynı proteini (SNAP-25)

kesmelerine karşın, BoNT/E hücre içinde bir kaç günlük yarılanma ömrüyle kısıtlıyken BoNT/A sitoplazmada 6 ay kadar aktif bir halde kalabilir. Yarılanma ömürleri arasındaki bu farkların altında yatan moleküler mekanizmalar henüz tam anlamıyla açığa çıkarılmamıştır. Buna karşın BoNT/A'nın nöronlardaki yıkıcı protein bozunma mekanizmasından nasıl kaçtığını anlamaya yönelik araştırma ilgisi artmıştır. Son çalışmalar E3 ligaz ve deubikitasyon enzimlerinin BoNT/A yıkımı için kritik önem arz ettiklerini ortaya çıkartmıştır. Spesifik olarak HECTD2 adındaki E3 ligaz BoNT/A hafif zincirinin ubikitasyonuna sebep olmaktadır ancak bu proteolitik degradasyon VCIP135 isimli deubikitasyon enziminin yoğun aktivitesiyle inhibe edilmektedir. Ayrıca USP9X isimli deubikitasyon enzimi indirekt olarak hücre içindeki BoNT/A stabilitesini etkilemektedir. Bu sebeple, Serotip A'nın hücre içindeki aktivitesinin sürekliliği spesifik düzenleyici faktörler kullanılarak modifiye edilebilir. BoNT/A'nın yıkımını düzenleyen küçük ilaç moleküllerinin geliştirilip tanımlanması, bu serotipe karşın etkili önemler geliştirmesi için önem arz etmektedir. Bu sebeple, bu tez çalışmasında ubikitin-proteazom sistemini hedef alan küçük molekül kütüphanelerinin taranması ile BoNT/A intoksitesi üzerinde etkili olan moleküllerin belirlenmesi ve toksinin katalitik olarak aktif kısımı olan hafif zinciri üzerindeki etkilerinin araştırılması hedeflenmektedir. Fare embriyonik kök hücreleri kullanılarak yapılan tarama sonucunda, BoNT/A aracılığı ile SNAP-25 kesilmesine karşı nöronlarda etkinliği olan öncü 10 adet potansiyel bileşik belirlenmiştir. Daha sonra, bu öncü bileşiklerin doza bağlı etkileri ve hücrelerdeki potansiyel toksik etkileri test edilmiştir. Bir sonraki aşamada öncü bileşiklerin hücre içerisindeki LC stabilitesi üzerindeki etkileri incelenmiştir. WP1130, b-AP15, NSC632839, PR-619, Celastrol, MDBN, PYR-41 ve SL-01 adlı küçük moleküller, BoNT/A LC etkilerine ve yarılanma ömrüne karşı etkili sonuç verdiler. Bunlar arasında, PR-619 adlı bileşik, BoNT/A LC'nin yarı ömrünü etkileyen çok önemli bir DUB olarak tanımlanan bir VCIP135 inhibitörü olduğu için oldukça önemli görünmektedir. Bu çalışma, hücrelerdeki küçük moleküllerin mekanik etkisini gösteren literatürdeki bilgimiz dahilinde olan

ilk çalışmadır, bu sebeple farklı BoNT serotipleri arasındaki stabilite farklarının sebeplerinin ortaya çıkartılması için önem arz etmektedir.

Anahtar Kelimeler: Botulizm, Botulinum Nörotoksini (BoNT), Synaptosomal-associated protein 25 (SNAP25), Motor Neuron, Synaptic Transmission

To the beauty of optimistic nihilism

ACKNOWLEDGMENTS

I would like to express my deep gratitude to my supervisor, Assoc. Prof. Dr. Erkan Kiriş for his scientific guidance, enthusiastic encouragement, useful critiques, and consistent support throughout this research. The door to his office was always open whenever I faced a challenging situation or had a question about my research. I will always be grateful for his counseling that took me forward and encourages me to become a well-rounded scientist.

I wanted to express my sincere gratitude to Prof. Dr. Mesut Muyan for his generous support and motivation in my whole academic life. Also, I would like to thank my jury member Asst. Prof. Onur Çizmecioğlu for his valuable suggestions.

I would also like to thank all Kiriş Lab members; Sena Ezgin, Dilara Koç, Onur Can Begentaş, Pari Başerat, Pınar Oduncu, Ceren Özdemir for their friendship and the fun times we spent together.

During the period of three years, many friends are helpful to color my life. I want to thank Pelin Yaşar and Kerim Yavuz for our therapy sessions, where I learn a lot, have fun and allow me to look at things from a different perspective. I am deeply grateful to Reyhan Yaka, Erinç Yurtman, Ozan Ertekin, Metin Acar, Meriç Öztürk, Irmak Gürcüoğlu for their endless support and for their friendship full of laughter, which is always special for me. I would like to thank my beloved friends Elif Özeren and Gözde Güven for their kind helps and friendship. I would like to thank Oğuz Atıcı, in particular, for his love and support.

Last but not least, I would like to thank to my family for their endless patience, support, and understanding. I cannot thank enough for my mother, who is my biggest supporter in this life. I always get strength from her, and none of this would have happened without her.

This work was supported by TÜBİTAK (Project No: 118C007)

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

LIST OF ABBREVIATIONS

ABBREVIATIONS

BoNT(s) =Botulinum Neurotoxin(s)

BoNT/A = Botulinum Neurotoxin Serotype A

LC = Light Chain

HC= Heavy Chain

SNAP-25 = Synaptosomal-Associated Protein of 25 kDa

SNARE = Soluble N-Ethylmaleimide- Sensitive Factor Attachment Protein Receptor

ACh = Acetylcholine

DUBs = Deubiquitinases

Ub= Ubiquitin

UPS = Ubiquitin-Proteasome System

UPP = Ubiquitin-Proteasome Pathway

IC50 = The half-maximal inhibitory concentration

MEF = Mouse embryonic fibroblast

FBS = Fetal Bovine Serum

LIF = Leukemia Inhibitory Factor

KSR = KnockOut™ Serum Replacement

EBs = Embryoid bodies

RA = Retinoic Acid

Shh = Sonic hedgehog

DMSO = Dimethyl Sulfoxide

µM = Micro-Molar

VAMP = Vesicle-Associated Membrane Protein

ES = Embryonic Stem

PBS = Phosphate-buffered saline

LB = Luria-Bertani

HEK293 = Human embryonic kidney 293

PDL = Poly-D-Lysine

CHX= Cycloheximide

CHAPTER 1

INTRODUCTION

1.1 Botulism

Botulism is a rare but life-threatening disease and caused by botulinum neurotoxin (BoNT) (Dickerson & Janda, 2006). It was first identified in Southern Germany approximately 200 years ago after the flaccid paralysis and death of people who shared contaminated ham and sausages (Rossetto, Pirazzini, Fabris, & Montecucco, 2020). There are different types of botulism, depending on the route of entry into the human body, and the most common type worldwide is foodborne botulism (Ambrozova, 2019). Other types include wound botulism, intestinal botulism, including infant botulism, inhalational botulism, botulism of unknown etiology, and iatrogenic botulism, which is inappropriate administration of botulinum toxin during its use as a pharmaceutical agent (Johnson & Montecucco, 2008). BoNT is produced by the bacterium *Clostridium botulinum*, which is a rod-shaped, gram-positive, spore-forming, and anaerobic bacterium (Peck, 2009). *Clostridium botulinum* is usually found in soils and water, on plants, and in the intestinal tracts of animals (Espelund & Klaveness, 2014). Weakness, blurred vision, feeling tired, and trouble speaking are among the common symptoms because it is a flaccid paralytic disease (Dickerson & Janda, 2006; Hambleton, 1992; Schantz & Johnson, 1992). Generally speaking, botulism may lead to death when the toxin affects the respiratory system with diaphragm muscles (Schantz & Johnson, 1992; Sobel, 2005). However, secondary infections or cardiac arrest would be the cause of death occasionally (Johnson & Montecucco, 2008). Therefore, hospitalization with intensive nursing care and mechanical ventilation can be mandatory to keep patients alive by preventing respiratory failure and

controlling secondary infections even though the recovery process is tedious and slow (Arnon et al., 2001; Johnson & Montecucco, 2008; Sobel, 2005).

1.2 Botulinum Neurotoxin (BoNT)

Botulinum neurotoxin is a proteinaceous toxin, which targets cholinergic nerve terminals (Dickerson & Janda, 2006). It is synthesized as an inactive polypeptide with ~150 kDa molecular weight (Dressler & Adib Saberi, 2005). The polypeptide is formed as two chains: one is a heavy chain (HC) with ~100 kDa molecular weight and is essential for the entrance of the toxin into cells (Dickerson & Janda, 2006; Dressler & Adib Saberi, 2005; Hambleton, 1992; Rossetto, Pirazzini, & Montecucco, 2014). The other one is a light chain (LC) with ~50 kDa molecular weight and is the catalytically active part of the toxin (Dickerson & Janda, 2006; Dressler & Adib Saberi, 2005; Hambleton, 1992; Rossetto et al., 2014). A single disulfide bond links these two chains (Dickerson & Janda, 2006; Rossetto et al., 2014). The bond is cleaved, and two chains are separated after the toxin enters the motor neurons (Winner, Bodt, & McNutt, 2020).

There are eight biochemically distinct serotypes of botulinum toxin (BoNT/A–G, BoNT/X) (Dickerson & Janda, 2006; Johnson & Montecucco, 2008; Montal, 2010; Rossetto et al., 2020). Serotypes A, B, E, and F cause the disease in humans, while other serotypes rarely affect humans (Johnson & Montecucco, 2008; Montal, 2010). BoNT/A is widely studied in research because it affects mostly humans, and its half-life is relatively longer than different serotypes (Walker & Dayan, 2014). After BoNT/A enters motor neurons, it cleaves the Synaptosomal-associated protein 25 (SNAP25), which is one of the Soluble N–ethylmaleimide sensitive factor (NSF) attachment protein receptor (SNARE) Complex protein and essential for the release of the content of vesicles into the synaptic cleft (Blasi et al., 1993; Dickerson & Janda, 2006). When the acetylcholine (ACh) molecules cannot be released into the neuromuscular junction from motor neurons, muscle cells cannot be contracted, and this eventually causes muscle paralysis (Pellizzari,

Rossetto, Schiavo, & Montecucco, 1999; Sobel, 2005). If diaphragm muscles are affected due to BoNT poisoning, this situation can lead to death in the absence of mechanical ventilation because of respiratory system failure (Simpson, 2000; Tacket, Shandera, Mann, Hargrett, & Blake, 1984).

Although BoNTs are extremely toxic, they are used at small doses as FDA approved therapeutic options in clinics for various conditions, such as lower urinary tract dysfunction, overactive bladder syndrome, limb spasticity, and they have been extensively utilized for cosmetic purposes to remove wrinkles (Chen & Kuo, 2020; Pirazzini, Rossetto, Eleopra, & Montecucco, 2017). However, there might be accidents in clinics during the toxin application causing iatrogenic botulism (Johnson & Montecucco, 2008). Additionally, these toxins are present in nature and occasionally cause food or liquid poisoning (Lonati et al., 2020; Peck, 2009). Because there is no approved treatment for BoNT intoxication after the toxin enters motor neurons, developing drugs and therapy for the intoxication of BoNTs is a critical concern to prevent devastating consequences (Kiris, Kota, et al., 2014).

1.3 Molecular Mechanism of BoNT Intoxication

Acetylcholine (ACh) is an excitatory neurotransmitter that plays a role in numerous physiological functions, for example, skeletal muscle contraction (Sam & Bordoni, 2020). ACh is released from motor neurons into the neuromuscular junction when the axon terminal is depolarized by an action potential, leading to muscle contraction (Sam & Bordoni, 2020). On the other hand, SNARE complex proteins are important for neurotransmitter release (Sam & Bordoni, 2020). As mentioned before, BoNT is composed of two chains, a heavy chain (HC) and a light chain (LC) (Rossetto et al., 2014). When BoNT reaches the target tissue, the heavy chain of it binds to specific glycoproteins, which are found on motor neurons, and internalization of the toxin occurs through receptor-mediated endocytosis (Dressler & Adib Saberi, 2005; Hambleton, 1992; Rossetto et al.,

2014). After that, the heavy chain and light chain are separated, and the light chain binds with high specificity and cleaves specific SNARE complex proteins depending on the serotypes (Dickerson & Janda, 2006; Montal, 2010).

In summary, the molecular mechanism of BoNT intoxication and ACh release inhibition follows a complex multistep process (Montecucco, Papini, & Schiavo, 1994). The process involves (1) the binding of BoNT HC to the receptor on the cell membrane; (2) the receptor-mediated endocytosis within synaptic vesicles; (3) endosome acidification; (4) translocation of BoNT LC from the acidified vesicle to the cytoplasm; and (5) the enzymatic activity of the BoNT LC on SNARE complex proteins (Johnson & Montecucco, 2008; Kiris, Burnett, Kane, & Bavari, 2014).

1.4 Drug Discovery against BoNT Intoxication

It is crucial to elucidate the cellular mechanism of BoNT intoxication to reveal the therapeutic targets for drug development. There are several potential steps to focus on for small molecule intervention in the mechanism of BoNT intoxication (*Figure 1*). Such potential steps can be divided into two main categories: inhibition of toxin entry into the cells or neutralizing the already internalized toxin inside the cells. The neutralizing antibodies and inhibitors interfering with BoNT entry into neurons can be used as therapeutic agents before the toxin enters motor neurons (Fan et al., 2015; Kiris, Kota, et al., 2014). After the toxin enters neurons, endocytosis, endosome acidification, and translocation BoNT/LC can be initial targets (Kiris, Burnett, et al., 2014; Pirazzini & Rossetto, 2017). After the catalytically active part of the toxin was translocated to the cytosol, LC inhibitors can be potential modulators to be investigated together with their effects on the ubiquitin-proteasome system (UPS) (Kiris, Burnett, et al., 2014). Therefore, the elucidation of the cellular mechanisms involved in intoxication and/or recovery in detail is important to find new potential targets to develop druggable molecules.

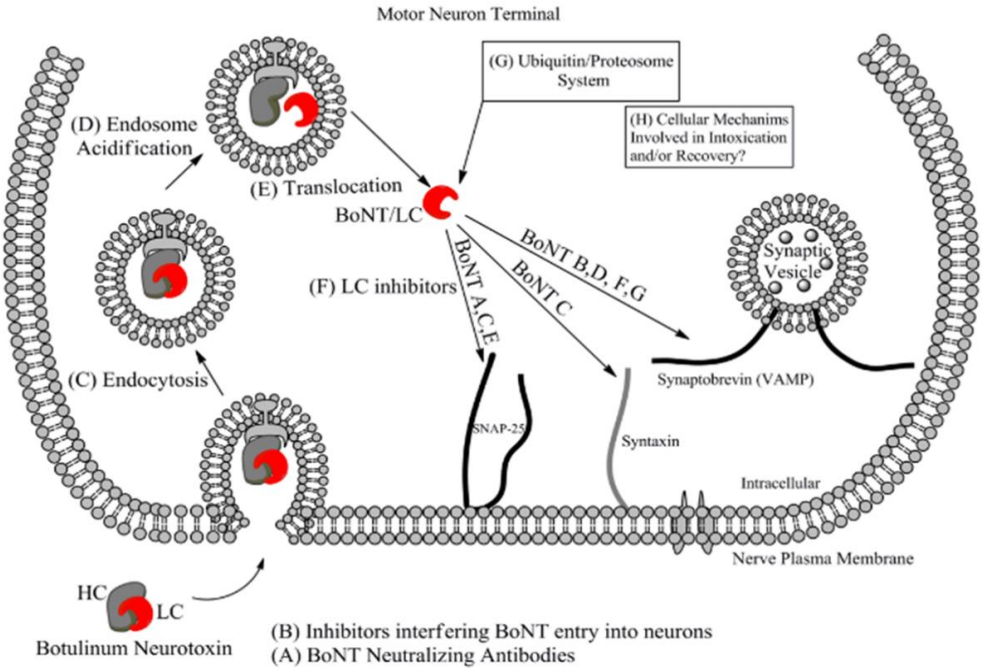


Figure 1. Potential steps of small molecule intervention for BoNT intoxication (Kiris, Burnett, Kane, & Bavari, 2014)

1.5 Inhibitors against Botulinum Neurotoxins

As mentioned above, there are various stages in the molecular mechanism of BoNT intoxication to develop drug molecules. The enzymatic part of the toxin, the light chain (BoNT LC), is the potential target for most of the previous drug development efforts (Fan et al., 2015). Inhibition of the enzymatic components of BoNTs can be a logical approach because the cleavage of SNARE complex proteins by BoNT LC leads to inhibition of the linkage between motor neurons and muscles (Pirazzini & Rossetto, 2017). Therefore, the development of many biochemical assays has been done to measure the proteolytic activity of BoNTs (Boldt, Dickerson, & Janda, 2006; Burnett et al., 2007; Opsenica et al., 2011). Such

efforts lead to identifying several chemically different LC inhibitors, including hydroxamates and aminoquinoline small molecules (Konstantinovic et al., 2018; Videnovic et al., 2014). Despite comprehensive research and development, there have been very few inhibitors to be tested in animal experiments (Pirazzini & Rossetto, 2017). Unfortunately, they all failed at various stages, so there have not been any small molecules that have progressed to human clinical trials (Lin et al., 2020). However, some of the molecules tested in animal studies like Quinolin-8-ol and EGA have demonstrated some efficacy, but their effects are limited (Azarnia Tehran et al., 2015; Bremer, Adler, Phung, Singh, & Janda, 2017). Some other studies show that Cu (II) and dithiocarbamate, and bis (thiosemicarbazone) complexes might be effective for BoNT/A intoxication, but their effects are limited to the delaying of deaths due to complications of intoxication (Bremer, Pellett, et al., 2017). Likewise, there is another molecule, Dyngo-4a, which has limited survival efficiency of 30% for only 24 hours (Seki et al., 2015). To sum up, all these molecules have limited potential for recovery, so it remains of an urgent need to develop innovative and more effective solutions to prevent the devastating consequences of BoNTs.

