INVESTIGATION OF THE EFFECT OF KX2-361 MOLECULE ON POST-INTOXICATION OF BOTULINUM NEUROTOXIN SEROTYPE A, USING MOTOR NEURONS DIFFERENTIATED FROM MOUSE EMBRYONIC STEM CELL LINE HBG3

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

BY

DİLARA KOÇ

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

AUGUST 2022

Approval of the thesis:

INVESTIGATION OF THE EFFECT OF KX2-361 MOLECULE ON POST-INTOXICATION OF BOTULINUM NEUROTOXIN SEROTYPE A, USING MOTOR NEURONS DIFFERENTIATED FROM MOUSE EMBRYONIC STEM CELL LINE HBG3

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ABSTRACT

INVESTIGATION OF THE EFFECT OF KX2-361 MOLECULE ON POST-INTOXICATION OF BOTULINUM NEUROTOXIN SEROTYPE A, USING MOTOR NEURONS DIFFERENTIATED FROM MOUSE EMBRYONIC STEM CELL LINE HBG3

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August 2022, 60 pages

Botulinum neurotoxins (BoNTs) are the most poisonous neurotoxins known, and intoxication by this toxin leads to botulism, a potentially lethal disease. BoNT/A, one of the 7 serotypes of BoNTs, is the most common serotype that causes human botulism. BoNT/A primarily targets the peripheral cholinergic nerves, especially motor neurons, and causes inhibition of acetylcholine release by cleaving SNAP-25 protein. Once BoNTs are internalized into motor neurons, there are no therapeutic options to neutralize them within the cytoplasm. Our previous studies evaluated libraries of small molecules for their inhibitory activities against BoNT/A. Such work led to the discovery of an Src inhibitor, KX2-391 (Tirbanibulin), as a BoNT/A inhibitor in motor neuron assays. Despite the promising features of the compound, unfortunately, KX2-391 failed to protect significantly against BoNT/A intoxication in mice. One possible reason for results obtained from *in vivo* studies may be the insufficient penetration capacity of KX2-391 to cross the Blood-Brain Barrier (BBB). Recently, a structural analog of KX2-391, KX2-361, specifically designed to cross BBB, has been developed and published. Therefore, in this thesis work, our

primary goal was to evaluate the inhibitory effects of KX2-361 against BoNT/A. Towards this goal, we evaluated both KX2-391 and KX2-361 molecules for their potential effects on cell viability in PC12 cells via MTT assay, and our findings show no significant cell viability differences, especially with lower doses. We then generated motor neurons from HBG3 mouse embryonic stem cells and tested the possible effects on cell viability of KX2-361 on motor neurons with imaging-based assays. Following this, we tested the compound's inhibitory effects against BoNT/A in mESC-derived motor neurons in pre-intoxication conditions, and excitingly, KX2-361 provided dose-dependent protection against the toxin. We then evaluated the compound in post-intoxication conditions, which exhibited activity against the toxin. Furthermore, KX2-361 inhibited BoNT/A LC enzymatic components in PC12 cells transfected with BoNT/A LC. The results of this work may open new avenues to develop structural analogs of KX2-361 to increase its efficacy against BoNT/A, which may provide a critical lead compound for drug development efforts against BoNT intoxication.

Keywords: Mouse Embryonic Stem Cells, Motor Neuron, KX2-361, KX2-391, Botulinum Neurotoxin

ÖZ

KX2-361 MOLEKÜLÜNÜN BOTULINUM NÖROTOKSİNİ SEROTİP A İLE ZEHİRLENME SONRASI KOŞULLARA ETKİSİNİN HBG3 FARE KÖK HÜCRELERİNDEN FARKLILAŞTIRILMIŞ MOTOR NÖRONLARDA İNCELENMESİ

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Botulinum Nörotoksinleri (BoNTs) bilinen en zehirli bakteri toksini olup ölümle sonuçlanabilen botulizm hastalığına sebep olur. 7 BoNT serotipinden biri olan BoNT/A insan botulizmine sebep olan en yaygın serotiptir. BoNT/A, öncelikli olarak periferik kolinerjik nöronları, özellikle de motor nöronları hedef alır ve SNAP-25 proteinini keserek asetilkolin salınımını inhibe eder. BoNTlar motor nöronların içine nüfuz ettikten sonra toksini hücre içinde nötralize edebilecek bir tedavi bulunmamaktadır. Önceki çalışmalarımız, küçük molekül kütüphanelerini BoNT/A'ya karşı inhibe edici aktiviteleri açısından değerlendirmiştir. Bu çalışma, motor nöron deneylerinde, bir BoNT/A inhibitörü olarak bir Src inhibitörü olan KX2-391'in (Tirbanibulin) keşfedilmesine yol açmıştır. Bileşiğin umut verici özelliklerine rağmen, ne yazık ki, KX2-391 farelerde BoNT/A zehirlenmesine karşı önemli ölçüde koruma sağlayamamıştır. *in vivo* çalışmalardan elde edilen sonuçların olası bir nedeni, KX2-391'in Kan-Beyin Bariyerini (BBB) geçmek için yetersiz penetrasyon kapasitesi olabilir. Son zamanlarda, özellikle BBB'yi geçmek için tasarlanmış KX2-391'in yapısal bir analoğu olan KX2-361 geliştirildi ve yayınlandı.

Bu nedenle, bu tez çalışmasında öncelikli amacımız KX2-361'in BoNT/A'ya karşı inhibe edici etkilerini değerlendirmekti. Bu amaç doğrultusunda, hem KX2-391 hem de KX2-361 moleküllerini, PC12 hücrelerinde hücre canlılığı üzerindeki potansiyel etkileri açısından MTT tahlili ile değerlendirdik ve bulgularımız özellikle düşük dozlarda hücre canlılığında önemli bir farklılık göstermedi. Daha sonra, HBG3 fare embriyonik kök hücrelerinden farklılaştırılmış motor nöronlar elde ettik ve KX2-361'in motor nöronlar üzerindeki olası toksik etkilerini görüntüleme tabanlı tahlillerle test ettik. Bunu takiben, mESC'den farklılaştırılan motor nöronlarda bileşiğin BoNT/A'ya karşı inhibe edici etkilerini zehirlenme öncesi koşullarda test ettik ve heyecan verici bir şekilde KX2-361'in, toksine karşı doza bağlı koruma sağladığı görüldü. Daha sonra bileşiği, zehirlenme sonrası koşullarda değerlendirdik ki bu koşullarda da bileşiğin toksine karşı aktivite sergilediği görüldü. Ayrıca, KX2-361, BoNT/A LC ile transfekte edilmiş PC12 hücrelerinde BoNT/A LC enzimatik bileşenlerini inhibe edebildi. Bu çalışmanın sonuçları, BoNT/A'ya karşı etkinliğini artırmak için KX2-361'in yapısal analoglarını geliştirmek için yeni yollar açabilir ki bu da BoNT zehirlenmesine karşı ilaç geliştirme çabaları için kritik bir öncü bileşik sağlayabilecektir.