The modulation of UPS would be another approach for drug discovery. In other words, activation of protein degradation mechanisms of cells might be an alternative method for treating BoNT intoxication. Foreign proteins in normal functioning cells can be labeled with ubiquitin molecules for degradation by the UPS (Landre, Rotblat, Melino, Bernassola, & Melino, 2014). However, BoNT/A can escape from this system (Tsai et al., 2017). Serotype A is the longest-lasting serotype in human botulism; it can survive within the neurons for up to 6 months (Shoemaker & Oyler, 2013). However, serotype E can be active for just a few days, although it targets the same protein with BoNT/A (Shoemaker & Oyler, 2013). A recent study shows that the E3 ubiquitin ligase TRAF2 mediated ubiquitination destroys BoNT/E in neurons (Tsai et al., 2010). However, BoNT/A degradation is modulated by the E3 ligase HECTD2 and the deubiquitinase VCIP135 (Tsai et al., 2017). VCIP135 has a crucial role in escaping BoNT/A from proteasomal

degradation mechanisms because the removal of ubiquitin molecules limits the polyubiquitination of the toxin, so it stabilizes the toxin by preventing degradation of it (Tsai et al., 2017). Therefore, HECTD2 and VCIP135 activities appear to be essential for determining the lifetime of BoNT/A and the duration of its action in neurons. It is also important to mention that VCIP135 may not be the only DUB affecting LC half-life as it is shown that there is another DUB, USP9X, which might have an indirect effect on BoNT/A LC degradation (Tsai et al., 2017). The half-life of BoNT/A in cells might be potentially controlled if the E3 ligase and/or the DUBs can be manipulated. Therefore, we focused on a small molecule library targeting the ubiquitin-proteasome pathway (UPP) to identify effective molecules against BoNT activity and its half-life.

1.6 The Ubiquitin-Proteasome System (UPS)

The principal mechanism for the short-lived, damaged, and misfolded protein catabolism is the ubiquitin (Ub)–proteasome pathway (UPP) in the mammalian cells (Hershko & Ciechanover, 1998). It plays an essential role in various cellular processes, so the defects in UPP can cause the pathogenesis of several diseases (Dantuma & Bott, 2014). Therefore, to be able to develop druggable molecules targeting UPP, the understanding of this system is very crucial.

UPP consists of two main steps: ubiquitylation and proteasomal degradation (Hershko & Ciechanover, 1998). Ubiquitin is a small regulatory protein and is found in eukaryotes (Finley, 2009). It plays a role in tagging the targeted protein by covalently binding the ε-amino group of lysine in the targeted protein for degradation (Finley, 2009). For polyubiquitination, a similar binding pattern is formed between the carboxy terminus of ubiquitin and the ε-amino group of lysine of another ubiquitin molecule (Shabek et al., 2012). The ubiquitin patterns and distributions are essential for the fate of the labeled protein. Polyubiquitination is the signal for protein degradation as well as transcription factor activation, kinase

activation, and DNA repair (Thrower, Hoffman, Rechsteiner, & Pickart, 2000). There are three critical steps for the protein ubiquitination process. The first one is that the E1 ubiquitin-activating enzyme activates ubiquitin at the C-terminus (Finley, 2009; Hershko & Ciechanover, 1998). The second one is that the E2 ubiquitin-conjugating enzyme conjugates the ubiquitin (Finley, 2009; Hershko & Ciechanover, 1998). The final one is that the E3 ubiquitin ligase transfers Ub to the target protein (Finley, 2009; Hershko & Ciechanover, 1998). In the end, the 26S proteasome, which has the catalytic 20S core and the 19S regulator, recognizes the ubiquitinated proteins, unfold them, and threaded into the 20S proteolytic chamber (Coux, Tanaka, & Goldberg, 1996; Hershko & Ciechanover, 1998; Schmidt & Finley, 2014). As a result, they are cleaved into short peptide fragments (Bhattacharyya, Yu, Mim, & Matouschek, 2014). Following this, they rapidly enter into the process of the cleavage of amino acids from peptides by cellular aminopeptidases, and they are recycled (Reits et al., 2003).

1.7 The Ubiquitin-Proteasome Pathway Inhibitor Library

The UPP is an important target for developing potential therapeutic agents because the aberration of proteasome-mediated protein degradation might lead to various diseases (Dantuma & Bott, 2014). There are various steps to block the UPP, but most inhibitors specifically target the activity of the 20S proteasome (Myung, Kim, & Crews, 2001). However, there are efforts to identify modulators of specific E3 ligases and DUBs.

Generally speaking, targeting UPP is considered as a challenging drug target. However, recent studies have identified various highly promising small molecules that either work on DUBs or E3 ligases. For example, Thalidomide Immunoprin, Lenalidomide Revlimid, and Pomalidomide Pomalyst are FDA-approved E3 modulators marketed by Celgene (Ito et al., 2010; Kronke et al., 2014; G. Lu et al., 2014; Wertz & Wang, 2019). VLX1570 is a proteasome-associated deubiquitinase inhibitor that is recently published in clinical

development (Wang et al., 2016; Wertz & Wang, 2019). There are also several E3 and DUB modulators in preclinical testing, such as Auxin, CC-885, ML364, P22077 (Davis et al., 2016; Matyskiela et al., 2016; Pozhidaeva et al., 2017; X. Tan et al., 2007; Wertz & Wang, 2019).

In our study, we focused on UPP pathway modulators to potentially affect ubiquitination and/or deubiquitination of the LC to affect the duration of botulinum neurotoxin type A light chain directly.

The ubiquitin-proteasome pathway inhibitor library utilized in this study was purchased from LifeSensors (SI9032), and it contains 32 ubiquitin-proteasome pathway inhibitors (10 mM in DMSO). The map of the library is shown in *Figure 2*

	1	2	3	4	5	6	7	8	9	10	11	12
A	Empty	SI9629 IU1	SI9639 LDN-57444	SI9659 WP1130	SI9669 b-AP15	SI9679 TCID	SI9689 NSC-632839	SI9649 1,10-Phen.	SI9619 PR-619	SI9699 P22077	Empty	Empty
B	Empty	SI9710 MG-132	SI9715 MLN9708	SI9720 PS-341	SI9725 MLN2238	SI9730 Aclacinomycin	SI9735 Oprozomib	SI9740 Celastrol	SI9790 Carfilzomib	SI9750 DbeQ	SI9770 MDBN	Empty
C	Empty	SI9810 PYR-41	SI9815 Serdemetan	SI9820 Thalidomide	SI9825 NSC-207895	SI9830 MLN-4924	SI9835 RITA	SI9840 TAME-HCl	SI9845 SKPin C1	SI9850 NSC-66811	SI9860 SL-01	Empty
D	Empty	SI9870 Nutlin-3	SI9880 SMER3	SI9890 AT-406	DMSO	Empty	Empty	Empty	Empty	Empty	Empty	Empty
E	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
F	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
G	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
H	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty

Figure 2 The map of the UPP-Inhibitor Library (Life sensor, SI9032).

In detail, the library contains seven proteasome inhibitors, IU1, LDN-57444, MG132, MLN9708, PS-341, MLN2238, and Oprozomib (Adams, 2002; Guo & Peng, 2013; Kupperman et al., 2010; Lee et al., 2010; Schrader et al., 2016; Y. Y. Tan, Zhou, Wang, & Chen, 2008; Zhu et al., 2019). There are also DUB inhibitors, which are WP1130, b-AP15, TCID, NSC 632839, PR-619, in this library (Aleo, Henderson, Fontanini, Solazzo, & Brancolini, 2006; Altun et al., 2011; D'Arcy & Linder, 2012; Driessen et al., 2015; Tian et al., 2011). Among these, WP1130 and PR-619 are very important for this study because they target USP9X and VCIP135, respectively (Altun et al., 2011; Driessen et al., 2015; Kapuria et al., 2010). The other DUB inhibitor is P22077, which inhibits the ubiquitin-specific protease USP7 and the closely related DUB, USP47 (Altun et al., 2011; Tian et al., 2011). Thalidomide and SMER3 target E3 ligase activity (Aghajan et al., 2010; Ito, Ando, & Handa, 2011; Ito et al., 2010). On the other hand, there is a small molecule in the library, 1,10-phenanthroline (o-phenanthroline), which is a chelator for divalent metal ions and affects UPP indirectly (Cooper et al., 2009). Aclacinomycin A, Celastrol, and Carfilzomib are specific inhibitors of the 20S proteasome chymotrypsin-like activity, and it is important to note that Carfilzomib is an FDA-approved drug (Figueiredo-Pereira, Chen, Li, & Johdo, 1996; Kiaei et al., 2005; Landgren et al., 2019; Sethi, Ahn, Pandey, & Aggarwal, 2007; Westerheide et al., 2004). The other compounds affect different pathways; for example, DBeQ acts as a reversible inhibitor of the AAA-ATPase p97 and blocks both ubiquitin-dependent and endoplasmic reticulum-associated degradation pathways, protein clearance pathways (Auner et al., 2013; Bastola, Neums, Schoenen, & Chien, 2016). MLN-4924 inhibits of NEDD8-dependent pathways, which have a critical role in mediating ubiquitination (Soucy, Dick, Smith, Milhollen, & Brownell, 2010). On the other hand, MDBN is known as an irreversible inhibitor of p97/valosin-containing protein that plays a vital role in cells by using its ATPase activity (Mori-Konya et al., 2009). NSC-207895 is an inhibitor of the oncogene, MDMX, which is a critical negative regulator of the tumor suppressor p53 (Yu et al., 2020). Serdemetan is an oral, phase 1 HDM2

antagonist, which also a key negative regulator of the tumor suppressor p53 (Jones et al., 2013). NSC-66811, Nutlin-3, and SL-01 inhibit the p53-MDM2 interaction (Li, Zhang, Gao, Chen, & Xie, 2011; Y. Lu et al., 2006; Vassilev et al., 2004). PYR-41 irreversibly inhibits E1, but it has little or no activity against E2s or E3s (Brahemi, Burger, Westwell, & Brancale, 2010; Yang et al., 2007). RITA blocks p53-HDM2 interaction and p53 ubiquitination (Chuang et al., 2014; Wanzel et al., 2016). TAME-HCl inhibits the ubiquitin ligase anaphase-promoting complex/cyclosome (APC/C) (Zeng & King, 2012; Zeng et al., 2010). The small molecule, SKPin C1, inhibits the cullin-RING ubiquitin E3 ligase SCF-Skp2 (Rico-Bautista & Wolf, 2012; Wu et al., 2012). Finally, AT-406 is known as an inhibitor of apoptosis proteins (Brunckhorst, Lerner, Wang, & Yu, 2012).

1.8 The aim of this study

There are no therapeutic agents for BoNT/A intoxication after the toxin enters into motor neurons (Sobel, 2005). Therefore, it is crucial to develop a therapeutic approach to prevent the devastating consequences of BoNT/A after the entrance to motor neurons. Previous studies identified specific E3 ligase and DUBs that affect BoNT/A LC degradation (Tsai et al., 2017). In this study, we aimed to screen a small molecule ubiquitin-proteasome system library to identify compounds that modulate BoNT/A LC half-life and thereby its activity in cells. Identification of such modulators could be important for basic research as molecular tools to better understand intoxication and/or recovery mechanisms. Additionally, such BoNT/A LC half-life modulators can be important for its clinical usage as well as drug development efforts against the toxins.

CHAPTER 2

MATERIALS AND METHODS

2.1 Differentiation of Mouse Embryonic Stem Cells to Motor Neurons

This work utilized a mouse embryonic stem (ES) cell line and previously established, well-characterized protocols to generate motor neurons from the ES cells (Kiris et al., 2011). Directed differentiation of the ES cells to motor neurons weres based on Retinoic Acid and Sonic Hedgehog induction method (Wichterle, Lieberam, Porter, & Jessell, 2002) Briefly, HBG3 ES cells were co-cultured with mitomycin-inactivated mouse embryonic fibroblast (MEF) cells in DMEM for ES cells supplemented with 15% fetal bovine serum (FBS), 1000 Unit/ml Leukemia Inhibitory Factor (LIF), β-mercaptoethanol (final concentration 0.1 mM), 1% Penicillin-Streptomycin, 1% Glutamax, and 1% Non-essential amino acids. For motor neuron differentiation, the mouse ES cells were separated from the feeder cells using enzymatic methods and then resuspended in the differentiation medium containing 1:1 Advanced DMEM-F12 and Neurobasal medium supplemented with 1% Penicillin-Streptomycin, 1% Glutamax, β-mercaptoethanol (final concentration 0.1 mM), and 15% Knockout Serum Replacement (KSR). To form embryoid bodies (EBs), the dissociated ES cells were seeded onto sterile low-attachment dishes. The next day as Day 1, EBs were harvested and cultured in a fresh differentiation medium for 24 hours. Retinoic Acid (1 μM, Sigma) was added following two days for 24 hours to induce neuralization. Then, EBs have treated with Hh-Ag1.5 (Cellagentech, C4412-2s) on day 3 to induce motor neuron specification. On day 4, 5 more mL of fresh ADFNK media supplemented with fresh Hh-Ag 1.5 compound (1 μM final concentration) was added to each plate. On day 5, EBs were transferred to new dishes containing fresh differentiation medium including 1:1 Advanced DMEM/ F12 and Neurobasal medium supplemented with 1% Penicillin-Streptomycin, 1%

Glutamax, and 2% B27 serum-free supplement (Invitrogen), brain-derived neurotrophic factor (10 ng/ml, Chemicon), glial cell-derived neurotrophic factor (100 ng/ml, R&D Systems), cliary-derived neurotrophic factors (10 ng/ml, Chemicon), and Neurotrophin3 (10 ng/ml, Chemicon). After two days, Day 7, the dissociation of EBs was done using accutase (Thermo Fisher Scientific, A1110501) based method, and dissociated EBs were plated onto matrigel (BD Biosciences, San Jose, CA) coated dishes. To allow the elongation of neurites, they were incubated for an additional three days.

To ensure whether the differentiated cells are motor neurons are not, the cell line with motor neuron-specific transcription factor Hb9 promoter was used. GFP expression is driven in that cell line with the specific promoter. They were cultured and differentiated into motor neurons as described in detail above.

2.2 Compound Treatment and BoNT/A Intoxication

Compound stock concentrations were 10 mM, dissolved in dimethyl sulfoxide (DMSO). All studies utilizing BoNT/A holotoxin (Metabiologics) were conducted by highly trained professionals in the United States (Frederick, MS, USA) through collaboration. Only LC transfection studies have been performed at METU. For holotoxin experiments, ES cell-derived motor neurons were cultured in 24-well plate formats and pre-treated with compounds before the toxin administration. They were incubated with compounds for 30 minutes at 37 °C with 5 % CO_2. All procedures were performed under biosafety level II conditions, and working areas were decontaminated before and after experiments. Cells were incubated with BoNT/A in a humidified incubator with 5% CO_2 at 37°C. At the end of the experiments, the toxin was removed through approved protocols, and cell lysis was obtained by scraping the cells in lysis buffer.

2.3 Transformation and Plasmid Purification

The pE-YFPC1 plasmid encoding YFP-tagged LCA (50 ng) was transformed into XL1-Blue competent cells. The transformed cells were grown on Luria-Bertani (LB) agar plate containing Kanamycin. After 18 hours, a single colony was selected using a sterile pipette tip from the LB agar plate containing Kanamycin, and it was dropped in the liquid LB medium. The bacterial culture was loosely covered with sterile aluminum foil and incubated at 37°C for 12-18 hr in a shaking incubator. For plasmid purification, ZymoPURE II Plasmid Midiprep Kit was used. The plasmids were purified according to the manufacturer's protocol.

2.4 Cell Culture

Cell culture conditions of the mouse ES cells are given in section 2.1. The other utilized cell line in this work was the human embryonic kidney cell line, HEK293. HEK293 cells were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, and 2% penicillin-streptomycin. They were grown at 37°C with 5% CO_2 and passaged when 70% confluent.

2.5 Transfection

TurboFect Transfection Reagent was used for transfecting pE-YFPC1 plasmid expressing BoNT/A LC into HEK293 cells. The cells were seeded on a 12-well or 24 well tissue culture plate depending on the experimental design and then allowed to attach to the surface and reach approximately 70-80% confluency for 24 hours. After the cells reached 70-80% confluency, they were transfected with pE-YFPC1 plasmid expressing BoNT/A LC genes. For HEK293 cells, the WT SNAP25 plasmid was also co-transfected with the pE-YFPC1 plasmid expressing BoNT/A LC in a 1:4 ratio of DNA: transfection reagent (Turbofect Transfection Reagent, Thermo Scientific Inc.) according to the manufacturer's instructions. The cells were

collected at specified time points by using a cell scraper in NP-40 lysis buffer containing fresh protease and phosphatase inhibitors for Western blot.

2.6 MTT Assay

The proliferation assay, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, was performed to look at the effects of selected compounds on the viability of HEK293 cells.

80.000 cells were seeded per well in a 96-well plate and then incubated for 24 hours at 37^{o}C, 5% CO_2 for the cells to attach to the surface of the plate. Then, 10 lead compounds (WP1130, b-AP15, NSC 632839, PR-619, P22077, Celastrol, MDBN, PYR-41, NSC 66811, SL-01) were added in specified concentrations for different wells. After 4 hours, the MTT solution, which is dissolved in PBS free from Ca^{2+} and Mg^{2+}, was added to the cells. After 4 hours of incubation, a 1% SDS-0.01M HCl (hydrochloric acid) solution was added for each well to dissolve formazan crystals. The plate was incubated for 4-18 hours. Finally, the absorbance was measured in a microplate reader (Thermo Fisher Scientific, USA) at 570 nm.