Anahtar Kelimeler: Fare Embriyonik Kök Hücre, Motor Nöron, KX2-361, KX2-391, Botulinum Nörotoksin

To the beauty of the science

ACKNOWLEDGMENTS

I would like to express my deepest gratitude to my supervisor, Assoc. Prof. Dr. Erkan Kiriş for his support, encouragement, guidance, and criticism during my time in Kiriş Lab. I have always felt his reliance on the path to becoming an independent scientist. When I faced a problem, he was always available and supportive, encouraging me to be even better than I was the day before. Thanks to him, I take my steps in science much more confidently today.

I wish to express my gratitude to Prof. Dr. Mesut Muyan for his support and motivation throughout my academic life. I would also like to thank my jury members, Prof. Dr. Kemal Can Akçalı and Prof. Dr. Sreeperna Banerjee, for their valuable time and comments.

I would like to thank Prof. Dr. Erden Banoğlu and his group for their support in the KX2-361 studies.

I would like to thank all current and former members of Kiriş Lab for their support. I want to thank my dear lab members Peri Beşarat and Sena Ezgin exclusively for not only being good lab partners but also great friends. I am fortunate to have the privilege of walking this path with them. I especially thank Peri Beşarat for her friendship, support, and good taste in music, which always makes my day. She has always been reliable whenever I need help. I also have special thanks to Sena Ezgin for always being by my side during all the good and bad times throughout this process and for sharing these moments with me. I want to thank Bilgehan İbibik for her help in the most unexpected moments, invaluable friendship, and support. I would also like to thank İsmail Güderer for his unconditional love, endless understanding, always being by my side, and being a light for me in my most desperate moments.

Last but not least, I would like to thank my mother and my sister because I wouldn't have gotten to this point without their support and love. I would like to thank my mother, from whom I learned to ask questions, research, and find solutions, for giving me the courage to be a woman who can stand on her own two feet.

This study was supported by the Middle East Technical University Scientific Research Projects Coordination Unit under grant numbers GAP-108-2021-10672 and Thesis Project TEZ-YL-108-2022-10856.

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

LIST OF ABBREVIATIONS

ABBREVIATIONS

Ach Acetylcholine

BBB Blood-Brain Barrier

BoNT Botulinum Neurotoxin

BoNT/A Botulinum Neurotoxin Serotype A

EB Embryoid Body

FBS Fetal Bovine Serum

FDA Food and Drug Administration

GFP Green Fluorescent Protein

HC Heavy Chain

LC Light Chain

MC Mitomycin C

MEF Mouse Embryonic Fibroblast

mESC Mouse Embryonic Stem Cell

PVDF Polyvinylidene Fluoride

RA Retinoic Acid

Shh Sonic Hedgehog

SNAP-25 Synaptosomal-Associated Protein of 25 kDa

SNARE Soluble N-Ethylmaleimide-Sensitive Factor Attachment Protein Receptor

CHAPTER 1

INTRODUCTION

1.1 Botulinum Neurotoxin and Botulism

Botulinum neurotoxins (BoNTs) are toxins produced by the anaerobic bacteria Clostridium botulinum (Smith, Hill, & Raphael, 2015). These toxins cause botulism, causing flaccid paralysis in those it infects (Rossetto, Pirazzini, & Montecucco, 2014). BoNTs target the peripheral cholinergic nerve endings of the human nervous system with high affinity while also targeting the autonomic nervous system (Rossetto et al., 2014; Rummel, 2015). Botulinum neurotoxins specifically target vertebrates (Peck, 2006). BoNTs can enter the body in different ways. The most common method of transmission is food poisoning. Today, although the developments in food technology have prevented bacteria from growing in food, botulism due to botulinum neurotoxins is still a severe problem for human life (Peck, Stringer, & Carter, 2011). The mechanism of action of BoNTs and peripheral nerve palsy observed as a result of poisoning, including the muscles and autonomic nervous system, is the same regardless of the method of entry into the body. Paralysis begins on the face, follows the eyes, and progresses to the respiratory system (Cherington, 1998; Johnson & Montecucco, 2008). If respiratory support cannot be provided to the patient under these conditions, poisoning can result in death due to respiratory failure due to BoNT intoxication following muscle paralysis (N. Thirunavukkarasu et al., 2018). Although it is possible to keep the patient alive with respiratory support, botulism is a severe disease that threatens human health in the long term and on a broad scale, as it will take months for the patient to fully recover (Dashtipour & Pedouim, 2016).

There are 7 known serotypes of botulinum neurotoxin (A to G). Among these serotypes, A, B, E, and F cause human botulism, while other serotypes affect humans very little. In particular, BoNT serotype A (BoNT/A) is the serotype that most commonly causes disease in humans (Rossetto et al., 2014). Although their sequences differ, the molecular structure is conserved among all BoNT serotypes. BoNTs are synthesized as single-chain, inactive polypeptides of 150 kDa. The active toxin consists of a 100 kDa heavy chain (HC) and a 50 kDa light chain (LC), and these two chains are linked by a disulfide bond (Cenciarelli, Riley, & Baka, 2019) (Dong, Masuyer, & Stenmark, 2019; Rossetto et al., 2014). Reducing the disulfide bond between these two chains releases the metalloprotease activity of BoNT (Simpson, Maksymowych, Park, & Bora, 2004).

The BoNT heavy chain consists of two 50 kDa parts, the amino-terminal part (HC-N) and the carboxy-terminal part (HC-C) (Fischer et al., 2008). From these parts, HC allows the toxin to interact with the unmyelinated region of motor neuron terminals and also to bind the toxin to the polysialoganglioside (PSG) and the luminal domain of synaptic vesicle protein (J. O. Dolly, Black, Williams, & Melling, 1984; Rummel, 2013). This binding causes the toxin to be taken up and reach the endocytic part (Rummel, 2013). The HN part of BoNT allows the LC, the catalytic domain of the toxin, to exit through the endocytic vesicle and pass into the cytosol. The LC, which enters the cytosol and releases the heavy chain, quickly finds its target protein, the SNARE complex elements, as described below.

1.2 Molecular Mechanisms of Botulinum Neurotoxin

Botulinum neurotoxin, similar to other bacterial exotoxins, has evolved to introduce the metalloprotease domain into the host cell. The poisoning of the nerve endings of BoNT takes place following specific steps. First, BoNT binds with high affinity to its primary target, the presynaptic plasma membrane at the nerve endings of motor neurons (J. O. Dolly et al., 1984). During this binding, hundreds of BoNT molecules are found per μm^2, which shows how specific this toxin is to its target, and this

specificity against motor neurons makes BoNT particularly dangerous. BoNT initiates the intracellular entry process by interacting with two receptors on the presynaptic membrane, polysialoganlioside (PSG) and synaptic vesicle protein (Montecucco & Schiavo, 1995)(Montecucco, 1986). The robust binding of BoNT to the membrane is accomplished by PSG, while the SV ensures the toxin uptake by the endocytic vesicle.

While the synaptic vesicle performs endocytosis, the v-ATPase proton pump increases the amount of hydrogen in the vesicle. It acidifies the environment inside so that the vesicle can be reused and recycled (Parsons, 2000). During the uptake of BoNT by the SV, this acidification caused by the v-ATPase creates a suitable environment for the LC to go out of the vesicle. This decrease in intra-vesicle pH causes a conformational change involving LC, HC, and even membrane lipids. As a result of this change, ion channels are formed (Montecucco, Schiavo, & Dasgupta, 1989) in the membrane, and HC acts as a transmembrane chaperone, passing the LC through the membrane and releasing it into the cytosol (Montal, 2010).

Breaking the disulfide bond that connects the LC and HC is the most critical step in botulinum neurotoxin poisoning. The time and place the disulfide bond is broken are crucial for the LC to release its metalloprotease activity (Fischer & Montal, 2007). If the disulfide bond is broken before the LC enters the cytosol, the translocation of the LC is impaired because the LC must be bound with HC to pass through the vesicle membrane to the cytosol (Pirazzini, Rossetto, Bolognese, Shone, & Montecucco, 2011). For this reason, the disulfide bond is not broken at low pH. After the disulfide bond is broken, the LC rapidly turns to its target protein, SNARE complex elements, and performs its protease activity.

After BoNT/A enters the cell, it exerts its effect in motor neurons by cleaving Synaptosomal associated protein 25 (SNAP25), one of the N-ethylmaleimide sensitive factor (NSF) binding protein receptor (SNARE) complex protein, from its C-terminal region. This cleavage causes a 9 amino acid break from the 206 amino acid SNAP-25 protein (J. Oliver Dolly, Lawrence, Meng, Wang, & Ovsepian, 2009;

Huang X, 2019; Walker & Dayan, 2014). With this interruption, the connection between motor neurons and muscles is paralyzed (Holmberg, Krogseth, Grude, & Wian, 2018), and the regulation of SNARE proteins' release of Acetylcholine (ACh) molecules from motor neurons to the neuromuscular junction and the realization of muscle movements, which occurs under normal conditions, is interrupted (Huang X, 2019). As a result, the muscles cannot contract, and poisoning can result in death due to respiratory failure as a result of BoNT intoxication following muscle paralysis (Holmberg et al., 2018; Marco Pirazzini, Ornella Rossetto, Roberto Eleopra, & Cesare Montecucco, 2017). BoNT serotypes other than the BoNT/A serotype prevent the formation of a stable SNARE complex by cleaving their specific target SNARE proteins. On the other hand, although BoNT/A cleaves the SNAP-25 protein, the truncated SNAP-25 remains part of a stable SNARE complex. However, cleavage of SNAP-25 completely inhibits exocytosis by itself. Even cleaving only 10-15% of the total SNAP-25 is sufficient for paralysis (M. Pirazzini, O. Rossetto, R. Eleopra, & C. Montecucco, 2017).

1.3 Pharmaceutical Usage of BoNTs

Essential features of BoNTs make them a potential threat to human health; they are highly toxic, neuron-specific, have a long half-life, and have an enzymatic nature that can constantly inactivate their substrate, making them a very dangerous toxin. In addition to their harmful effects, BoNTs are used extensively in clinics for various conditions, studies, and cosmetic purposes to remove wrinkles (Marco Pirazzini et al., 2017).

Different BoNT formulations are available for the therapeutic use of botulinum neurotoxin. The two most well-known and reliable products are BOTOX (Allergan), which contains onabotulinum toxin A, and Dysport, which contains abobotulinumtoxin A. Masport, a Dysport counterpart, is also widely used and, like the other two products, it is FDA-approved. Since Masport and BOTOX products contain different BoNT/A formulations, dose equivalency is also essential in using

these products. A 1:3 dose conversion is known between BOTOX and Dysport/Masport products (Dashtipour & Pedouim, 2016; Scaglione, 2016).

BoNT is widely available, albeit for therapeutic purposes, and caution should be exercised against misuse or accident because there is no cure for BoNT poisoning after the toxin has penetrated into motor neurons (Nagarajan Thirunavukkarasu et al., 2018). The long half-life of BoNT results in prolonged poisoning time and effects; therefore, long-term respiratory support and intensive care application are required (Marco Pirazzini et al., 2017; Nagarajan Thirunavukkarasu et al., 2018). Due to the long duration of action, it may not be possible to offer the intensive treatment needed for BoNT/A poisoning to many patients; this is a significant problem to be solved, as intensive care capacities are limited in hospitals (Dashtipour & Pedouim, 2016). For these reasons, discovering and developing a treatment that will inactivate the already internalized BoNT is an important scientific gap.

The only known target of BoNT/A in the cell is the SNAP-25 protein, and after poisoning, LC is known to cleave this protein at a specific site (Fernandez-Salas et al., 2012). In addition, multiple studies have shown that BoNT-induced SNAP-25 cleavage alone can stop nerve conduction. For these reasons, specific cleavage of the SNAP-25 protein is widely used in the literature as a read-out for detecting the activity of BoNT.

The inhibition of possible inhibitors of LC-induced SNAP-25 cleavage is used to measure the inhibition potential of the molecule. For this reason, LC-induced SNAP-25 interruption was used as a read-out in our study.

1.4 Drug Development Against Botulinum Neurotoxin Intoxication

Antibodies targeting BoNTs have been developed as a potential treatment method, but this method is effective before the toxin enters the cell, so it only has a therapeutic feature against circulating BoNT (Dembek, Smith, & Rusnak, 2007). Antibody therapy cannot prevent the effect of the toxin that has already entered the cell, as

patients affected by BoNT naturally only go to the hospital for treatment after symptoms appear (Nagarajan Thirunavukkarasu et al., 2018). Therefore, therapeutic studies developed to prevent poisoning after the entry of the toxin into the cell are needed. One of the prominent approaches in these studies is to target the signaling pathways that may play a role in the activity of the toxin and/or its entry into the cell by using small molecules.