For the interpreting of results, the absorbance reading of the blank was subtracted from all absorbance reading of samples. Then, absorbance readings from test samples are divided by those of the control and multiplied by 100. This gives the percentage of cell viability or proliferation. The formula is given below.

$$\%\ Viable\ Cells = \frac{(abs_{sample} - abs_{blank})}{(abs_{control} - abs_{blank})} x100$$

2.7 Cycloheximide and Compound Treatment

Before cell seeding, the plates were coated first with 0.1% gelatin for 30 minutes. Then, the plates were double-coated with Poly-D-Lysine (PDL) (Sigma-Aldrich, P0899) solution. HEK293 cells were seeded onto a double-coated 24-well

plate in 500 µl of growth medium 24 hours before transfection. After 24 hours, the cells were transfected with 1 µg plasmid encoding YFP-tagged LC/A. Cycloheximide (25 µg/ml) (Sigma-Aldrich, C7698) diluted in DMEM was added to the transfected cells to inhibit new protein synthesis 24 h after transfection. For each condition, the cells were collected at that time point as a control. The rest of the plate was incubated for 2 hours in a humidified incubator with 5% CO_2 at 37°C. After 2 hours, the media containing the cycloheximide (CHX) was removed. The selected compounds were diluted to 20 µM concentration in DMEM and then given to the cells. For each condition, the cells were collected at that time point as well. At the specified time points, cells were lysed in NP-40 lysis buffer containing freshly added protease and phosphatase inhibitors and processed for western blotting.

2.8 Sample Preparation for SDS-PAGE

The cells were collected in NP-40 lysis buffer containing freshly added protease inhibitor (cOmplete Protease Inhibitor Cocktail, Roche) and phosphatase inhibitor (PhosSTO, Roche). After that, the cell lysates were stored at -80ºC. Before SDS-PAGE, the cell lysates were thawed on ice and centrifuged at 12000 rpm for 20 minutes (Nüve, NF800R) at +4ºC. The supernatant contains total protein extract, so it was separated from the pellet and mixed with 6X Laemmli Sample Buffer in a new tube.

2.9 Western Blot

The proteins were separated by the SDS-PAGE method at 220 V in 12% polyacrylamide gel after denaturation at 95ºC for 5 minutes. As a marker, PageRuler Plus Prestained Protein Ladder, 10 to 250 kDa (Thermo Scientific, 26619), was used. After that, the proteins on the gel were transferred to a PVDF (Polyvinylidene Fluoride) membrane (Serva, 4251401) with 0.45 µM pore size at 30 V for 2 hours in wet conditions. Then, the membrane was blocked in 5% skimmed milk dissolved in

0.01 % TBS-T. After that, the membrane was incubated in the primary antibody solution overnight at 4^{o}C. The membrane was washed two times for 10 minutes in 0.01% TBS-T and blocked in 5% skimmed milk dissolved in 0.01% TBS-T for 20 minutes at room temperature. Then, the membrane was incubated in a secondary antibody solution for 1 hour at room temperature. The membrane was washed three times for 10 minutes in 0.01% TBS-T. The visualization of it was carried out by using the SynGene GeneGnome Chemiluminescence Imaging System. The enhanced chemiluminescence (ECL) kit (WesternBright ECL HRP substrate, Advansta, K-12045-D50) was used for visualization.

Table 1 Antibodies that are used in this study

Target Protein	**Host**	**Brand**	**Dilutions**	**Media**
SNAP-25	Mouse	BioLegend	1:2500	5% skimmed milk
GFP	Rabbit	Invitrogen	1:2500	5% skimmed milk
GFP	Mouse	Santa Cruz	1:2000	5% skimmed milk
GAPDH	Mouse	Santa Cruz	1:2000	5% skimmed milk
β-actin	Mouse	Santa Cruz	1:4000	5% skimmed milk

2.10 Buffers and Solutions

The recipes of buffers used in this study and their preparation of them are given in Appendix C.

2.11 Statistical Analysis

Data were analyzed using Student's *t*-test and reported as the mean ± SEM (GraphPad Prism Software 9.0 and Microsoft Excel). $p<0.05$ was stated as statistically significant.

RESULTS

3.1 Preliminary Studies

3.1.1 Western Blot Analysis of Stem Cell-Derived Motor Neurons Treated with BoNT/A Holotoxin in Indicated Doses

Mouse ES cell-derived motor neurons are suitable model systems to screen compounds against BoNT intoxication (Kiris et al., 2011). We first sought to determine the sensitivity of ES cell-derived motor neurons to the toxin to be utilized in the study. To do so, we intoxicated the motor neurons derived from ES cells with BoNT/A holotoxin in increasing concentrations (0.5 nM, 1 nM, 5 nM, and 20 nM) to determine the effect of the toxin on SNAP-25 protein cleavage. It is well established that SNAP-25 is the only known biological target of BoNT/A, and the toxin-mediated SNAP-25 protein cleavage is routinely used as a read-out in the field (Kiris, Kota, et al., 2014). The percentage of full-length SNAP-25 was determined in each condition and was compared to DMSO control. DMSO treatment was used as a negative control. In the literature, it has been demonstrated that Toosendanin can be an effective cure for experimental botulism (Shi & Wang, 2004). Therefore, Toosendanin treatment, together with 0.5 nM BoNT/A, was used as a positive control group. The full protection was observed after Toosendanin treatment as expected. β-Actin was used as the loading control. Our results show that the percentage of full-length SNAP-25 decreases while the toxin concentration increase (*Figure 3*). The decrease in the percentage of full-length SNAP-25 was statistically significant for each dose. Based on our results, 0.5 nM was considered optimal concentrations for the rest of the study because approximately 50% cleavage was observed, which should allow detection of increased or decreases SNAP-25 cleavage detection upon compound treatments.

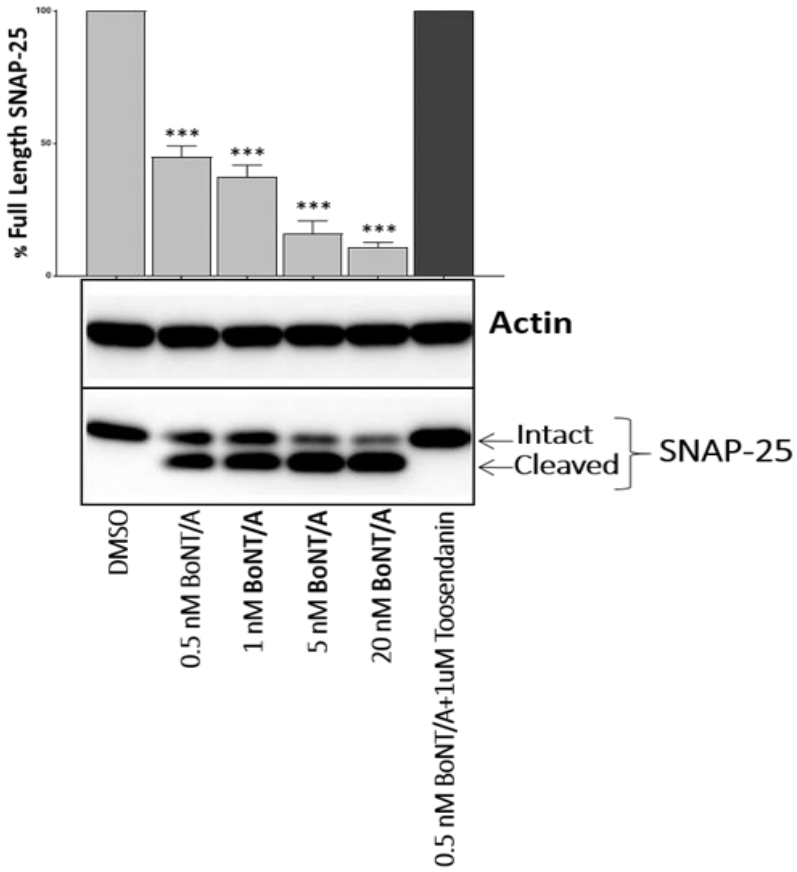


Figure 3 Western blot analysis of SNAP-25 in ES cell-derived motor neurons treated with BoNT/A holotoxin in indicated doses. Mouse ES cell-derived motor neurons were treated with BoNT/A holotoxin with 0.5 nM, 1 nM, 5 nM and 20 nM concentrations and the percentage of full length SNAP-25 was determined. DMSO was used as a negative control. 1 μM Toosendanin treatment with 0.5 nM BoNT/A was used as a positive control group. β-Actin was used as a loading control. Data are presented as means ± SEM calculated from three independent samples and compared by unpaired t-test to DMSO control condition.

3.1.2 The Initial Screen of Small Molecule Library Targeting the UPP

After determining optimal toxin concentration for approximately 50% SNAP-25 cleavage, the initial screen of small molecules targeting the UPP was conducted. The effects of small molecules in the library on 500 pM BoNT/A intoxication were investigated by western blotting. Mouse ES-derived motor neurons were treated with the compounds 30 minutes before intoxication, and then cells were intoxicated with 500 pM BoNT/A for a total of 4 hours. Similar to Figure3, SNAP-25 cleavage was utilized as a read-out to determine the efficacies of the compounds. Based on the entire library screening (total 32 compounds), WP1130, b-AP15, NSC-632839, PR619, P22077, Celastrol, MDBN, PYR-41, NSC-66811, SL-01 were selected for further analysis because of their statistically significant effects on the protection of SNAP-25 cleavage. DbeQ also showed a significant effect, but it was not selected for further analysis because of the decrease in the level of β-Actin. The decrease might be caused by potential cell death after DbeQ treatment. Also, Serdemetan was not selected for further analysis because of its possible toxic effects on neurons under the fluorescence microscope, although it showed a statistically significant effect. DMSO treatment and DMSO treatment with administration of BoNT/A were used as control groups because the compounds were solved in DMSO. Also, Toosendanin treatment was used as a positive control group, and as expected, it demonstrated full protection against the toxin. β-Actin was used as a loading control.

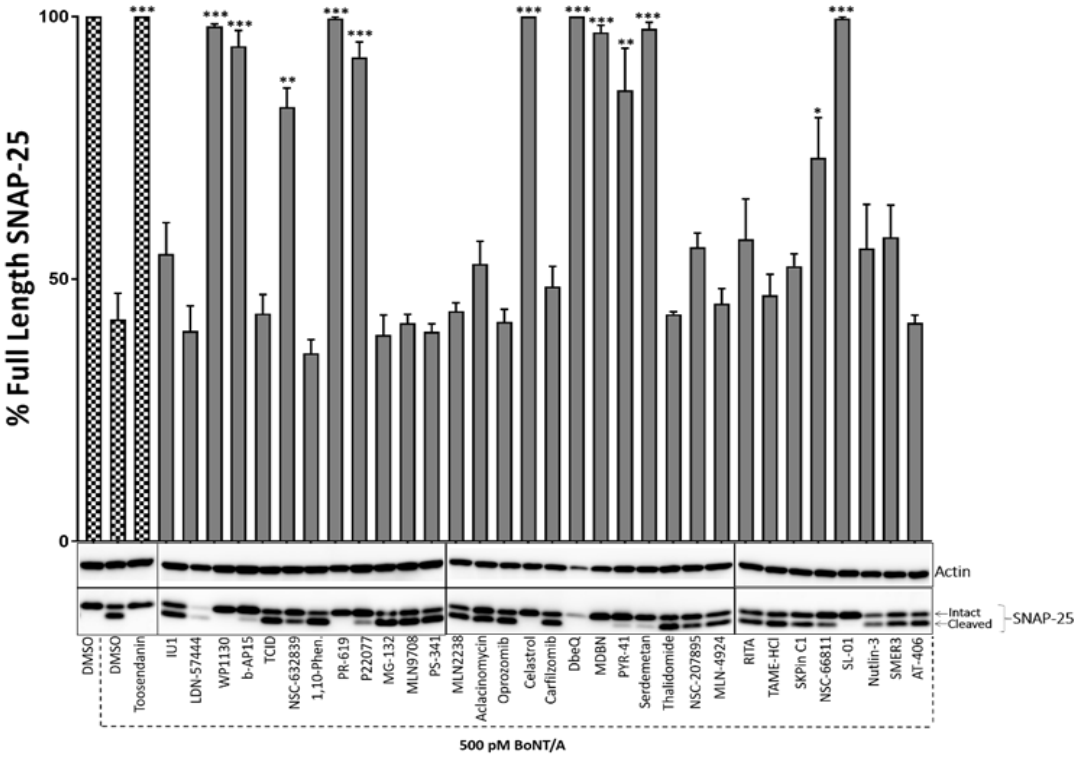


Figure 4 Initial screen of UPS library with western blot anaylsis. The differences in the percentage of full length SNAP-25 was shown under the effects of compounds in 500 pM BoNT/A intoxication. Control groups were treated with DMSO and Toosendanin. β-Actin was used as loading control. Data are presented as means ± SEM calculated from three independent experiments and compared by unpaired t-test to DMSO+500pM BoNT/A condition.

3.1.3 The Selected Compounds

After the initial screening of the UPP-inhibitor library (Life sensor, SI9032), the rest of our work focused on selected lead compounds (WP1130, b-AP15, NSC632839, PR-619, P22077, Celastrol, MDBN, PYR-41, NSC66811, and SL-01), which were tested in further investigations. Their 2D- structures were shown in *Figure 6*. Following the subtitles of 3.1.3 provides literature information on selected lead compounds.

3.1.3.1 WP1130

WP1130, also known as degrasyn, is a cell-permeable small molecule (Kapuria et al., 2010). It is a deubiquitinase (DUB) inhibitor and directly inhibits DUB activity of USP9X, USP14, USP5, UCH37 (Driessen et al., 2015; Kapuria et al., 2010). Identification of WP1130 in our screen against BoNT/A might be highly important as it was shown that one of WP1130 targets, USP9X, affects the turnover of BoNT/A LC (Tsai et al., 2017). It also blocks Jak2 signaling through Jak2 ubiquitination (Kapuria et al., 2011).

3.1.3.2 b-AP15

b-AP15 is a cell-permeable small molecule and a deubiquitinase (DUB) inhibitor (Ward et al., 2020). It inhibits USP14 and UCHL5, which are two proteasome-associated DUBs (D'Arcy & Linder, 2012; Schmidt et al., 2019; Zhang et al., 2020). It affects upstream of the ubiquitin-proteasome cascade by suppressing both proteasomal regulatory 19S subunits (Schmidt et al., 2019; Zhang et al., 2020).

3.1.3.3 NSC632839

NSC632839 is a small molecule and DUB inhibitor, specifically inhibits USP2, USP7, and SENP2 (Nicholson et al., 2008). It also inhibits deSUMOylases (Nicholson et al., 2008). This molecule has a capacity for sustaining caspase-3/caspase-7 activity when caspase-9 has no functionality and activates apoptosis when there is no functional apoptosome (Aleo et al., 2006).

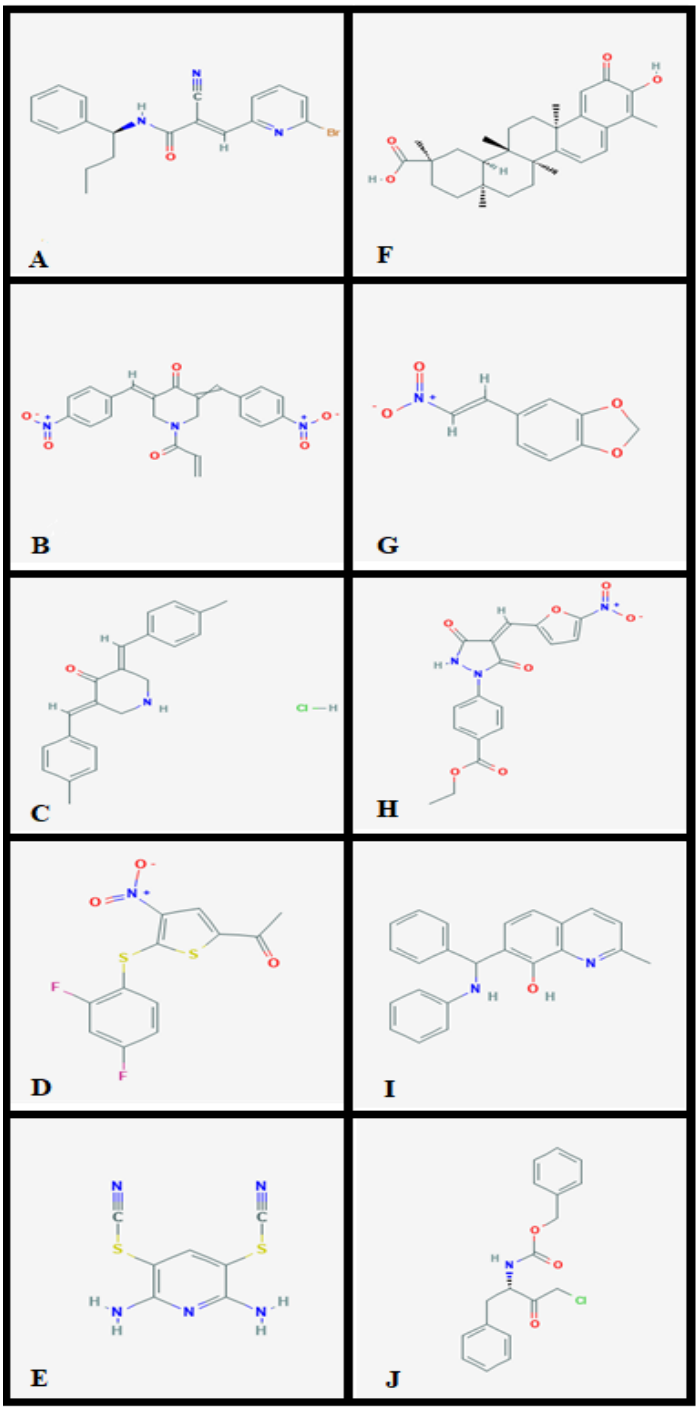


Figure 5 2D- Chemical structures of lead compounds. **A.** WP-1130 **B.** bAP-15 **C.** NSC632839 **D.** PR-619 **E.** P22077 **F.** Celastrol **G.** MDBN **H.** PYR-41 **I.** NSC66811 **J.** SL-01 (National Center for Biotechnology Information, 2020)

3.1.3.4 PR-619

PR-619 is a novel cell-permeable small molecule and a broad-spectrum active ubiquitin/ UbL isopeptidase inhibitor (Altun et al., 2011; Cowell, Ling, Swan, Brooks, & Austin, 2019; Tian et al., 2011). It reversibly inhibits DUBs in *in vitro* assays (Altun et al., 2011; Tian et al., 2011). Because it is a broad-spectrum inhibitor, it inhibits many DUBs, including VCIP135 (Altun et al., 2011; Tian et al., 2011). VCIP135 is an important target because it affects the half-life of BoNT/A LC (Tsai et al., 2017). Therefore, identification of PR-619 is crucial for this study.

3.1.3.5 P22077

P22077 (1-(5-((2, 4-difluorophenyl) thio)-4-nitrothiophen-2-yl) ethenone) is a cell-permeable small molecule, which is a DUB inhibitor, specifically for ubiquitin-specific-processing protease 7 (USP7), and it also inhibits the closely related deubiquitinase USP47 (Altun et al., 2011; Tian et al., 2011). It has weak or no inhibitory effects on proteases (Altun et al., 2011; Tian et al., 2011). P22077 treatment with 20-40 μM doses leads to the accumulation of K48-linked ubiquitination (Altun et al., 2011; Tian et al., 2011).

3.1.3.6 Celastrol

Celastrol is a naturally occurring cell-permeable small molecule derived from the Thunder of God Vine (Boridy, Le, Petrecca, & Maysinger, 2014). It has anti-inflammatory activity (Sethi et al., 2007). The IC50 value of it is 270 nM for the inhibition of NFκB (Kiaei et al., 2005; Sethi et al., 2007; Westerheide et al., 2004). On the other hand, its IC50 value is 2.5 μM to inhibit 20S proteasome chymotrypsin-like activity (Kiaei et al., 2005; Sethi et al., 2007; Westerheide et al., 2004). Celastrol also enhances cytoprotection and heat shock response in numerous cells (Kiaei et al., 2005; Sethi et al., 2007; Westerheide et al., 2004).

3.1.3.7 3,4-Methylenedioxy-beta-nitrostyrene (MDBN)

MDBN is a cell-permeable small molecule and inhibits p97 irreversibly (Chou & Deshaies, 2011). p97 plays a role in the disassembly of SNARE proteins after membrane fusion, and it is also known as Syk inhibitor III (Chou & Deshaies, 2011).

3.1.3.8 PYR-41

PYR-41 is a cell-permeable small molecule and inhibits ubiquitin-activating enzyme E1 irreversibly while it does not have any effects on E2 and E3 (Chou & Deshaies, 2011). It also enhances total sumoylation in cells in addition to blocking ubiquitylation (Yang et al., 2007). It also blocks the degradation of p53, attenuates NFκB-dependent pathways (Brahemi et al., 2010; Yang et al., 2007).

3.1.3.9 NSC-66811

NSC66811 is a non-peptide, cell-permeable small molecule and inhibits MDM2, which is known as E3 ubiquitin-protein ligase (Y. Lu et al., 2006). It activates p53 by disrupting Human Murine Double Minute 2 (MDM2)-p53 interaction (Y. Lu et al., 2006). The inhibitor constant (Ki) is 120 nM for NSC-66811 (Golubovskaya & Cance, 2013).

3.1.3.10 SL-01

SL-01 is a cell-permeable small molecule (Li et al., 2011). It primarily inhibits the interaction of the transcription factor p53 and the oncoprotein MDM2 (Li et al., 2011). It is known that p53 is a tumor suppressor regulating apoptosis and cell growth inhibition (Aubrey, Kelly, Janic, Herold, & Strasser, 2018). On the other hand, MDM2 is known as an oncoprotein that suppresses p53 activity by inhibiting

transcriptional activity directly and enhancing p53 degradation via the UPP (Brooks & Gu, 2006). SL-01 targets that interaction and inhibits it by affecting UPP (Li et al., 2011).

3.1.4 Dose-dependent Effects of Selected Compounds on SNAP-25 Degradation

Selected compounds were evaluated for their potential dose-dependent effects on the inhibition of BoNT/A mediated SNAP-25 cleavage. The ES-cell-derived motor neurons were treated with selected compounds at 1.25 μM, 2.5 μM, 5 μM, 10 μM, and 20 μM concentrations 30 minutes before 500 pM BoNT/A holotoxin intoxication initiated. Similar to the initial screen, SNAP-25 cleavage was utilized as a read-out, and the effectiveness of the compounds was tested by determining the percentage of full-length SNAP-25 in each experimental condition, compared to controls. SMER3, which did not show significant protection in the initial screen (Figure 4), was utilized as a control, and it did not lead to dose-dependent protection. DMSO treatment without any other treatment was used as a negative control. DMSO treatment, together with BoNT/A, was used as a control to determine the total SNAP-25 cleavage of the toxin in the experiment. Toosendanin treatment was used as a positive control group showing full recovery. β-III Tubulin was used as a loading control on western blots. Our results showed that 20 μM is the most effective dose for the most efficacious compounds to inhibit BoNT/A mediated SNAP-25 cleavage. Excitingly, many of our lead compounds, including PR-619 and WP1130, led to dose-dependent protection against the BoNT/A challenge.

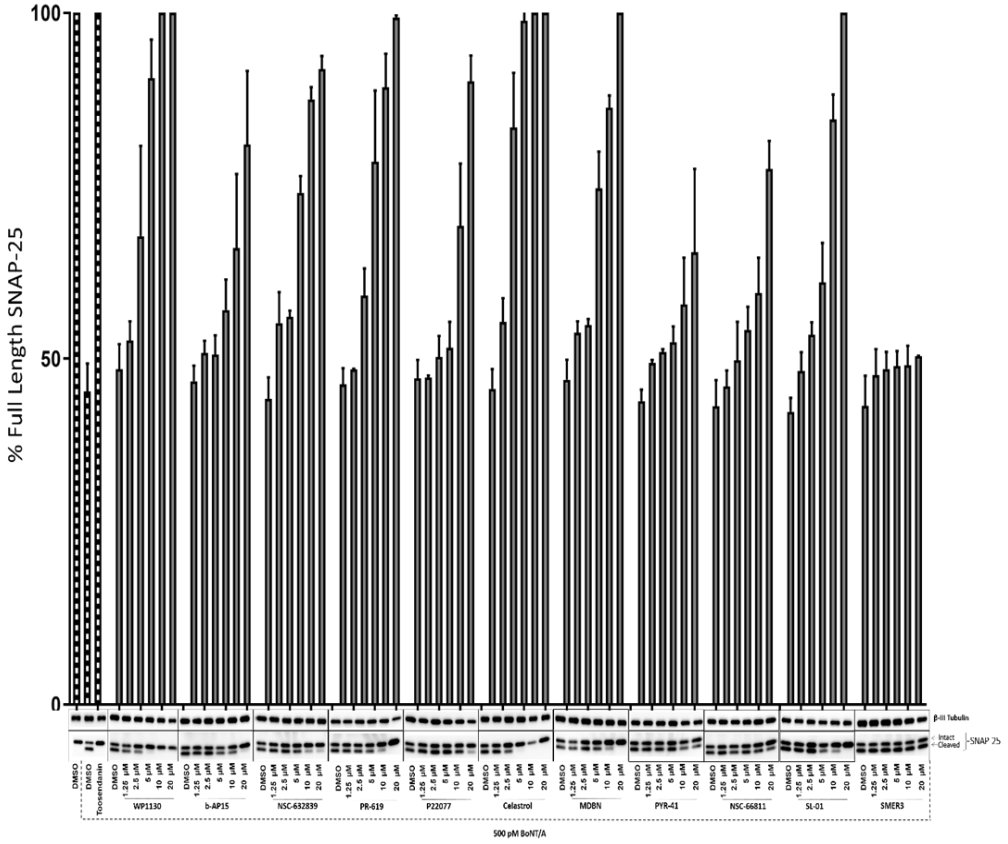


Figure 6 Western blot analysis of ES cell-derived motor neurons treated with selected compounds in 500 pM BoNT/A intoxication. The differences in the percentage of full length SNAP-25 after 500 pM BoNT/A intoxication and treatment with selected compounds in specified doses. Control groups were treated with DMSO and Toosendanin. β-III Tubulin was used as a loading control. Data are presented as means ± SEM.

3.2 The Effects of Selected Compounds on the Cell Viability in Different Doses

Cell viability assays are frequently performed for the assessment of compound cytotoxicity, evaluation of growth conditions, and determination of cell health (Class et al., 2015). Therefore, we performed an MTT assay to determine cell viability upon compound treatments to determine whether the compounds have any toxic effects on cells. The fact that HEK293 cells are

easy to grow and maintain with high reproducibility brings about them to use in cell viability assays extensively (Class et al., 2015).

HEK293 cells were treated with selected compounds at 1.25 μM, 2.5 μM, 5 μM, 10 μM, and 20 μM concentrations for 4 hours. Because compounds were dissolved in DMSO, the control group was treated with DMSO. Based on our results, we have not detected any significant viability differences between the selected compounds and the control groups.

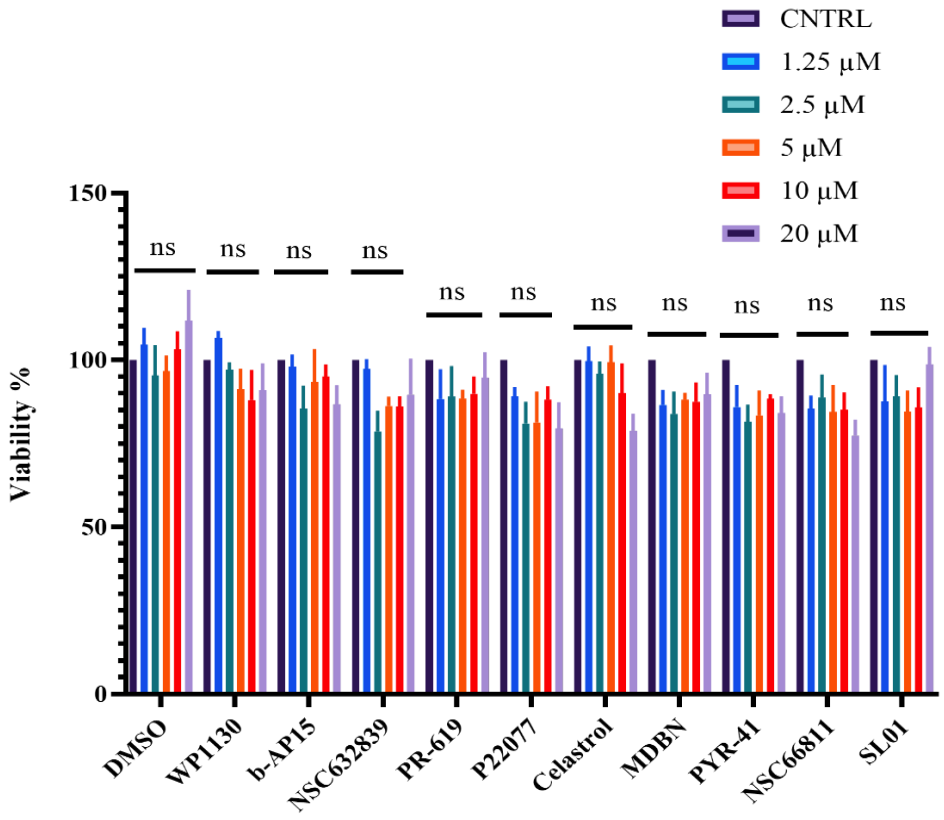


Figure 7 The percentage of HEK293 cell viability. MTT assay was performed by using HEK293 cells treated with selected compounds at 1.25 µM, 2.5 µM, 5 µM, 10 µM, and 20 µM concentrations. Data are presented as means ± SEM calculated from three independent samples and compared by unpaired t-test.

3.3 The Effects of Selected Compounds on the Degradation of BoNT/A LC in Cells

3.3.1 Determination of the Optimal amount of YFP tagged BoNT/A LC Plasmid and the Optimal Post-transfection Incubation Time

Before investigating the effects of the selected compounds on BoNT/A LC degradation, we sought to establish our cell culture system with BoNT/A LC transfection. To do so, we determined the optimal plasmid amount and time for

transfection of A/LC plasmid tagged with YFP to HEK293 cells. We tested 0.1 µg, 0.5 µg, and 1 µg A/LC transfections for 12 hours and 24 hours. The result showed that the transfection of HEK293 cells for 12 hours was not sufficient to see YFP-LCA expression. Based on our results shown in *Figure 8*, we decided to utilize 1 µg YFP-LCA plasmid transfection for 24 hours in HEK293 cells for our stability experiments.

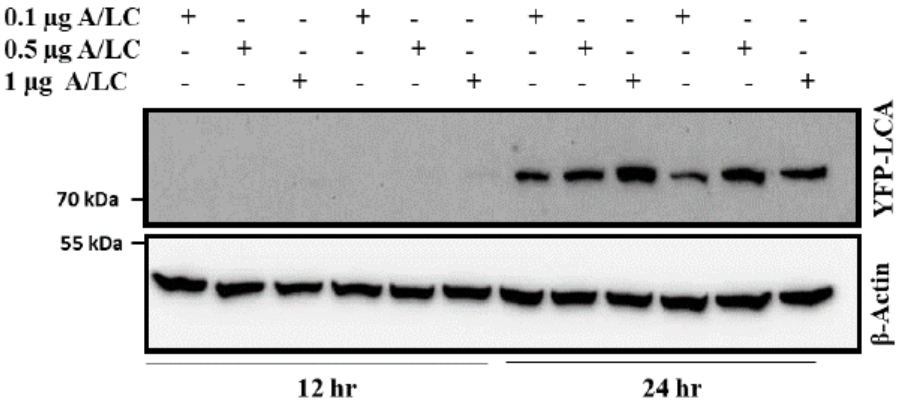


Figure 8 Comparisons of LC/A Stability in different dosage in indicated time points. HEK293 Cells transfected with YFP-LCA in indicated concentrations at 12 hr and 24 hr. β-Actin was used as a loading control.

3.3.2 Transfection Efficiency in HEK293 Cells

We utilized FLoid Cell Imaging Station to observe transfection efficiently in both control and transfected conditions based on the selected dose and time of the plasmid shown in *Figure 8*. Fluorescent images of HEK293 cells in the non-transfected and transfected state are shown in *Figure 9*. As our BoNT/A LC plasmid is YFP tagged, we were able to determine transfection efficiency using FLoid Cell Imaging Station, and our results suggest that the conditions we chose led to a quite

high transfection efficiency. We have regularly checked the transfection efficiency using FLoid in each experiment we run.

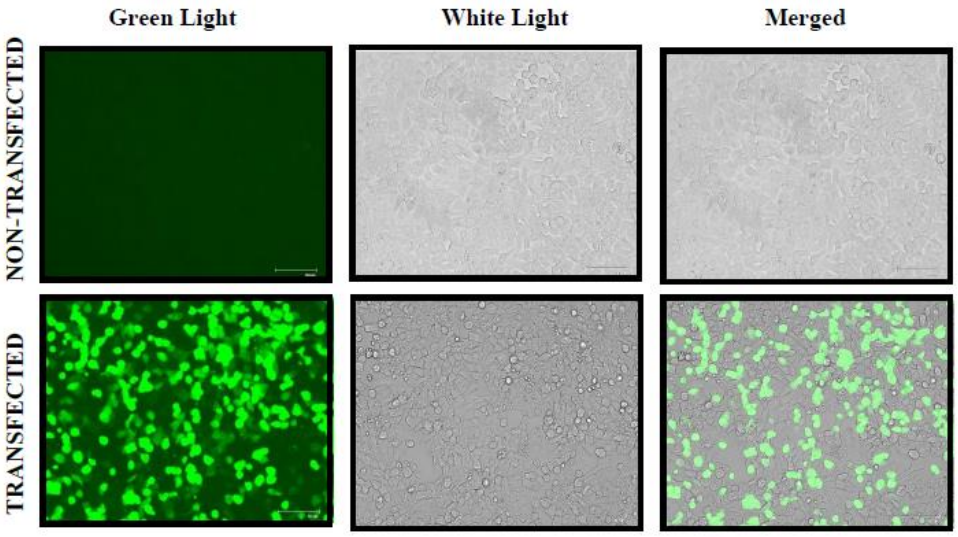
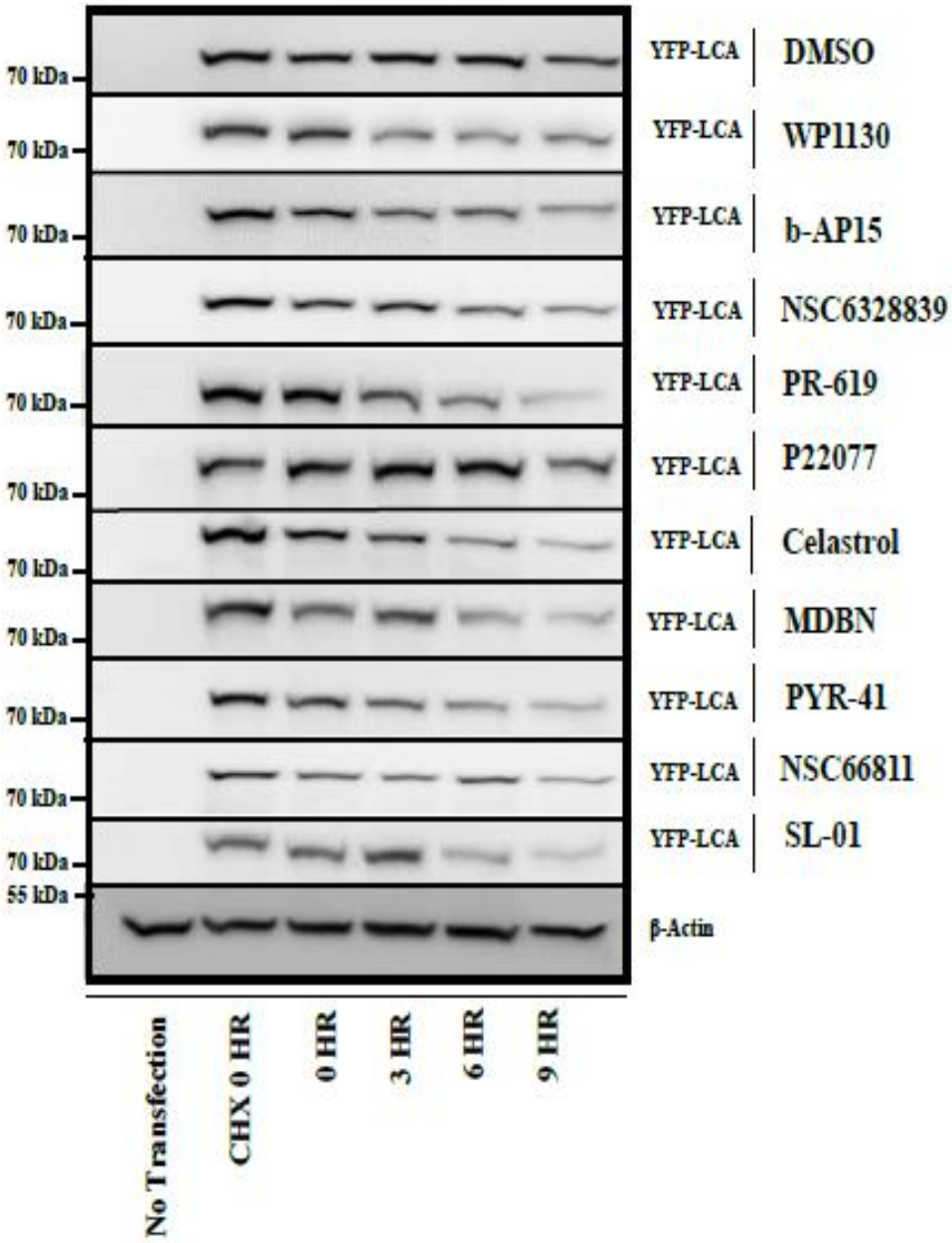


Figure 9 Fluorescent cell images of transfected and non-transfected HEK293 cells. They were seeded in growth medium 24 hours before transfection. 1 μg YFP-LCA plasmid was transfected. They were visualized after 24 hours post-transfection.