In our previous studies, we screened various libraries and identified several compounds with inhibitory activity against BoNT/A, which is the serotype responsible for most cases of human botulism (Erkan Kiris et al., 2015). Notably, the compounds we evaluated were not just BoNT/A LC active site inhibitors; their cellular targets were mainly known. From this pool of compounds, we finally selected the KX2-391 molecule for *in vivo* studies based on the drug-like properties of the molecule and our extensive data on motor neuron activity, pharmacokinetics, and tolerability. As a result, the KX2-391 molecule, a well-established Src family kinase inhibitor, was selected for further testing in *in vivo* studies.

1.5 Src Family Kinases

Src Family Kinases (SFKs) are a family of proteins also known as non-receptor tyrosine kinases. SKFs with 4 members, Src, Fyn, Yes, and Lyn, have a protected domain organization among their members. While the SH1 domain of SFKs, which consists of 3 basic domains, is responsible for catalytic tyrosine kinase activity, the SH2 and SH3 domains are responsible for target recognition and binding (Ohnishi, Murata, Okazawa, & Matozaki, 2011). SFKs are primarily associated with cancer, but they also play important roles in the nervous system, such as neurotransmission, synaptic plasticity, axon guidance, and development and maintenance of neurons (Kao, Palmesino, & Kania, 2009; Ohnishi et al., 2011). In addition, SFKs have high gene expression in motor neurons and play a role in critical processes such as neurotransmitter release, axonal growth, and modulation of ion channels (Wiesner & Fuhrer, 2006). Modulation of SFKs is achieved through phosphorylation. The

auto-phosphorylation of the tyrosine 418 (Y416 in chicken) domain (Koga et al., 2006) plays a role in activating protein kinase activity. The phosphorylation of this region plays a critical role in SFK activation and modulation. Related to this, it has been shown that BoNTs can be phosphorylated by Src after they enter the cell, and this event may affect the activity of the toxin (Ibanez, Blanes-Mira, Fernandez-Ballester, Planells-Cases, & Ferrer-Montiel, 2004). As mentioned above, our studies have shown that Src family kinase inhibitors can be used to antagonize the toxicity of BoNT in human embryonic stem cell-derived motor neurons.

1.6 KX2-391 Small Molecule

The KX2-391 (N-benzyl-2-(5-(4-(2-morpholinoethoxy) phenyl) pyridin-2-yl) or known as Tirbanibulin, is a non-ATP competitive Src kinase inhibitor. It targets the substrate-binding domain of Src kinase instead of the ATP binding site as with most Src kinase inhibitors. This molecule was tested in Phase 3 clinical trials of an actinic keratosis skin disease and received FDA approval in 2021 (Nagai et al., 2007). , it was tested in clinical trials against solid tumors (Antonarakis et al., 2013; Naing et al., 2013) and for topical and oral administration (Ciesielski et al., 2018). It has been shown in previous studies that the KX2-391 molecule, as an inhibitor of Src kinase, can act on cells in a non-ATP-competitive manner and affect tubulin polymerization (Smolinski et al., 2018). Although this molecule, which has the potential to be a drug in several aspects, showed higher success in our *in vitro* experiments than other molecules, it did not show a significant effect in *in vivo* experiments (i.e., in toxin-treated mouse models). It was evaluated that this situation could be potentially caused by the inability of the KX2-391 molecule to cross the blood-brain barrier.

1.7 KX2-361 Small Molecule

There are 10 structural analogs of KX2-391 generated in the literature, and among these derivatives, KX2-361 was designed explicitly to increase BBB penetration.

KX2-361 has been investigated by various groups, and it has been demonstrated that it has higher lipophilicity and lower ionic character and these features of KX2-361 provide a better capacity for BBB penetration demonstrated by mouse experiments. It is also being investigated in clinical trials (Phase 1) in brain-related diseases (Ciesielski et al., 2018). To the best of our knowledge, the efficacy of KX2-361 against BoNT/A has never been investigated. Determining whether KX2-361 affects the biological action of BoNT in neurons may be essential for the progression of drug discovery against botulinum neurotoxin intoxication. KX2-391 is known to be the first Src inhibitor used in clinical trials, and although it received FDA approval for actinic keratosis in 2021, KX2-361 may be a more suitable molecule for central nervous system use than KX2-391.

1.8 Aim of the Study

This study aimed to evaluate the protective effect of KX2-361, compared to KX2-391 against BoNT/A intoxication, in motor neurons derived from mouse embryonic stem cells and PC12 cells. This is technically a follow-up study from a previous publication from our group that demonstrated the KX2-391 might be a promising small molecule against BoNT/A. For the drug discovery efforts against BoNT/A, literature has shown that it is crucial to perform cell culture studies rather than *in vitro* test tube experiments (enzymatic assays) because compounds that were successful in *in vitro* experiments often did not show the same success in cells. For cell culture studies, however, it is vital to use physiologically meaningful cultures of motor neurons because these cells are the main target of the toxin. Given that KX2-361 is shown to cross BBB efficiently and we have shown that the parent compound, KX2-391 is a potent BoNT inhibitor, the results of this study may open new avenues for BoNT drug discovery efforts.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Culture

2.1.1 Production of Mitotically Inactivated Mouse Embryonic Fibroblasts

For use in stem cell culture, primary mouse embryonic fibroblasts (mouse embryonic fibroblasts, MEF) were cultured and mitotically inactivated, and then cell stocks were generated. Inactivated MEFs used as feeder cells were used instead of other more expensive techniques. They are frequently used in the literature to allow stem cells to retain their pluripotent properties (Amit & Itskovitz-Eldor, 2009). Although it is possible to purchase inactivated MEFs from abroad from commercial sources, it is cheaper and more efficient to generate our own mitotically inactivated MEFs in our laboratory. For this, primary MEF cell stocks that were obtained from Dr. Lino Tessarollo's lab (NCI-Frederick, NIH, USA) were inactivated by Mitomycin C treatment in light of the protocols we used before (Tessarollo, 2001). Briefly, primary MEFs in their early passages (such as P3) were grown in 150 mm cell culture dishes in a MEF medium containing DMEM (Thermo Fisher, catalog no. 41966029), 10% Fetal Bovine Serum (FBS) (Thermo Fisher, catalog no. 10270106), 1% GlutaMAX (Thermo Fisher, catalog no.) 35050061) and 1% Pen/Strep (Thermo Fisher, catalog no. 15140122)) at 5% CO 2 and 37°C.

After cells reached approximately 80% confluency, they were treated for 3 hours with 10 μg/mL Mitomycin C (Cayman Chemicals, catalog no. 11435) diluted in MEF medium. Cells kept at 5% CO_2 and 37°C temperature during incubation were

washed 3 times with PBS (Biological Industries, catalog no. BI02-023-1A) to take cell stocks after 3 hours. Then cell stocks were prepared using a freezing medium containing 50% MEF medium, 40% FBS, and 10% DMSO (Serva, catalog no. SE3975701)) and stored in the vapor phase in nitrogen tanks for use in stem cell culture. These cells were used throughout the study in our mouse embryonic stem cell cultures.

2.1.2 Directed Differentiation of Motor Neurons from HBG3 Mouse Embryonic Stem Cells

2.1.2.1 Cultivation of HBG3 Mouse Embryonic Stem Cells

The mouse embryonic stem cell line used in this study, HBG3 (a kind gift from Thomas Jessell, Columbia University, USA), contains a green fluorescence protein (GFP) controlled by the transcription factor HB9. The transcription factor HB9, active in motor neurons, synthesizes GFP after cells differentiate into motor neurons. This specific property of the HBG3 line has been used as an indicator for the successful differentiation of motor neurons throughout the study (Wichterle & Peljto, 2008). For HBG3 embryonic stem cell culture, 150 mm cell culture plates were coated with 0.1% gelatin (Serva, catalog no. 221501.02) and subjected to UV light for 30 minutes. After the coating process, mitotically inactivated MEF (mitoMEF) cells were seeded on gelatin-coated plates with stem cell growth medium containing DMEM, 15% FBS, 1% GlutaMAX, 1% Penicillin/Streptomycin, 1% Non-essential Amino Acids, 0.1 mM β-mercaptoethanol, and 1000 units/mL Leukemia Inhibitory Factor (LIF). MitoMEF cells were incubated 3-4 hours before stem cell introduction in a cell culture incubator. When mitoMEFs were attached to the plate, cells from HBG3 cell stocks in our nitrogen tank in the METU Department of Biological Sciences were seeded on which these gelatin-coated and inactive MEFs were cultivated using the stem cell medium. Cells were cultured under conditions of 5%

CO2 and 37°C. The cell medium was changed daily until stem cell colonies grew in size and number suitable for differentiation.

2.1.2.2 Directed Differentiation of HBG3 Cells to Motor Neurons

In the differentiation process from HBG3 stem cells to motor neurons, optimized and successful protocols were followed by our group and different groups working in this field (Wichterle & Peljto, 2008).

As stated above, the successful differentiation of this cell line into motor neurons can be visualized by eGFP synthesis, and these neurons have been characterized in detail in our previous studies (Kiris et al., 2011). Thanks to this feature of the HBG3 cell line, the motor neurons we produced were regularly checked during the differentiation processes using the FLOID Cell Imaging System in the department.

When the mouse embryonic stem cell colonies reached sufficient size and number, the cells were put into the differentiation process. First, stem cell colonies need to be detached from inactive MEFs. For this purpose, stem cells were incubated with the enzyme Dispase (Thermo Fisher, catalog no. 17105041), which is known to cut the connection between embryonic epithelium and fibroblasts and separate stem cells from the feeder cell line. At the end of the incubation, the cells were checked under the microscope to confirm that the stem cell colonies and MEFs were dissociated from each other. Then, the stem cell colonies were collected and centrifuged, and the supernatant was removed without disturbing the pellet so that the Dispase enzyme could be removed from the medium. Cells were washed with serum-free DMEM and centrifuged again, and re-suspended in ADFNK medium (1:1 Advanced DMEM/F12 (Thermo Fisher, catalog no. 12634028) and Neurobasal Medium (Miltenyi Biotec, catalog no. 130-093-570), 1% Pen /Strep, 1% GlutaMAX, 10% Knock-out Serum Replacement (KSR) (Thermo Fisher, catalog no. 10828028), 0.1mM β-mercaptoethanol) and seeded in low-binding surface cell plates. Embryoid bodies are expected to form in these plates as cells cannot adhere to the surface. This process

was considered as Day 0 of differentiation. On Day 2, EBs were collected and centrifuged at 6500 rpm for 1.5 minutes. The cell pellet was dissolved in 12 ml of ADFNK medium containing 1.5 μM Retinoic acid (RA) (Sigma, catalog no. 2625) and transferred to a new low-binding surface plate. On day 3, EBs were collected in the center of the cell plate by circular motions, and the RA-containing medium from the day before was aspirated from the edges of the plate. The use of centrifuges was avoided in order not to break up the EBs formed on the second day. After the medium was aspirated, 12 ml of ADFNK medium containing 1 μM Sonic-hedgehog protein Ag.Hh 1.5 (Cellagentech, catalog no. C4412-2s), prepared just prior to aspiration, was administered to the cells. On the fourth day of the differentiation, considering the half-life of Ag.Hh 1.5, 5 ml of ADFNK – Ag.Hh 1.5 solution was added to the plate so that the final concentration in the plate was still 1 μM. No medium change was performed on this day. On Day 5, EBs were collected in the center of the cell plate by circular motions, and the medium containing Ag.Hh 1.5 was aspirated and replaced with 12 ml of ADFNB medium (1:1 Advanced DMEM/F12 ((Thermo Fisher, catalog no. 12634028) and Neurobasal Medium (Miltenyi Biotec, catalog no. 130-093). -570), 1% Pen/Strep, 1% Glutamax, and 2% B27 serum-free supplement (Gibco, ref. 12587-010)). On Day 6, The medium of embryoid spheroids was changed as on day 5, and 12 ml of fresh ADFNB medium was added. On Day 7, EBs were collected and centrifuged at 200g for 2 minutes. The supernatant was aspirated, and the cell pellet was washed with 5ml of PBS and centrifuged again at 200g for 2 minutes. The supernatant was removed from the medium, and the pellet was re-suspended with 1ml Accutase (Biolegend, catalog no. 42320) enzyme and kept in a water bath at 37˚C for 5 minutes to decompose the EBs. Then, in order to get rid of the toxic effect of the enzyme, the cells were centrifuged at 6500 rpm for 1 minute, and the pellet was dissolved in DMEM containing 10% FBS. Cells were centrifuged at 200g for 4 minutes. After removing the supernatant, the cell precipitate was finally dissolved in 4ml of ADFNB medium by gentle pipetting. Overly lysing cells at this step will result in significant cell death and failure of the differentiation process. 50 μl of the cell suspension was taken, and cells were counted and seeded at $5x10^5$ cells

per well in a 24-well cell plate pre-coated with Matrigel (Corning, catalog no. 354277). The plates were then incubated in cell culture incubators, and to promote neurite growth, cells were cultured for an additional 3 days before toxicity experiments, and experiments were started on the 10th day of differentiation. During this maturation process, GFP signals coming from motor neurons were regularly checked via FLOID Cell Imaging System.