3.3.3 The effects of the lead compounds on the degradation of BoNT/A LC in cells.

To investigate the effects of selected compounds on the degradation of the catalytic domain of BoNT/A, we measured the changes in the protein level of YFP tagged BoNT/A LC at various time points after compound treatment. CHX 0 hr indicates the time point when cycloheximide (CHX) was added after 24 hours of post-transfection incubation time. CHX is a protein synthesis inhibitor that blocks new protein synthesis by inhibiting translation elongation in eukaryotes (Schneider-Poetsch et al., 2010). After 2 hours of CHX treatment, selected compounds were administered to transfected HEK293 cells, and the time point was considered as 0 hr. Then, the effects of compounds on BoNT/A LC protein levels were examined at three-time points (3 hours, 6 hours, and 9 hours). *Figure 11* shows representative YFP-LCA expression levels at indicated time points detected by Western blotting using anti-GFP antibody for each selected compound. β-Actin was used as a loading control.

An all the compounds were solubilized in DMSO and therefore, DMSO only treatment condition served as a negative control. As a result, PR-619 shows a highly significant time-dependent effect on the degradation of LC/A. This is an important finding, as stated earlier, PR619 is an inhibitor of VCIP135 that has been identified as a crucial DUB affecting the half-life of BoNT/A LC. Similarly, Celastrol, WP1130, b-AP15, NSC632839, MDBN, and SL-01 also show a significant effect on YFP-LCA degradation even at 3 hours. After 6 hours of compound treatment, b-AP15, PR-619, and Celastrol show a highly significant effect, while WP1130, NSC632839, and SL-01 show a very significant effect. On the other hand, MDBN and PYR-41 show a significant effect at 6 hours compared to CHX 0 hr. At 9 hours, WP1130, b-AP15, PR-619, and Celastrol show a highly significant effect. NSC632839 and SL-01 show a very significant effect on the degradation of the catalytic domain of BoNT/A, while PYR-41 and MDBN show significant effects after 9 hours compared to CHX 0 hr.


70 kDa
70 kDa
70 kDa
70 kDa
70 kDa
70 kDa
70 kDa
70 kDa
70 kDa
70 kDa
70 kDa
55 kDa
YFP-LCA
DMSO
YFP-LCA
WP1130
YFP-LCA
b-AP15
YFP-LCA
NSC6328839
YFP-LCA
PR-619
YFP-LCA
P22077
YFP-LCA
Celastrol
YFP-LCA
MDBN
YFP-LCA
PYR-41
YFP-LCA
NSC66811
YFP-LCA
SL-01
β-Actin
No Transfection
CHX 0 HR
0 HR
3 HR
6 HR
9 HR

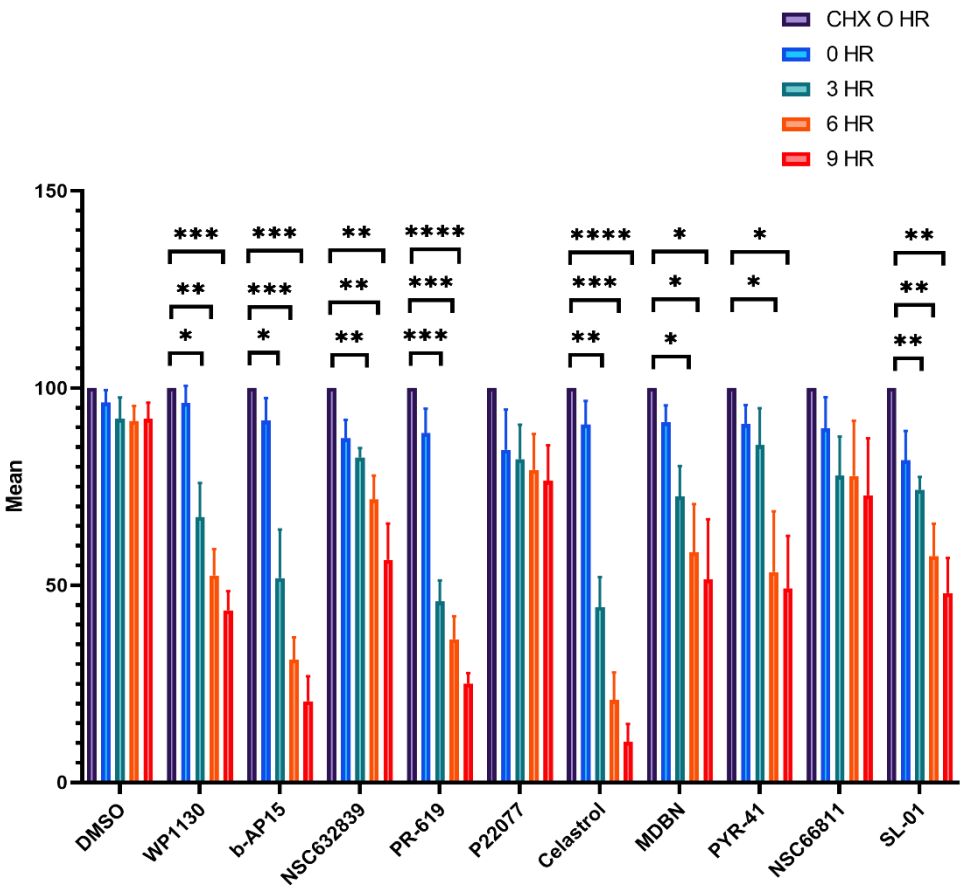


Figure 10 Western blot analysis of YFP-LCA protein expression in transfected HEK293 cells after compound treatment. (a) Representative Western blot images showing YFP-LCA protein expression at the indicated time points under the effects of selected compounds. 24 hrs post-transfection, CHX was added. After 2 hrs, CHX was removed, and HEK293 cells were treated with selected compounds with 20 μM concentration. CHX 0 hr indicates the time point when CHX was added. 0 hr indicated the time point when compound treatment was started. 3 hrs, 6 hrs, and 9 hrs indicate how long the cells have been exposed to the compound. Cell extracts were prepared and resolved by SDS-PAGE. YFP-LCA expression levels were detected by Western blotting by using anti-GFP antibody. β-Actin was used as a loading control and representative Western blot image was shown in the figure. (b) Quantitative analysis of YFP-LCA protein expressions for each compound at the indicated time points. Data are presented as means ± SEM calculated from three independent experiments and compared by unpaired t-test.

3.4 STRING Protein Interaction Analysis

The previous studies show that VCIP135, also known as VCPIP1, directly affects the duration of BoNT/A, whereas USP9X might have an indirect effect (Tsai et al., 2017). We also confirmed that VCIP135 and USP9X are critical targets for BoNT/A LC stability in our study. Both WP1130 and PR-619 show significant effects on the activity and half-life of BoNT/A LC, and it is known that the compounds affect USP9X and VCIP135, respectively (Altun et al., 2011; Driessen et al., 2015; Kapuria et al., 2010). In addition, some other compounds such as b-AP15, NSC632839, Celastrol, MDBN, PYR-41, and SL-01 also show significant effects, although they do not target USP9X and VCIP135. This may be an important finding as the identification of the targets of the compounds can reveal additional molecular player(s) important for the stability of BoNT/A LC. Such potential targets can be the focus of future studies.

Therefore, we conducted a STRING pathway analysis to look at the other possible protein targets which might interact with USP9X and VCIP135 commonly. They were used as input for STRING analysis, and a network was built based on medium confidence (0.4) evidence. The analysis showed that there is not a direct relationship between VCIP135 and USP9X. They interact with the proteins; CDK1, USP34, PEX12, UBC, UBA52, SNCA, PEX10, PEX6, PEX26, and SMAD4. However, none of them are targetted by b-AP15, NSC632839, Celastrol, MDBN, PYR-41, and SL-01. Regardless, further bioinformatics analyses and data mining studies can identify potential targets for future studies.

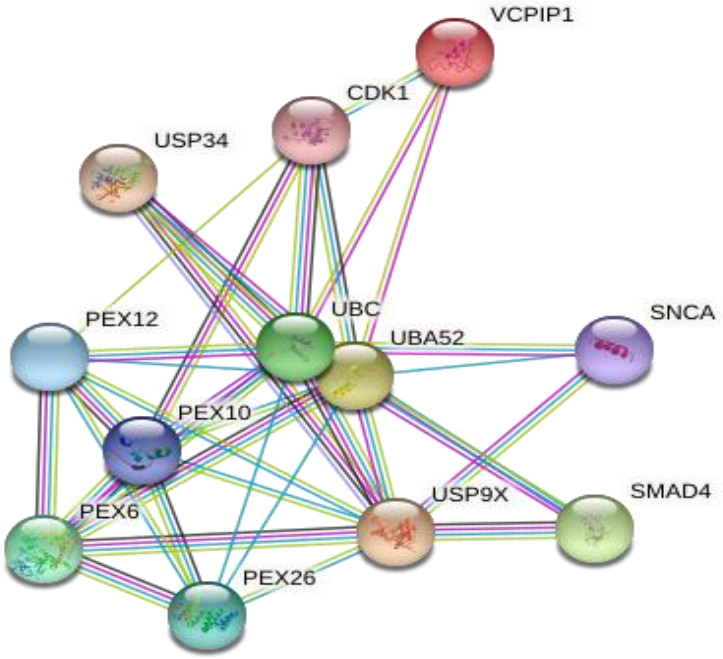


Figure 11 STRING Protein Interaction Analysis VCIP135/VCPIP1 and USP9X were used as input for STRING analysis.

CHAPTER 3

DISCUSSION

Botulism is a rare but potentially deadly disease caused by one of the category A bioterror agents, botulinum neurotoxin (Arnon et al., 2001; Osborne et al., 2007; Willis, Eubanks, Dickerson, & Janda, 2008). It is a highly poisonous toxin; for example, BoNT/A lead lethal effect in humans with an estimated dose of ~1 ng/kg (Arnon et al., 2001; Schantz & Johnson, 1992). However, there are no effective therapeutics against the intoxication of BoNTs after their entrance of motor neurons (Sobel, 2005). Supportive treatments, including mechanical breathing and intensive care, are the life-saving options for intoxicated patients until nerve cells clear out the toxin naturally (Rossetto et al., 2020; Sobel, 2005). There are botulinum antitoxin antibodies, but they can interact only with toxins in the circulation, and therefore, they should be applied in the earlier stage of poisoning, i.e., before toxin enters into motor neurons (Lin, Olson, Eubanks, & Janda, 2019; Sobel, 2005). Otherwise, they cannot work effectively. However, botulism symptoms are generally observed after toxins enter into the motor neurons, meaning that when patients are admitted to the hospitals, it may be too late for antitoxin application to neutralize the toxin (Sobel, 2005). Therefore, the identification of effective therapeutic approaches for already internalized toxin is very crucial for the treatment of botulism.

Botulinum neurotoxins can naturally cause disease botulism through food or liquid poisoning, and such cases occasionally are reported in many countries, including Turkey (Karsen, Ceylan, Bayindir, & Akdeniz, 2019). However, there is also concern regarding the deliberate usage of these toxins with ill-intents as they were used as a bioterror agent in the past (Arnon et al., 2001). In such a potential incident of mass intoxication, the availability of mechanical ventilation for large numbers of patients may not be possible. Additionally, hospitalization of BoNT

intoxicated patients can take a long time, which may cripple the health systems. For example, serotype A can be active up to 6 months, and intoxicated patients might need mechanical ventilation and intensive care during this period (Sobel, 2005). However, the capacities and facilities of hospitals might not be sufficient for such a long time for a large number of people. Therefore, drug discovery and development against botulism might prevent many problematic consequences in a possible terror attack.

On the other hand, BoNTs are frequently used in clinics as miracle drugs to treat many diseases and conditions (Pirazzini et al., 2017). Therefore, the enhancement of the duration of BoNT action can be another critical aspect for longer-lasting effects. For example, to compensate for the effects of damaged neurons, growth factors used in clinics can be possible molecules to change the duration of BoNT action (Tighe & Schiavo, 2013). An antibody to insulin growth factor I-receptor (anti-IGFIR) or a corticotropin-releasing factor can be given as examples for preventing functional upregulation of neuromuscular junction density that is associated with the return of muscle function after BoNT treatment (Harrison et al., 2011). Another concern is the accidental overdosing of BoNTs in clinics, and the development of effective anti-BoNT therapeutics could be important for such cases.

Different classes of small molecules have been developed against botulinum neurotoxin, with carrying efficacies. However, unfortunately, none of them has ever been graduated to clinical trials. Therefore, there is a need for novel classes of small molecules that can be effective against BoNT/A. In this study, we focused on the modulation of the ubiquitin-proteasome system (UPS) based on previous studies and screened a small molecule UPS library to evaluate compounds targeting E3 ligases and DUBs on the half-life of BoNT/A LC (Tsai et al., 2017; Tsai et al., 2010). The library that we selected included small molecules with known targets. For example, small molecule PR-619, which is identified as one of the lead compounds in this work, affects our main target, VCIP135 (Altun et al., 2011). Another target for us was USP9X, as it was shown that it might indirectly affect

BoNT/A LC degradation (Tsai et al., 2017). Excitingly, another lead compound identified in this study, WP1130, is known to target USP9X (Tsai et al., 2017).

In our screening experiments in this study, we utilized a well-characterized ES cell-derived motor neuron culture system. Previously, many different cell model systems have been utilized for BoNT studies, with varying degrees of success. For example, neuroblastoma cell lines can be used, but they are not sensitive to BoNTs as much as neurons (Kiris, Kota, et al., 2014). Also, mouse primary motor neurons are highly sensitive cellular models for the investigation of BoNT (Kiris, Kota, et al., 2014). However, they are not suitable for drug screening studies because the isolation and culturing of mouse primary motor neurons on a large scale are a challenging and costly process. Therefore, ES cell-derived motor neurons can be an ideal model for drug screening studies against BoNT intoxication as they are physiologically relevant, sensitive, and well-established.

In some experiments in our study, the transfection of plasmids that encode BoNT/A LC was used rather than BoNT/A holotoxin. We used a codon-optimized for mammalian expression plasmid encoding BoNT/A LC to study BoNT/A persistence (Fernandez-Salas, Ho, Garay, Steward, & Aoki, 2004; Fernandez-Salas, Steward, et al., 2004; Kuo, Oyler, & Shoemaker, 2011; Tsai et al., 2010). This approach was highly useful for us as the BoNT/A LC was tagged with YFP, which allowed us to monitor LC levels in cells in a relatively easy manner. We utilized Toosendanin (TSN) treatment as the positive control group because TSN is a traditional Chinese medicine that has been shown to be effective against BoNT/A mediated SNAP-25 cleavage (Shi & Wang, 2004).

Our initial screening of the UPS small molecule library in stem cell-derived motor neurons shows that WP1130, b-AP15, NSC632839, PR619, P22077, Celastrol, MDBN, PYR-41, Serdemetan, DbeQ, NSC 66811, SL-01 decrease SNAP-25 cleavage from BoNT/A significantly. However, Serdemetan and DbeQ were excluded from further experiments because of their possible toxicity. Therefore, we focused on the effects of the other compounds on BoNT/A

intoxication in a dose-dependent manner. According to the results, 20 μM was selected as an effective dose, and it was used in further experiments.

MTT assay is frequently used as a cytotoxicity assay method (Tolosa, Donato, & Gomez-Lechon, 2015). Therefore, the viability of cells under the effects of these selected compounds was analyzed with an MTT assay to look whether they have significant cytotoxic effects or not. Several concentrations were tested to look at the effects on the proliferation of HEK293 cells. As a result, none of them leads to any significant decrease in the viability of HEK293 cells (*Figure 7*). Low toxicity is an essential point for drug discovery to prevent damage to cells while repairing intoxication. Therefore, the result is so crucial to demonstrate that the lead compounds have no significant toxic effects on mammalian cells.

Finally, we investigated how these selected compounds affect the stability of LC using a time-course experiment. Cycloheximide was used to inhibit new protein synthesis after a specified time point. Because we used a construct containing BoNT/A LC fused with YFP, the YFP expression level was compared to each other in indicated time points. There is a difference between GFP and YFP because of only T203Y mutation; however, the antibodies raised against full-length GFP might also detect YFP (Day & Davidson, 2009). Therefore, we used the optimized GFP antibody, which should also detect YFP in our western blot experiments. Results show that WP1130, b-AP15, NSC632839, PR619, Celastrol, MDBN, PYR-41, and SL-01 decrease BoNT/A LC stability. It is important to note that the compounds WP1130 and PR619, which target USP9X and VCIP135, significantly affect LC stability (Altun et al., 2011; Kapuria et al., 2010). The result is highly important because it confirmed that VCIP135 and USP9X are the key DUBs to regulate BoNT/A LC stability (Tsai et al., 2017). Our findings showed that the small molecules targetting them might be good therapeutic agents to manage BoNT/A intoxication. On the other hand, the findings of the other compounds showing significant effects on BoNT/A LC are very important because some other factors might also play crucial roles in the stability of the catalytically active part of the toxin. Overall, the eight compounds were stated as promising

compounds to inhibit BoNT/A intoxication and can be investigated for further analysis to reveal the mechanisms of action.

To sum up, we aimed to screen a small molecule library targeting UPS to reveal compounds that can enhance the clearance of BoNT/A LC, based on our previous studies showing that VCIP135 and USP9X might be the primary targets to induce the degradation of BoNT/A LC. As a result, WP1130, b-AP15, NSC632839, PR619, Celastrol, MDBN, PYR-41, and SL-01 show promising results against BoNT/A LC. Further studies to reveal their mechanisms of action can potentially enable developing therapeutic options that can be effective on already intoxicated motor neurons and accelerate the recovery by inducing the degradation of BoNT/A LC.

CHAPTER 5

CONCLUSION AND FUTURE STUDIES

In this study, we screened a small molecule library that targets the UPS to potentially identify molecules that can affect BoNT/A LC activity and half-life in cells. Based on the previous findings, DUBs might affect BoNT/A LC stability (Tsai et al., 2017; Tsai et al., 2010). The significant findings in this study are listed below.

1. Screening of the UPP library led to the discovery of initial 10 lead compounds. More specifically, WP1130, b-AP15, NSC-632839, PR619, P22077, Celastrol, MDBN, PYR-41, NSC-66811, SL-01 show promising results because of their significant effects on the inhibition of BoNT/A mediated SNAP-25 cleavage.
2. 10 of lead compounds (WP1130, b-AP15, NSC-632839, PR619, P22077, Celastrol, MDBN, PYR-41, NSC-66811, SL-01) demonstrated dose-dependent protection against BoNT/A activity in mouse ES-derived motor neurons.
3. The lead compounds did not exhibit any negative effects on the viability of HEK293 cells.
4. Our stability experiments following cycloheximide chase demonstrated that WP1130, b-AP15, NSC632839, PR-619, Celastrol, MDBN, PYR-41 and SL-01 treatments lead to a reduction of LC/A protein in cells.

However, there are still several questions that remain to be answered. The compounds are UPP modulators, but it should be revealed whether their activity on BoNT/A LC degradation is indeed through ubiquitination mediated processes.

Therefore, ubiquitination differences after compound treatments should be investigated in further experiments.

In this study, we focus on the inhibition of deubiquitination of BoNT/A LC. However, after the library screen and investigation of compounds on SNAP-25 cleavage and BoNT/A LC degradation, some compounds such as P22077 and NSC66811 did not show a significant effect. However, these compounds can be used for the investigation of molecular destruction mechanisms for BoNTs in further studies to understand better.

Based on previous findings of the BoNT/A LC stability depending on VCIP135 and USP9X, those DUBs were our primary targets. It is known that WP1130 inhibits USPX and PR-619 inhibits VCIP135. Our results also confirmed that their activities are important for the stability of BoNT/A LC because their inhibition leads to the degradation of BoNT/A LC. However, the results showed that there are other molecules like b-AP15, NSC632839, Celastrol, MDBN, PYR-41, and SL-01, which do not target USP9X or VCIP135 but show significant effects on degradation of BoNT/A LC. These outcomes may be also very crucial because other possible mechanisms might be important for the stability of BoNT/A LC, different than VCIP135 and USP9X. Therefore, those compounds and their potential targets within the context of BoNT/A should be investigated in further studies.

REFERENCES

Adams, J. (2002). Development of the proteasome inhibitor PS-341. *Oncologist, 7*(1), 9-16. doi:10.1634/theoncologist.7-1-9

Aghajan, M., Jonai, N., Flick, K., Fu, F., Luo, M., Cai, X., . . . Huang, J. (2010). Chemical genetics screen for enhancers of rapamycin identifies a specific inhibitor of an SCF family E3 ubiquitin ligase. *Nat Biotechnol, 28*(7), 738-742. doi:10.1038/nbt.1645

Aleo, E., Henderson, C. J., Fontanini, A., Solazzo, B., & Brancolini, C. (2006). Identification of new compounds that trigger apoptosome-independent caspase activation and apoptosis. *Cancer Res, 66*(18), 9235-9244. doi:10.1158/0008-5472.CAN-06-0702

Altun, M., Kramer, H. B., Willems, L. I., McDermott, J. L., Leach, C. A., Goldenberg, S. J., . . . Kessler, B. M. (2011). Activity-based chemical proteomics accelerates inhibitor development for deubiquitylating enzymes. *Chem Biol, 18*(11), 1401-1412. doi:10.1016/j.chembiol.2011.08.018

Ambrozova, H. (2019). Botulism - a rare but still present, life-threatening disease. *Epidemiol Mikrobiol Imunol, 68*(1), 33-38.

Arnon, S. S., Schechter, R., Inglesby, T. V., Henderson, D. A., Bartlett, J. G., Ascher, M. S., . . . Working Group on Civilian, B. (2001). Botulinum toxin as a biological weapon: medical and public health management. *JAMA, 285*(8), 1059-1070. doi:10.1001/jama.285.8.1059

Aubrey, B. J., Kelly, G. L., Janic, A., Herold, M. J., & Strasser, A. (2018). How does p53 induce apoptosis and how does this relate to p53-mediated tumour suppression? *Cell Death Differ, 25*(1), 104-113. doi:10.1038/cdd.2017.169

Auner, H. W., Moody, A. M., Ward, T. H., Kraus, M., Milan, E., May, P., . . . Dillon, N. (2013). Combined inhibition of p97 and the proteasome causes lethal disruption of the secretory apparatus in multiple myeloma cells. *PLoS One, 8*(9), e74415. doi:10.1371/journal.pone.0074415

Azarnia Tehran, D., Zanetti, G., Leka, O., Lista, F., Fillo, S., Binz, T., . . . Pirazzini, M. (2015). A Novel Inhibitor Prevents the Peripheral Neuroparalysis of Botulinum Neurotoxins. *Sci Rep, 5*, 17513. doi:10.1038/srep17513

Bastola, P., Neums, L., Schoenen, F. J., & Chien, J. (2016). VCP inhibitors induce endoplasmic reticulum stress, cause cell cycle arrest, trigger caspase-mediated cell death and synergistically kill ovarian cancer cells in combination with Salubrinal. *Mol Oncol, 10*(10), 1559-1574. doi:10.1016/j.molonc.2016.09.005

Bhattacharyya, S., Yu, H., Mim, C., & Matouschek, A. (2014). Regulated protein turnover: snapshots of the proteasome in action. *Nat Rev Mol Cell Biol, 15*(2), 122-133. doi:10.1038/nrm3741

Blasi, J., Chapman, E. R., Link, E., Binz, T., Yamasaki, S., De Camilli, P., . . . Jahn, R. (1993). Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. *Nature, 365*(6442), 160-163. doi:10.1038/365160a0

Boldt, G. E., Dickerson, T. J., & Janda, K. D. (2006). Emerging chemical and biological approaches for the preparation of discovery libraries. *Drug Discov Today, 11*(3-4), 143-148. doi:10.1016/S1359-6446(05)03697-4

Boridy, S., Le, P. U., Petrecca, K., & Maysinger, D. (2014). Celastrol targets proteostasis and acts synergistically with a heat-shock protein 90 inhibitor to kill human glioblastoma cells. *Cell Death Dis, 5*, e1216. doi:10.1038/cddis.2014.182

Brahemi, G., Burger, A. M., Westwell, A. D., & Brancale, A. (2010). Homology Modelling of Human E1 Ubiquitin Activating Enzyme. *Lett Drug Des Discov, 7*(1), 57-62. doi:10.2174/157018010789869316

Bremer, P. T., Adler, M., Phung, C. H., Singh, A. K., & Janda, K. D. (2017). Newly Designed Quinolinol Inhibitors Mitigate the Effects of Botulinum Neurotoxin A in Enzymatic, Cell-Based, and ex Vivo Assays. *J Med Chem, 60*(1), 338-348. doi:10.1021/acs.jmedchem.6b01393

Bremer, P. T., Pellett, S., Carolan, J. P., Tepp, W. H., Eubanks, L. M., Allen, K. N., . . . Janda, K. D. (2017). Metal Ions Effectively Ablate the Action of Botulinum Neurotoxin A. *J Am Chem Soc, 139*(21), 7264-7272. doi:10.1021/jacs.7b01084

Brooks, C. L., & Gu, W. (2006). p53 ubiquitination: Mdm2 and beyond. *Mol Cell, 21*(3), 307-315. doi:10.1016/j.molcel.2006.01.020

Brunckhorst, M. K., Lerner, D., Wang, S., & Yu, Q. (2012). AT-406, an orally active antagonist of multiple inhibitor of apoptosis proteins, inhibits progression of human ovarian cancer. *Cancer Biol Ther, 13*(9), 804-811. doi:10.4161/cbt.20563

Burnett, J. C., Opsenica, D., Sriraghavan, K., Panchal, R. G., Ruthel, G., Hermone, A. R., . . . Bavari, S. (2007). A refined pharmacophore identifies potent 4-amino-7-chloroquinoline-based inhibitors of the botulinum neurotoxin serotype A metalloprotease. *J Med Chem, 50*(9), 2127-2136. doi:10.1021/jm061446e

Chen, J. L., & Kuo, H. C. (2020). Clinical application of intravesical botulinum toxin type A for overactive bladder and interstitial cystitis. *Investig Clin Urol, 61*(Suppl 1), S33-S42. doi:10.4111/icu.2020.61.S1.S33

Chou, T. F., & Deshaies, R. J. (2011). Quantitative cell-based protein degradation assays to identify and classify drugs that target the ubiquitin-proteasome system. *J Biol Chem, 286*(19), 16546-16554. doi:10.1074/jbc.M110.215319

Chuang, H. C., Yang, L. P., Fitzgerald, A. L., Osman, A., Woo, S. H., Myers, J. N., & Skinner, H. D. (2014). The p53-reactivating small molecule RITA induces senescence in head and neck cancer cells. *PLoS One, 9*(8), e104821. doi:10.1371/journal.pone.0104821

Class, B., Thorne, N., Aguisanda, F., Southall, N., McKew, J. C., & Zheng, W. (2015). High-throughput viability assay using an autonomously bioluminescent cell line with a bacterial Lux reporter. *J Lab Autom, 20*(2), 164-174. doi:10.1177/2211068214560608

Cooper, E. M., Cutcliffe, C., Kristiansen, T. Z., Pandey, A., Pickart, C. M., & Cohen, R. E. (2009). K63-specific deubiquitination by two JAMM/MPN+

complexes: BRISC-associated Brcc36 and proteasomal Poh1. *EMBO J, 28*(6), 621-631. doi:10.1038/emboj.2009.27
Coux, O., Tanaka, K., & Goldberg, A. L. (1996). Structure and functions of the 20S and 26S proteasomes. *Annu Rev Biochem, 65*, 801-847. doi:10.1146/annurev.bi.65.070196.004101
Cowell, I. G., Ling, E. M., Swan, R. L., Brooks, M. L. W., & Austin, C. A. (2019). The Deubiquitinating Enzyme Inhibitor PR-619 is a Potent DNA Topoisomerase II Poison. *Mol Pharmacol, 96*(5), 562-572. doi:10.1124/mol.119.117390
D'Arcy, P., & Linder, S. (2012). Proteasome deubiquitinases as novel targets for cancer therapy. *Int J Biochem Cell Biol, 44*(11), 1729-1738. doi:10.1016/j.biocel.2012.07.011
Dantuma, N. P., & Bott, L. C. (2014). The ubiquitin-proteasome system in neurodegenerative diseases: precipitating factor, yet part of the solution. *Front Mol Neurosci, 7*, 70. doi:10.3389/fnmol.2014.00070
Davis, M. I., Pragani, R., Fox, J. T., Shen, M., Parmar, K., Gaudiano, E. F., . . . Simeonov, A. (2016). Small Molecule Inhibition of the Ubiquitin-specific Protease USP2 Accelerates cyclin D1 Degradation and Leads to Cell Cycle Arrest in Colorectal Cancer and Mantle Cell Lymphoma Models. *J Biol Chem, 291*(47), 24628-24640. doi:10.1074/jbc.M116.738567
Day, R. N., & Davidson, M. W. (2009). The fluorescent protein palette: tools for cellular imaging. *Chem Soc Rev, 38*(10), 2887-2921. doi:10.1039/b901966a
Dickerson, T. J., & Janda, K. D. (2006). The use of small molecules to investigate molecular mechanisms and therapeutic targets for treatment of botulinum neurotoxin A intoxication. *ACS Chem Biol, 1*(6), 359-369. doi:10.1021/cb600179d
Dressler, D., & Adib Saberi, F. (2005). Botulinum toxin: mechanisms of action. *Eur Neurol, 53*(1), 3-9. doi:10.1159/000083259
Driessen, S., Berleth, N., Friesen, O., Loffler, A. S., Bohler, P., Hieke, N., . . . Stork, B. (2015). Deubiquitinase inhibition by WP1130 leads to ULK1 aggregation and blockade of autophagy. *Autophagy, 11*(9), 1458-1470. doi:10.1080/15548627.2015.1067359
Espelund, M., & Klaveness, D. (2014). Botulism outbreaks in natural environments - an update. *Front Microbiol, 5*, 287. doi:10.3389/fmicb.2014.00287
Fan, Y., Geren, I. N., Dong, J., Lou, J., Wen, W., Conrad, F., . . . Marks, J. D. (2015). Monoclonal Antibodies Targeting the Alpha-Exosite of Botulinum Neurotoxin Serotype/A Inhibit Catalytic Activity. *PLoS One, 10*(8), e0135306. doi:10.1371/journal.pone.0135306
Fernandez-Salas, E., Ho, H., Garay, P., Steward, L. E., & Aoki, K. R. (2004). Is the light chain subcellular localization an important factor in botulinum toxin duration of action? *Mov Disord, 19 Suppl 8*, S23-34. doi:10.1002/mds.20006
Fernandez-Salas, E., Steward, L. E., Ho, H., Garay, P. E., Sun, S. W., Gilmore, M. A., . . . Aoki, K. R. (2004). Plasma membrane localization signals in the

light chain of botulinum neurotoxin. *Proc Natl Acad Sci U S A, 101*(9), 3208-3213. doi:10.1073/pnas.0400229101

Figueiredo-Pereira, M. E., Chen, W. E., Li, J., & Johdo, O. (1996). The antitumor drug aclacinomycin A, which inhibits the degradation of ubiquitinated proteins, shows selectivity for the chymotrypsin-like activity of the bovine pituitary 20 S proteasome. *J Biol Chem, 271*(28), 16455-16459. doi:10.1074/jbc.271.28.16455

Finley, D. (2009). Recognition and processing of ubiquitin-protein conjugates by the proteasome. *Annu Rev Biochem, 78*, 477-513. doi:10.1146/annurev.biochem.78.081507.101607

Golubovskaya, V. M., & Cance, W. G. (2013). Targeting the p53 pathway. *Surg Oncol Clin N Am, 22*(4), 747-764. doi:10.1016/j.soc.2013.06.003

Guo, N., & Peng, Z. (2013). MG132, a proteasome inhibitor, induces apoptosis in tumor cells. *Asia Pac J Clin Oncol, 9*(1), 6-11. doi:10.1111/j.1743-7563.2012.01535.x

Hambleton, P. (1992). Clostridium botulinum toxins: a general review of involvement in disease, structure, mode of action and preparation for clinical use. *J Neurol, 239*(1), 16-20. doi:10.1007/BF00839205

Harrison, A. R., Berbos, Z., Zaldivar, R. A., Anderson, B. C., Semmer, M., Lee, M. S., & McLoon, L. K. (2011). Modulating neuromuscular junction density changes in botulinum toxin-treated orbicularis oculi muscle. *Invest Ophthalmol Vis Sci, 52*(2), 982-986. doi:10.1167/iovs.10-6427

Hershko, A., & Ciechanover, A. (1998). The ubiquitin system. *Annu Rev Biochem, 67*, 425-479. doi:10.1146/annurev.biochem.67.1.425

Ito, T., Ando, H., & Handa, H. (2011). Teratogenic effects of thalidomide: molecular mechanisms. *Cell Mol Life Sci, 68*(9), 1569-1579. doi:10.1007/s00018-010-0619-9