2.1.3 Cultivation of PC12 Cells

Before starting the PC12 culture, the 100 mm cell culture plate was first coated with 0.1% gelatin, followed by 0.01mg/ml collagen coating (Sigma, C7661-25MG). The collagen-coated plate was incubated in a cell culture incubator for 3 hours, then collagen was removed, and the plate was left to dry under UV light for 40 minutes. PC12 cells were seeded using PC12 Growth Medium (High-Glucose DMEM, 10% Horse Serum (Thermo Fisher, catalog no. 26050088), 5% FBS, 1% Pen/strep) and incubated under conditions of 37˚C and 5% CO2 in cell culture incubator. Medium change was performed daily, and the morphology of cells was checked under a microscope daily to prevent clump formation of cells. When cells reached 70% confluency, they were either passaged or taken as cell stocks for future usage throughout the study.

2.2 MTT Assay

Both small molecules (KX2-391 and KX2-361) were analyzed for their effects on cell viability at 7 different doses by MTT assays using PC12 cells. For this aim, a 96-well plate was coated with 0.1% gelatin and then with 0.01 mg/ml collagen. After coating, PC12 cells were seeded on the plate at a density of $5x10^4$ cells per well, and both KX2-391 and KX2-361 were introduced to cells with a wide range of doses (0.1 μM, 1 μM, 10 μM, 20 μM, 30 μM, 40 μM, and 50 μM). First, the KX2-361 compound was dissolved with DMSO to obtain a 10mM stock concentration. The

stock was distributed into aliquots of 20 µl to avoid multiple freeze-thaw cycles. Since the drugs were dissolved using DMSO, the same volume of DMSO was used as a control (vehicle control) in each experiment. Since the biological half-life of the compounds is about 4 hours (Antonarakis et al., 2013), the cells were incubated in suspensions in a cell culture incubator for 4 hours. After 4 hours, MTT solution at a concentration of 5 mg/ml was added to each well and incubated for 4 hours. During this time, living cells were expected to produce formazan crystals by processing MTT. Formazan crystals formed at the end of the incubation were dissolved by introducing SDS-HCl solution, and the plate was measured at 570 nm using an ELISA plate reader. Results were analyzed using Student's t-test with GraphPad Prism. The tests were repeated with at least 3 different biological replicas.

2.3 Imaging-Based Analysis

As described above, the particular cell line used in this project contains the HB9::eGFP transgene, and these cells can give green signals under fluorescent light when successfully differentiated into motor neurons (Wichterle & Peljto, 2008). These properties of HBG3 cells were used to examine whether the small molecules KX2-391 and KX2-361 used in the project have any effect on the cell viability of motor neurons. In the experiments, motor neurons differentiated from HBG3 cells were seeded in 24-well plates and then exposed to KX2-391 or KX2-361 treatment at concentrations of 10 µM, 20 µM, 30 µM, and 40 µM. The GFP fluorescent signal in each condition was visualized with the FLOID Cell Imaging System as soon as the drugs were added, and this time was accepted as 0H. All the imaging conditions were kept identical for all wells. Cells were incubated with molecules at the indicated concentrations for 4 hours, and then the GFP signal was rechecked, and cell pictures were taken. GFP signal, a marker of motor neurons, was analyzed using the CellProfiler program (https://cellprofiler.org), and the cell viability difference between these molecules was compared based on dosage and incubation time. This procedure is abundantly used in literature (Ward, Janbandhu, Chapman, Dunwoodie,

& Harvey, 2022). For this, the "Identify Primary Objects" command available in the program is used. Thanks to this command, neuron bodies with at least 15-pixel units and at most 65-pixel units are marked as objects. The program counted these objects, and the outputs obtained were used in our analysis. These experiments were repeated in at least 4 different biological replicates.

2.4 BoNT/A Administration in Pre and Post-Intoxication Experimental Models

2.4.1 BoNT/A Administration in Pre-Intoxication Experimental Model

The pre-intoxication model system is extensively utilized in the BoNT literature, and it reflects experimental conditions in which the test molecule is provided to the cells before BoNT intoxication is initiated. Before BoNT/A Intoxication, BoNT/A (Masport 500, Lot # 010103) was reconstituted by adding 3.2 ml of 9% sodium chloride (normal saline) to obtain 500 units of BoNT/A solution. When the toxin is reconstructed, it is stable for about 4 months. Before BoNT/A administration, motor neurons derived from HBG3 stem cells were seeded in matrigel-coated 24-well cell plates at $5x10^5$ cells per well and checked regularly for GFP signal with the FLOID imaging system. One well of the seeded cells was used as a control group that was not exposed to toxins or compounds. Similarly, one well was treated with no compound, only BoNT/A, and used as BoNT/A activity control. In the pre-intoxication experimental design, cells were treated with the KX2-361 molecule before the toxin was introduced into the cells. In this process, KX2-361 was given to neurons at concentrations of 10 μM, 20 μM, 30 μM, and 40 μM and incubated in a cell culture incubator for 1 hour. At the end of 1 hour, 10 units of BoNT/A (Masport) diluted in DMEM were added to the wells other than the positive control and incubated for 24 hours. Experiments were repeated as 4 different biological replicates. Each BoNT/A vial contains 500 units (Units), and the concentration was chosen in the light of the literature (P. Chen et al., 2021; Dashtipour & Pedouim,

2016). Since the half-life of KX2-361 is 4 hours (Antonarakis et al., 2013), the same concentrations of the molecule were added to the wells every 4 hours until the end of the 24-hour incubation. After the incubation, cells were lysed in NP-40 lysis solution supplemented with protease (Pierce, catalog no. A32955) and phosphatase (Roche, catalog no. 04 906 837 001) inhibitors.

Figure 2.1. Schematic representation of the pre-intoxication experimental design

2.4.2 BoNT/A Administration in Post-Intoxication Experimental Model

Post-intoxication experimental model was designed to examine the inhibitory effect of KX2-361 in two different conditions. In both conditions, cells were intoxicated first, and then the compound was administrated. Similar to 2.3.1, HBG3 cells were seeded on a matrigel-coated 24-well plate. Before KX2-361 administration, cells were subject to BoNT/A at a concentration of 12.5 units for each well for 16 hours in the first condition. After incubation, KX2-361 was introduced to motor neurons at concentrations of 10 μM, 20 μM, 30 μM, and 40 μM without removing the medium that contained BoNT/A. Cells were incubated for 4 hours, considering the half-life of KX2-361. At the end of 4 hours of incubation, another set of KX2-361 was added to wells at the same concentrations until the whole incubation time reached 24 hours. After incubation, cell lysates were prepared by using NP-40 lysis buffer with protease and phosphates inhibitors.

In the second condition, cells were incubated with BoNT/A for 6 hours. Then, KX2-361 was added at the same concentrations used in the first condition. Considering the KX2-361 half-life, every 6 hours, KX2-361 was added until the total incubation time with BoNT/A reached 24 hours. At the end of the incubation, cells were lysed.

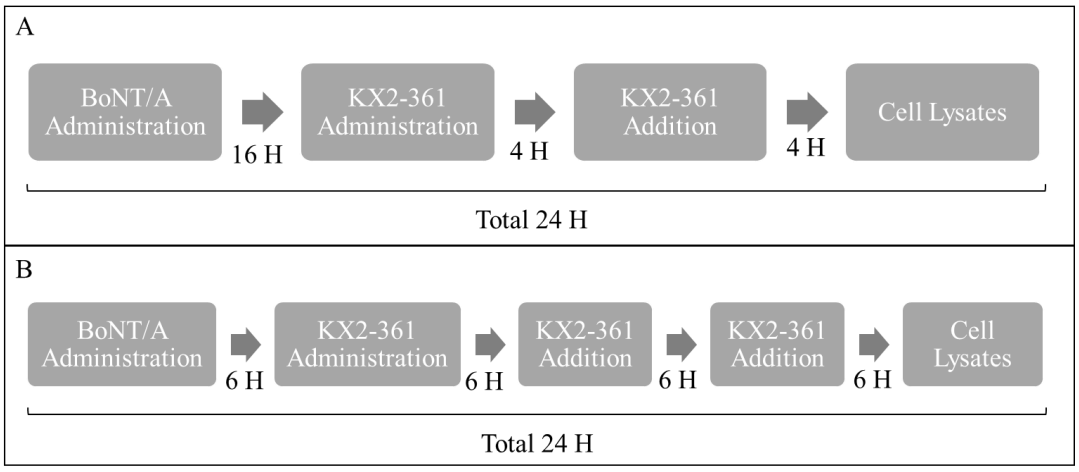

Figure 2.2. Schematic representation of the post-intoxication experimental design

(A) represents condition 1 and (B) represents condition 2

2.5 Transformation and Plasmid Purification

2.5.1 Transformation of XL1B cells with BoNT/A Light Chain Plasmid

The goal here was to purify BoNT/A LC plasmid (a kind gift from Dr. Yien Che Tsai, NCI-Frederick, NIH, USA). The BoNT/A light chain plasmid includes the YFP-tagged LCA (BoNT/A light chain, BoNT/A LC) as the pE-YFPC1 plasmid. For bacterial production of this plasmid, it was transformed into XL1B competent cells. Prior to transformation, agar plates supplemented with kanamycin antibiotics were prepared. For transformation, 50 ng of plasmid DNA was mixed into 50 µL of competent cells, and the DNA-bacteria mixture was incubated on ice for 1 hour. After incubation, the mixture was heat-shocked at 42˚C for 45 seconds; then, the tube was placed on ice for 2 minutes. Then 200 µL of LB broth (without antibiotics) was added to the mixture, and the tube was placed in a shaking incubator with conditions of 37˚C for 45 minutes to allow bacteria to grow. After incubation, 50 µL of the mixture was plated onto an agar plate with kanamycin by spreading, and the plate was placed in a bacterial incubator with conditions of 37˚C and 5% CO2 for overnight incubation.

2.5.2 Colony Selection

Agar plates that contain plasmid-containing bacteria were checked to confirm successful bacterial growth. Before colony selection, 4.8 μL of kanamycin was added to 4 mL of LB broth. A well-separated, grown colony was identified and removed from the agar plate by a touch of a pipette tip or sterile forceps. The separated colony was dropped into prepared LB broth with kanamycin and placed in a shaking incubator for 45 minutes. Then 150 mL of LB broth with 180 μL of kanamycin was prepared, and at the end of the incubation, 2 mL of previously grown bacteria were transferred into 150 mL LB broth and incubated in a shaking incubator for overnight incubation.

2.5.3 Plasmid Isolation

ZymoPure MidiPrep Plasmid Isolation Kit was used for plasmid isolations. Centrifuge protocol of the kit was followed. After the elution step, obtained plasmids were measured in terms of their purity and concentration. A260/A280 and A260/A230 ratios were found as 1.9 and 2.1, while plasmid concentrations were 0.927 μg/μl.

2.6 Transfection PC12 Cells

Prior to transfection, PC12 cells were seeded at 4.5 x 10 5 cells per well in gelatin-collagen coated 24-well plates. At the end of 24 hours following cell seeding, the transfection mix was prepared at 1 μg of plasmid DNA per well. This mixture was prepared in serum-free DMEM, and the Lipofectamine 2000 transfection reagent was preferred for transfection because its success in PC12 cells was known. For each 1 μg plasmid DNA, 4μL of Lipofectamine 2000 reagent was used. The transfection mix was incubated at room temperature for 25 minutes to allow DNA-reagent complex. After incubation, the transfection mix was administrated to the cells

avoiding interrupting the complex. Cells were transfected for 24 hours. At the 16th and 20th hours following the transfection, 2 doses of KX2-391 at concentrations of 10μM, 20μM, 30μM, and 40μM; KX2-361 was added to the other part at the same concentrations. At the end of the incubation period, cell lysates were collected with NP-40 lysis solution containing protease and phosphatase inhibitors.

2.7 Western Blot

Prior to Western Blot, protein purification was performed. Samples lysed with lysis buffer were placed on ice until they completely melted. All samples were centrifuged at 12000 rpm at 4˚C for 20 minutes. After centrifugation, the supernatant was transferred to another tube to avoid disturbing the pellet. Then the appropriate amount of 6X Sample Buffer was added onto samples with respect to the amount of lysed sample. After that step, samples can be stored at -80˚C or be processed to Western Blot. An appropriate amount of sample was transferred to a mini centrifuge tube and heated at 95˚C for 5 minutes to degrade proteins. Samples were loaded into 12% SDS-PAGE gel with a protein ladder. Then proteins were run at 100V for 4 hours. After electrophoresis, proteins were transferred onto the PVDF membrane via one of the transfer methods, wet-transfer. When the transfer process was completed, the PVDF membrane was blocked with either 5% BSA or 5% skim milk-TBST solution at room temperature for 1 hour. After blocking, the membrane was probed with a primary antibody at 4˚C overnight. Following probing, the primary antibody was removed, and the membrane was washed with TBST 2 times to get rid of non-specific interactions prior to secondary antibody administration. After secondary antibody incubation, the membrane was washed 3 times with TBST and imaged with Syngene Western Blot Imaging Station. Results were analyzed with ImageJ software.

Table 2.1 List of antibodies used in this study

Name of Antibody	***Company***	***Molecular Weight***	***Blocking Agent***
SNAP-25	SMI-81	25	Skim milk
P-Src(Y418)	CellSignaling	50	BSA
β-Actin	Santa Cruz	43	Skim milk
Total Src	Cell Signaling	50	Skim milk
βIII Tubulin	R&D Systems	53	Skim milk

2.8 Statistical Analysis

Statistical analyses were performed by Graphpad Prism software, utilizing the *Student's t-test.**: $P \leq 0.05$; **: $P \leq 0.01$, ***: $P \leq 0.001$.