Ito, T., Ando, H., Suzuki, T., Ogura, T., Hotta, K., Imamura, Y., . . . Handa, H. (2010). Identification of a primary target of thalidomide teratogenicity. *Science, 327*(5971), 1345-1350. doi:10.1126/science.1177319

Johnson, E. A., & Montecucco, C. (2008). Botulism. *Handb Clin Neurol, 91*, 333-368. doi:10.1016/S0072-9752(07)01511-4

Jones, R. J., Gu, D., Bjorklund, C. C., Kuiatse, I., Remaley, A. T., Bashir, T., . . . Orlowski, R. Z. (2013). The novel anticancer agent JNJ-26854165 induces cell death through inhibition of cholesterol transport and degradation of ABCA1. *J Pharmacol Exp Ther, 346*(3), 381-392. doi:10.1124/jpet.113.204958

Kapuria, V., Levitzki, A., Bornmann, W. G., Maxwell, D., Priebe, W., Sorenson, R. J., . . . Donato, N. J. (2011). A novel small molecule deubiquitinase inhibitor blocks Jak2 signaling through Jak2 ubiquitination. *Cell Signal, 23*(12), 2076-2085. doi:10.1016/j.cellsig.2011.08.002

Kapuria, V., Peterson, L. F., Fang, D., Bornmann, W. G., Talpaz, M., & Donato, N. J. (2010). Deubiquitinase inhibition by small-molecule WP1130 triggers aggresome formation and tumor cell apoptosis. *Cancer Res, 70*(22), 9265-9276. doi:10.1158/0008-5472.CAN-10-1530

Karsen, H., Ceylan, M. R., Bayindir, H., & Akdeniz, H. (2019). Foodborne botulism in Turkey, 1983 to 2017. *Infect Dis (Lond), 51*(2), 91-96. doi:10.1080/23744235.2018.1524582

Kiaei, M., Kipiani, K., Petri, S., Chen, J., Calingasan, N. Y., & Beal, M. F. (2005). Celastrol blocks neuronal cell death and extends life in transgenic mouse model of amyotrophic lateral sclerosis. *Neurodegener Dis, 2*(5), 246-254. doi:10.1159/000090364

Kiris, E., Burnett, J. C., Kane, C. D., & Bavari, S. (2014). Recent advances in botulinum neurotoxin inhibitor development. *Curr Top Med Chem, 14*(18), 2044-2061. doi:10.2174/1568026614666141022093350

Kiris, E., Kota, K. P., Burnett, J. C., Soloveva, V., Kane, C. D., & Bavari, S. (2014). Recent developments in cell-based assays and stem cell technologies for botulinum neurotoxin research and drug discovery. *Expert Rev Mol Diagn, 14*(2), 153-168. doi:10.1586/14737159.2014.867808

Kiris, E., Nuss, J. E., Burnett, J. C., Kota, K. P., Koh, D. C., Wanner, L. M., . . . Bavari, S. (2011). Embryonic stem cell-derived motoneurons provide a highly sensitive cell culture model for botulinum neurotoxin studies, with implications for high-throughput drug discovery. *Stem Cell Res, 6*(3), 195-205. doi:10.1016/j.scr.2011.01.002

Konstantinovic, J., Kiris, E., Kota, K. P., Kugelman-Tonos, J., Videnovic, M., Cazares, L. H., . . . Solaja, B. A. (2018). New Steroidal 4-Aminoquinolines Antagonize Botulinum Neurotoxin Serotype A in Mouse Embryonic Stem Cell Derived Motor Neurons in Postintoxication Model. *J Med Chem, 61*(4), 1595-1608. doi:10.1021/acs.jmedchem.7b01710

Kronke, J., Udeshi, N. D., Narla, A., Grauman, P., Hurst, S. N., McConkey, M., . . . Ebert, B. L. (2014). Lenalidomide causes selective degradation of IKZF1 and IKZF3 in multiple myeloma cells. *Science, 343*(6168), 301-305. doi:10.1126/science.1244851

Kuo, C. L., Oyler, G. A., & Shoemaker, C. B. (2011). Accelerated neuronal cell recovery from Botulinum neurotoxin intoxication by targeted ubiquitination. *PLoS One, 6*(5), e20352. doi:10.1371/journal.pone.0020352

Kupperman, E., Lee, E. C., Cao, Y., Bannerman, B., Fitzgerald, M., Berger, A., . . . Bolen, J. (2010). Evaluation of the proteasome inhibitor MLN9708 in preclinical models of human cancer. *Cancer Res, 70*(5), 1970-1980. doi:10.1158/0008-5472.CAN-09-2766

Landgren, O., Sonneveld, P., Jakubowiak, A., Mohty, M., Iskander, K. S., Mezzi, K., & Siegel, D. S. (2019). Carfilzomib with immunomodulatory drugs for the treatment of newly diagnosed multiple myeloma. *Leukemia, 33*(9), 2127-2143. doi:10.1038/s41375-019-0517-6

Landre, V., Rotblat, B., Melino, S., Bernassola, F., & Melino, G. (2014). Screening for E3-ubiquitin ligase inhibitors: challenges and opportunities. *Oncotarget, 5*(18), 7988-8013. doi:10.18632/oncotarget.2431

Lee, B. H., Lee, M. J., Park, S., Oh, D. C., Elsasser, S., Chen, P. C., . . . Finley, D. (2010). Enhancement of proteasome activity by a small-molecule inhibitor of USP14. *Nature, 467*(7312), 179-184. doi:10.1038/nature09299

Li, J., Zhang, S., Gao, L., Chen, Y., & Xie, X. (2011). A cell-based high-throughput assay for the screening of small-molecule inhibitors of p53-MDM2 interaction. *J Biomol Screen, 16*(4), 450-456. doi:10.1177/1087057111399191

Lin, L., Olson, M. E., Eubanks, L. M., & Janda, K. D. (2019). Strategies to Counteract Botulinum Neurotoxin A: Nature's Deadliest Biomolecule. *Acc Chem Res, 52*(8), 2322-2331. doi:10.1021/acs.accounts.9b00261

Lin, L., Olson, M. E., Sugane, T., Turner, L. D., Tararina, M. A., Nielsen, A. L., . . . Janda, K. D. (2020). Catch and Anchor Approach To Combat Both Toxicity and Longevity of Botulinum Toxin A. *J Med Chem, 63*(19), 11100-11120. doi:10.1021/acs.jmedchem.0c01006

Lonati, D., Schicchi, A., Crevani, M., Buscaglia, E., Scaravaggi, G., Maida, F., . . . Locatelli, C. A. (2020). Foodborne Botulism: Clinical Diagnosis and Medical Treatment. *Toxins (Basel), 12*(8). doi:10.3390/toxins12080509

Lu, G., Middleton, R. E., Sun, H., Naniong, M., Ott, C. J., Mitsiades, C. S., . . . Kaelin, W. G., Jr. (2014). The myeloma drug lenalidomide promotes the cereblon-dependent destruction of Ikaros proteins. *Science, 343*(6168), 305-309. doi:10.1126/science.1244917

Lu, Y., Nikolovska-Coleska, Z., Fang, X., Gao, W., Shangary, S., Qiu, S., . . . Wang, S. (2006). Discovery of a nanomolar inhibitor of the human murine double minute 2 (MDM2)-p53 interaction through an integrated, virtual database screening strategy. *J Med Chem, 49*(13), 3759-3762. doi:10.1021/jm060023+

Matyskiela, M. E., Lu, G., Ito, T., Pagarigan, B., Lu, C. C., Miller, K., . . . Chamberlain, P. P. (2016). A novel cereblon modulator recruits GSPT1 to the CRL4(CRBN) ubiquitin ligase. *Nature, 535*(7611), 252-257. doi:10.1038/nature18611

Montal, M. (2010). Botulinum neurotoxin: a marvel of protein design. *Annu Rev Biochem, 79*, 591-617. doi:10.1146/annurev.biochem.051908.125345

Montecucco, C., Papini, E., & Schiavo, G. (1994). Bacterial protein toxins penetrate cells via a four-step mechanism. *FEBS Lett, 346*(1), 92-98. doi:10.1016/0014-5793(94)00449-8

Mori-Konya, C., Kato, N., Maeda, R., Yasuda, K., Higashimae, N., Noguchi, M., . . . Kakizuka, A. (2009). p97/valosin-containing protein (VCP) is highly modulated by phosphorylation and acetylation. *Genes Cells, 14*(4), 483-497. doi:10.1111/j.1365-2443.2009.01286.x

Myung, J., Kim, K. B., & Crews, C. M. (2001). The ubiquitin-proteasome pathway and proteasome inhibitors. *Med Res Rev, 21*(4), 245-273. doi:10.1002/med.1009

Nicholson, B., Leach, C. A., Goldenberg, S. J., Francis, D. M., Kodrasov, M. P., Tian, X., . . . Butt, T. R. (2008). Characterization of ubiquitin and ubiquitin-like-protein isopeptidase activities. *Protein Sci, 17*(6), 1035-1043. doi:10.1110/ps.083450408

Opsenica, I., Burnett, J. C., Gussio, R., Opsenica, D., Todorovic, N., Lanteri, C. A., . . . Bavari, S. (2011). A chemotype that inhibits three unrelated pathogenic

targets: the botulinum neurotoxin serotype A light chain, P. falciparum malaria, and the Ebola filovirus. *J Med Chem, 54*(5), 1157-1169. doi:10.1021/jm100938u

Osborne, S. L., Latham, C. F., Wen, P. J., Cavaignac, S., Fanning, J., Foran, P. G., & Meunier, F. A. (2007). The Janus faces of botulinum neurotoxin: sensational medicine and deadly biological weapon. *J Neurosci Res, 85*(6), 1149-1158. doi:10.1002/jnr.21171

Peck, M. W. (2009). Biology and genomic analysis of Clostridium botulinum. *Adv Microb Physiol, 55*, 183-265, 320. doi:10.1016/S0065-2911(09)05503-9

Pellizzari, R., Rossetto, O., Schiavo, G., & Montecucco, C. (1999). Tetanus and botulinum neurotoxins: mechanism of action and therapeutic uses. *Philos Trans R Soc Lond B Biol Sci, 354*(1381), 259-268. doi:10.1098/rstb.1999.0377

Pirazzini, M., & Rossetto, O. (2017). Challenges in searching for therapeutics against Botulinum Neurotoxins. *Expert Opin Drug Discov, 12*(5), 497-510. doi:10.1080/17460441.2017.1303476

Pirazzini, M., Rossetto, O., Eleopra, R., & Montecucco, C. (2017). Botulinum Neurotoxins: Biology, Pharmacology, and Toxicology. *Pharmacol Rev, 69*(2), 200-235. doi:10.1124/pr.116.012658

Pozhidaeva, A., Valles, G., Wang, F., Wu, J., Sterner, D. E., Nguyen, P., . . . Bezsonova, I. (2017). USP7-Specific Inhibitors Target and Modify the Enzyme's Active Site via Distinct Chemical Mechanisms. *Cell Chem Biol, 24*(12), 1501-1512 e1505. doi:10.1016/j.chembiol.2017.09.004

Reits, E., Griekspoor, A., Neijssen, J., Groothuis, T., Jalink, K., van Veelen, P., . . . Neefjes, J. (2003). Peptide diffusion, protection, and degradation in nuclear and cytoplasmic compartments before antigen presentation by MHC class I. *Immunity, 18*(1), 97-108. doi:10.1016/s1074-7613(02)00511-3

Rico-Bautista, E., & Wolf, D. A. (2012). Skipping cancer: small molecule inhibitors of SKP2-mediated p27 degradation. *Chem Biol, 19*(12), 1497-1498. doi:10.1016/j.chembiol.2012.12.001

Rossetto, O., Pirazzini, M., Fabris, F., & Montecucco, C. (2020). Botulinum Neurotoxins: Mechanism of Action. *Handb Exp Pharmacol*. doi:10.1007/164_2020_355

Rossetto, O., Pirazzini, M., & Montecucco, C. (2014). Botulinum neurotoxins: genetic, structural and mechanistic insights. *Nat Rev Microbiol, 12*(8), 535-549. doi:10.1038/nrmicro3295

Sam, C., & Bordoni, B. (2020). Physiology, Acetylcholine. In *StatPearls*. Treasure Island (FL).

Schantz, E. J., & Johnson, E. A. (1992). Properties and use of botulinum toxin and other microbial neurotoxins in medicine. *Microbiol Rev, 56*(1), 80-99.

Schmidt, M., Altdorfer, V., Schnitte, S., Fuchs, A. R., Kropp, K. N., Maurer, S., . . . Dorfel, D. (2019). The Deubiquitinase Inhibitor b-AP15 and Its Effect on Phenotype and Function of Monocyte-Derived Dendritic Cells. *Neoplasia, 21*(7), 653-664. doi:10.1016/j.neo.2019.03.001

Schmidt, M., & Finley, D. (2014). Regulation of proteasome activity in health and disease. *Biochim Biophys Acta, 1843*(1), 13-25. doi:10.1016/j.bbamcr.2013.08.012

Schneider-Poetsch, T., Ju, J., Eyler, D. E., Dang, Y., Bhat, S., Merrick, W. C., . . . Liu, J. O. (2010). Inhibition of eukaryotic translation elongation by cycloheximide and lactimidomycin. *Nat Chem Biol, 6*(3), 209-217. doi:10.1038/nchembio.304

Schrader, J., Henneberg, F., Mata, R. A., Tittmann, K., Schneider, T. R., Stark, H., . . . Chari, A. (2016). The inhibition mechanism of human 20S proteasomes enables next-generation inhibitor design. *Science, 353*(6299), 594-598. doi:10.1126/science.aaf8993

Seki, H., Xue, S., Hixon, M. S., Pellett, S., Remes, M., Johnson, E. A., & Janda, K. D. (2015). Toward the discovery of dual inhibitors for botulinum neurotoxin A: concomitant targeting of endocytosis and light chain protease activity. *Chem Commun (Camb), 51*(28), 6226-6229. doi:10.1039/c5cc00677e

Sethi, G., Ahn, K. S., Pandey, M. K., & Aggarwal, B. B. (2007). Celastrol, a novel triterpene, potentiates TNF-induced apoptosis and suppresses invasion of tumor cells by inhibiting NF-kappaB-regulated gene products and TAK1-mediated NF-kappaB activation. *Blood, 109*(7), 2727-2735. doi:10.1182/blood-2006-10-050807

Shabek, N., Herman-Bachinsky, Y., Buchsbaum, S., Lewinson, O., Haj-Yahya, M., Hejjaoui, M., . . . Ciechanover, A. (2012). The size of the proteasomal substrate determines whether its degradation will be mediated by mono- or polyubiquitylation. *Mol Cell, 48*(1), 87-97. doi:10.1016/j.molcel.2012.07.011

Shi, Y. L., & Wang, Z. F. (2004). Cure of experimental botulism and antibotulismic effect of toosendanin. *Acta Pharmacol Sin, 25*(6), 839-848.

Shoemaker, C. B., & Oyler, G. A. (2013). Persistence of Botulinum neurotoxin inactivation of nerve function. *Curr Top Microbiol Immunol, 364*, 179-196. doi:10.1007/978-3-642-33570-9_9

Simpson, L. L. (2000). Identification of the characteristics that underlie botulinum toxin potency: implications for designing novel drugs. *Biochimie, 82*(9-10), 943-953. doi:10.1016/s0300-9084(00)01169-x

Sobel, J. (2005). Botulism. *Clin Infect Dis, 41*(8), 1167-1173. doi:10.1086/444507

Soucy, T. A., Dick, L. R., Smith, P. G., Milhollen, M. A., & Brownell, J. E. (2010). The NEDD8 Conjugation Pathway and Its Relevance in Cancer Biology and Therapy. *Genes Cancer, 1*(7), 708-716. doi:10.1177/1947601910382898

Tacket, C. O., Shandera, W. X., Mann, J. M., Hargrett, N. T., & Blake, P. A. (1984). Equine antitoxin use and other factors that predict outcome in type A foodborne botulism. *Am J Med, 76*(5), 794-798. doi:10.1016/0002-9343(84)90988-4

Tan, X., Calderon-Villalobos, L. I., Sharon, M., Zheng, C., Robinson, C. V., Estelle, M., & Zheng, N. (2007). Mechanism of auxin perception by the

TIR1 ubiquitin ligase. *Nature, 446*(7136), 640-645. doi:10.1038/nature05731

Tan, Y. Y., Zhou, H. Y., Wang, Z. Q., & Chen, S. D. (2008). Endoplasmic reticulum stress contributes to the cell death induced by UCH-L1 inhibitor. *Mol Cell Biochem, 318*(1-2), 109-115. doi:10.1007/s11010-008-9862-x

Thrower, J. S., Hoffman, L., Rechsteiner, M., & Pickart, C. M. (2000). Recognition of the polyubiquitin proteolytic signal. *EMBO J, 19*(1), 94-102. doi:10.1093/emboj/19.1.94

Tian, X., Isamiddinova, N. S., Peroutka, R. J., Goldenberg, S. J., Mattern, M. R., Nicholson, B., & Leach, C. (2011). Characterization of selective ubiquitin and ubiquitin-like protease inhibitors using a fluorescence-based multiplex assay format. *Assay Drug Dev Technol, 9*(2), 165-173. doi:10.1089/adt.2010.0317

Tighe, A. P., & Schiavo, G. (2013). Botulinum neurotoxins: mechanism of action. *Toxicon, 67*, 87-93. doi:10.1016/j.toxicon.2012.11.011

Tolosa, L., Donato, M. T., & Gomez-Lechon, M. J. (2015). General Cytotoxicity Assessment by Means of the MTT Assay. *Methods Mol Biol, 1250*, 333-348. doi:10.1007/978-1-4939-2074-7_26

Tsai, Y. C., Kotiya, A., Kiris, E., Yang, M., Bavari, S., Tessarollo, L., . . . Weissman, A. M. (2017). Deubiquitinating enzyme VCIP135 dictates the duration of botulinum neurotoxin type A intoxication. *Proc Natl Acad Sci U S A, 114*(26), E5158-E5166. doi:10.1073/pnas.1621076114

Tsai, Y. C., Maditz, R., Kuo, C. L., Fishman, P. S., Shoemaker, C. B., Oyler, G. A., & Weissman, A. M. (2010). Targeting botulinum neurotoxin persistence by the ubiquitin-proteasome system. *Proc Natl Acad Sci U S A, 107*(38), 16554-16559. doi:10.1073/pnas.1008302107

Vassilev, L. T., Vu, B. T., Graves, B., Carvajal, D., Podlaski, F., Filipovic, Z., . . . Liu, E. A. (2004). In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science, 303*(5659), 844-848. doi:10.1126/science.1092472

Videnovic, M., Opsenica, D. M., Burnett, J. C., Gomba, L., Nuss, J. E., Selakovic, Z., . . . Solaja, B. A. (2014). Second generation steroidal 4-aminoquinolines are potent, dual-target inhibitors of the botulinum neurotoxin serotype A metalloprotease and P. falciparum malaria. *J Med Chem, 57*(10), 4134-4153. doi:10.1021/jm500033r

Walker, T. J., & Dayan, S. H. (2014). Comparison and overview of currently available neurotoxins. *J Clin Aesthet Dermatol, 7*(2), 31-39.

Wang, X., Mazurkiewicz, M., Hillert, E. K., Olofsson, M. H., Pierrou, S., Hillertz, P., . . . D'Arcy, P. (2016). The proteasome deubiquitinase inhibitor VLX1570 shows selectivity for ubiquitin-specific protease-14 and induces apoptosis of multiple myeloma cells. *Sci Rep, 6*, 26979. doi:10.1038/srep26979

Wanzel, M., Vischedyk, J. B., Gittler, M. P., Gremke, N., Seiz, J. R., Hefter, M., . . . Stiewe, T. (2016). CRISPR-Cas9-based target validation for p53-

reactivating model compounds. *Nat Chem Biol, 12*(1), 22-28. doi:10.1038/nchembio.1965

Ward, J. A., Pinto-Fernandez, A., Cornelissen, L., Bonham, S., Diaz-Saez, L., Riant, O., . . . Tate, E. W. (2020). Re-Evaluating the Mechanism of Action of alpha,beta-Unsaturated Carbonyl DUB Inhibitors b-AP15 and VLX1570: A Paradigmatic Example of Unspecific Protein Cross-linking with Michael Acceptor Motif-Containing Drugs. *J Med Chem, 63*(7), 3756-3762. doi:10.1021/acs.jmedchem.0c00144

Wertz, I. E., & Wang, X. (2019). From Discovery to Bedside: Targeting the Ubiquitin System. *Cell Chem Biol, 26*(2), 156-177. doi:10.1016/j.chembiol.2018.10.022

Westerheide, S. D., Bosman, J. D., Mbadugha, B. N., Kawahara, T. L., Matsumoto, G., Kim, S., . . . Morimoto, R. I. (2004). Celastrols as inducers of the heat shock response and cytoprotection. *J Biol Chem, 279*(53), 56053-56060. doi:10.1074/jbc.M409267200

Wichterle, H., Lieberam, I., Porter, J. A., & Jessell, T. M. (2002). Directed differentiation of embryonic stem cells into motor neurons. *Cell, 110*(3), 385-397. doi:10.1016/s0092-8674(02)00835-8

Willis, B., Eubanks, L. M., Dickerson, T. J., & Janda, K. D. (2008). The strange case of the botulinum neurotoxin: using chemistry and biology to modulate the most deadly poison. *Angew Chem Int Ed Engl, 47*(44), 8360-8379. doi:10.1002/anie.200705531

Winner, B. M., Bodt, S. M. L., & McNutt, P. M. (2020). Special Delivery: Potential Mechanisms of Botulinum Neurotoxin Uptake and Trafficking within Motor Nerve Terminals. *Int J Mol Sci, 21*(22). doi:10.3390/ijms21228715

Wu, L., Grigoryan, A. V., Li, Y., Hao, B., Pagano, M., & Cardozo, T. J. (2012). Specific small molecule inhibitors of Skp2-mediated p27 degradation. *Chem Biol, 19*(12), 1515-1524. doi:10.1016/j.chembiol.2012.09.015

Yang, Y., Kitagaki, J., Dai, R. M., Tsai, Y. C., Lorick, K. L., Ludwig, R. L., . . . Weissman, A. M. (2007). Inhibitors of ubiquitin-activating enzyme (E1), a new class of potential cancer therapeutics. *Cancer Res, 67*(19), 9472-9481. doi:10.1158/0008-5472.CAN-07-0568

Yu, D. H., Xu, Z. Y., Mo, S., Yuan, L., Cheng, X. D., & Qin, J. J. (2020). Targeting MDMX for Cancer Therapy: Rationale, Strategies, and Challenges. *Front Oncol, 10*, 1389. doi:10.3389/fonc.2020.01389

Zeng, X., & King, R. W. (2012). An APC/C inhibitor stabilizes cyclin B1 by prematurely terminating ubiquitination. *Nat Chem Biol, 8*(4), 383-392. doi:10.1038/nchembio.801

Zeng, X., Sigoillot, F., Gaur, S., Choi, S., Pfaff, K. L., Oh, D. C., . . . King, R. W. (2010). Pharmacologic inhibition of the anaphase-promoting complex induces a spindle checkpoint-dependent mitotic arrest in the absence of spindle damage. *Cancer Cell, 18*(4), 382-395. doi:10.1016/j.ccr.2010.08.010

Zhang, F., Xu, R., Chai, R., Xu, Q., Liu, M., Chen, X., . . . Liu, N. (2020). Deubiquitinase Inhibitor b-AP15 Attenuated LPS-Induced Inflammation via Inhibiting ERK1/2, JNK, and NF-Kappa B. *Front Mol Biosci, 7*, 49. doi:10.3389/fmolb.2020.00049

Zhu, H., Wang, T., Xin, Z., Zhan, Y., Gu, G., Li, X., . . . Liu, C. (2019). An oral second-generation proteasome inhibitor oprozomib significantly inhibits lung cancer in a p53 independent manner in vitro. *Acta Biochim Biophys Sin (Shanghai), 51*(10), 1034-1040. doi:10.1093/abbs/gmz093

APPENDICES

A. Time-Dependent LC/A Experiment

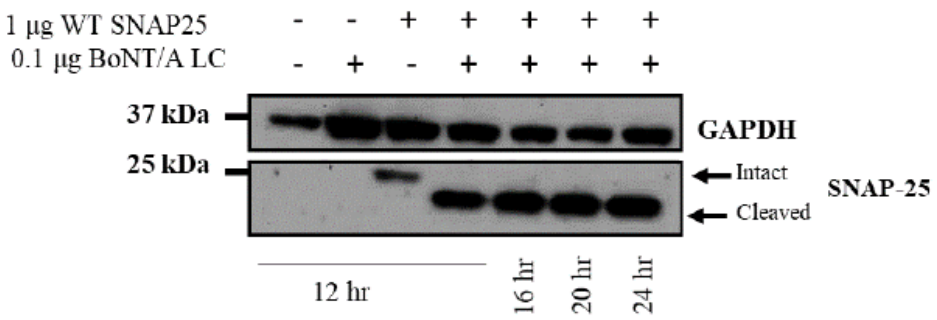


Figure 12 Western blot analysis of SNAP-25 protein expression under the effect of BoNT/A LC in co-transfected HEK293 cells in time dependent manner. GAPDH was used as a loading control. This experiment shows that LC we utilized is functional and can cleave SNAP-25 protein in HEK293 cells.

B. Vector Map

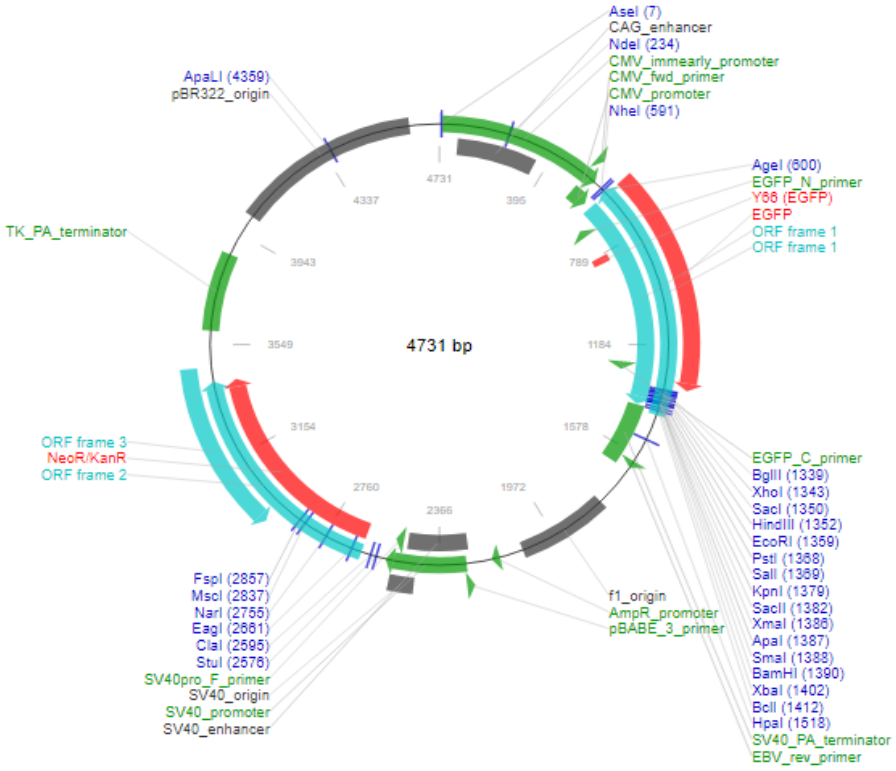


Figure 13 The map of pEYFP-C1 vector.

C. Buffer Contents

Luria Bertani (LB) Medium

20 g LB Medium Powder (Serva, 4850101) was dissolved in 800 ml deionized water and bring up to 1000 ml. Then autoclaved for 20 minutes.

Luria Bertani (LB) Agar

3 gr Agar (Sigma, 7002) was added in 200 ml LB medium and then autoclaved for 20 minutes.

NP-40 Lysis Buffer

Reagent	Volume
5 M NaCl	30 ml
10% NP-40	100 ml
1 M Tris (pH 8.0)	50 ml
Distilled H^2O	820 ml

All reagents were combined to top up the solution to 1 L. The NP-40 Lysis Buffer was stored at +4^oC.

10% Ammonium persulfate (APS)

1 g Ammonium persulfate was dissolved in 10 ml. Aliquots were stored at 20^oC.

6X Sample Loading Dye

Reagent	Volume
SDS (12%)	1.2 gr
β-mercaptoethanol (30%)	3 ml

Glycerol (60%)	6 ml
Bromophenol blue (0.012%)	0.0012 gr
Tris-HCl (0.375 M)	3.75 ml

5% Stacking Gel Mixture

Reagent	Volume (For 1 Mini Gel)
Acrylamide:Bisacrylamide (40%)	375 µl
0.5M Tris-HCl pH: 8.8	0.75 ml
SDS (10%)	30 µl
APS (10%)	30 µl
TEMED	3 µl
dH^2O	1.815 ml

10% Separating Gel Mixture

Reagent	Volume (For 1 Mini Gel)
Acrylamide – Bisacrylamide (40%)	1.5 ml
1.5M Tris-HCl pH: 8.8	1.5 ml
SDS (10%)	60 µl
APS (10%)	60 µl
TEMED	2.4 µl
dH^2O	2.88 ml

10X Running Buffer

Reagent	Volume
Tris Base	30.0 g
Glycine	144.0 g
SDS	10.0 g

30 g Tris Base, 144 g Glycine, and 10 g SDS were dissolved in 900 ml distilled water, and the final volume was adjusted to 1000 ml. No pH adjustment was required. Before use, it was diluted to 1X.

10X Transfer Buffer

Reagent	Volume
Tris Base	30.3 gr
Glycine	144.1 gr

Mild Stripping Buffer

Reagent	Volume
Glycine	15 g
SDS	1 g
Tween20	10 ml

15 g glycine, 1 g SDS and 10 ml Tween20 were dissolved in 800 ml distilled water. pH was adjusted to 2.2, and volume was brought up to 1 L with distilled water.

10X TBS

Reagent	Volume
Tris Base	24 g

NaCl	88 g

24 g Tris Base and 88 g NaCl was dissolved in 900 ml distilled water. pH was adjusted to 7.6, and volume was brought up to 1 L with distilled water.

0.01% TBS-T

Reagent	Volume
1X TBS	100 ml
Distilled H2O	900 ml
Tween-20	1 ml

100 ml 1X TBS was added on 900 mL dH2O, and finally, 1 ml Tween-20 was added to the solution.

Blocking Solution

2.5 g skimmed milk was dissolved in 50 ml 0.01% TBS-T.

TE Buffer pH 8.0

Reagent	Volume	Final concentration
1M Tris-Cl (pH 8.0)	1 mL	10 mM
0.5M EDTA (pH 8.0)	0.2 mL	1 mM
Distilled H2O	98.8 mL	

1 ml 1M Tris-Cl (pH 8.0) and 0.5M EDTA (pH 8.0) were added on 98.8 mL dH2O to top up the solution to 100 mL.

1 X Phosphate-Buffered Saline

Solution A (20X)

NaH_2PO_4	28.8 g
K_2HPO_4	4.8 g

Solution B (20X)

NaCl	160 g
KCl	4 g
$CaCl_2.2H_2O$	2.66 g
$MgCl_2.6H_2O$	2 g

Both solution A and solution B were prepared separately in 1000 ml distilled water.

In the preparation of 1X PBS, 50 mL of each 20X solution was taken and poured into 800 mL of deionized water. pH was stabilized at 7.4 with HCl, and then, the solution was completed to 1000 ml.

Phosphate-Buffered Saline without Calcium and Magnesium

Reagent	Volume
NaH2PO4	28.8 g
K2HPO4	4.8 g
NaCl	160 g
KCl	4 g

0.1% Gelatin Solution

0.5 g gelatin was dissolved in 500 ml dH_2O by microwaving on medium power for 2-4 minutes and then filtered with a 0.22 um sterile filter. Finally, the solution was autoclaved for 20 minutes.

0.1 mg/ml Poly-D-Lysine Solution

100 mg Poly-D-Lysine (70-150 kD molecular weight) (Sigma, P0899) was dissolved in 100 ml sterile distilled water to prepare a 1mg/ml stock solution. The

stock was diluted to 0.1 mg/ml with sterile distilled water before the coating procedure.

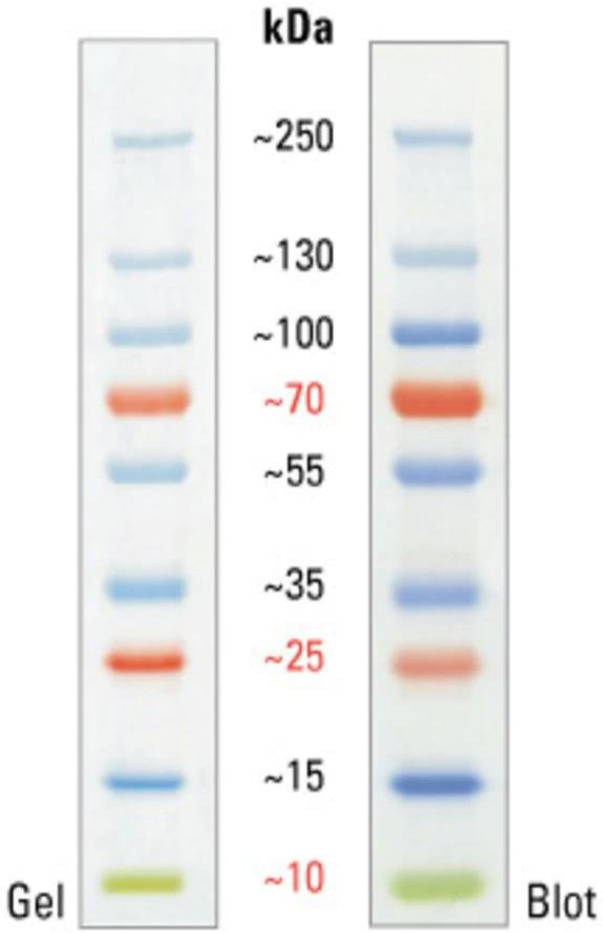
MTT Solution

5 mg MTT Powder (Sigma, M2003) was dissolved in Phosphate Buffer Saline (PBS). It was stored at 4°C and protected from the light.

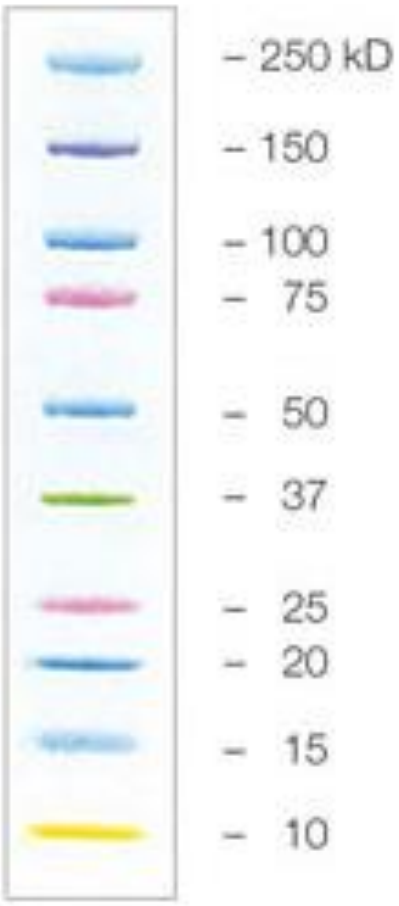
MTT Solubilization Solution

1 g Sodium Dodecyl Sulfate (SDS) was dissolved in 10 mL HCl (0.01 M) by vortexing.

D. Protein Ladders



PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa



Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standard