CHAPTER 3

RESULTS

3.1 Generation of Mitotically Inactivated Mouse Embryonic Fibroblast Cells for Pluripotent Cell Culture

This study aimed to analyze the potential inhibitory effect of KX2-361 against BoNT/A intoxication, and to do so, we chose to utilize physiologically relevant mouse ES cell (HBG3) derived motor neurons.

Under ideal conditions, the healthy culture of the HBG3 stem cell line used in this study has been critical for the success of motor neuron differentiation. For this purpose, feeder cell lines are frequently used to create the ideal growth environment for stem cells and to keep stem cells pluripotent (Amit & Itskovitz-Eldor, 2009). One of these lines, Mouse Embryonic Fibroblasts (MEFs), are cells that are widely used in stem cell culture. Co-culture of MEFs with mouse embryonic stem cells is critical because of the important factors that MEFs provide since these factors are essential to the healthy growth of stem cells to keep them pluripotent. However, MEFs must first be mitotically inactivated to be utilized in co-culture studies. This inactivation prevents MEFs from growing, dominating the culture environment, and competing with stem cells for the medium. During our study, a significant amount of mitotically inactivated MEFs was needed to ensure the continuation of the mouse ES cell culture. Although inactivated MEFs can be obtained commercially, the financial burden it will bring to the laboratory budget and the negative situations encountered during procurement hold researchers back from commercial purchases. For this reason, as described in section 2.1.1.2, the 'primary' MEFs obtained from mouse embryos (on the 13th embryonic day) were inactivated using Mitomycin C chemical

based on well-established protocols (Tessarollo, 2001). Mitotically inactivated MEF stocks were obtained in amounts to meet the need during the study and used in it (Figure 3.1).

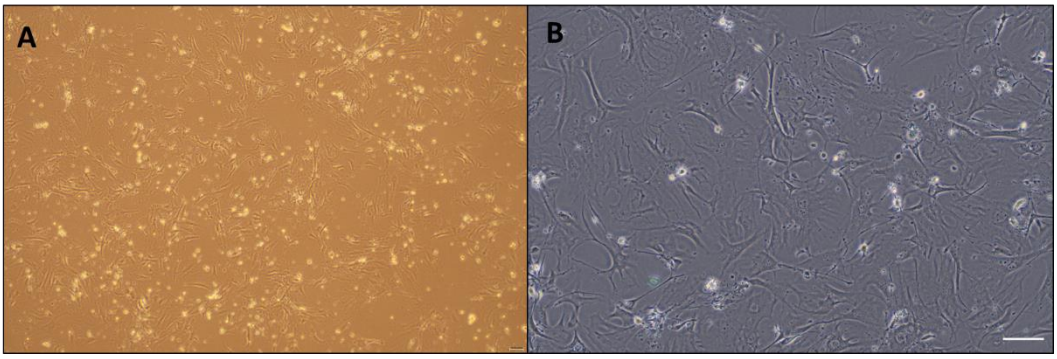

Figure 3.1. Cell culture microscope images of mitotically inactivated MEF cells at two different magnifications

MEF cells were grown in 150mm cell culture plates and visualized with 4X (A) and 10X (B) magnification using Olympus CX45 cell culture microscope and BAB camera system and imaging program.

3.2 Proliferation and Differentiation of HBG3 Pluripotent Mouse Embryonic Stem Cells into Motor Neurons

3.2.1 Cultivation of HBG3 Pluripotent Mouse Embryonic Stem Cells

The mitotically inactivated MEFs obtained in section 2.1.1.2 were used as a feeder layer for stem cells, and the HBG3 cell line was cultured under the conditions described in Section 2.1.2.1. The growth, number, and morphological health of the stem cells were regularly checked under the microscope daily. Continuity of the cell culture was ensured by making cell stocks from the grown stem cells in large quantities in different passages.

3.2.2 Differentiation and Use of HBG3 Pluripotent Mouse Embryonic Stem Cells into Motor Neurons

Our previously optimized protocols were used to obtain motor neurons from HBG3 mouse embryonic stem cells. The steps of the differentiation process are given in detail in Section 2.1.2.2. The stages of the differentiation protocol are also summarized in Figure 3.2. HBG3 mouse embryonic stem cells carry a transgene in which the Hb9 promotor drives eGFP expression (Wichterle & Peljto, 2008). It has been experimentally demonstrated that when the HBG3 line differentiates into motor neurons, eGFP is expressed, which allows visualization of the differentiation process under fluorescent light. This feature of the HBG3 line was used in the study to demonstrate the success of motor neuron differentiation. From the 6th day of differentiation, the eGFP signal could be observed in the embryoid bodies and at the end of the 7th day in the motor neurons that have completed their differentiation (Figure 3.2). Regularly, each differentiation set was examined at each stage under fluorescence and cell culture microscopes. Our group has extensive experience with this cell line and differentiation toward motor neurons, and our characterizations were published previously (Kiris et al., 2011). As demonstrated in the representative images in Figure 3.2., we generated GFP+ motor neurons upon directed differentiation, which was consistent with our previous studies.

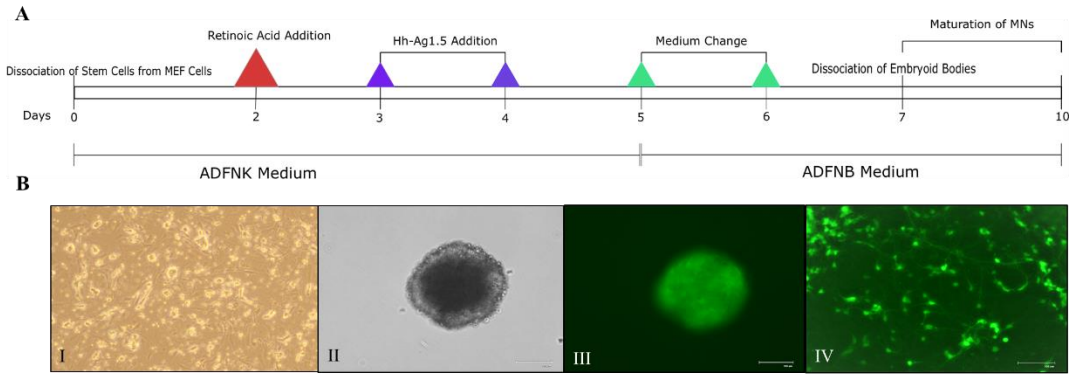

Figure 3.2. Differentiation process of motor neurons from HBG3 mouse embryonic stem cells

(A) Our motor neuron differentiation protocol is outlined schematically. (B) (I) Microscopic images of HBG3 mouse embryonic stem cells grown on MEF cells (brightfield), (II) embryoid body at day 4 (brightfield) and (III) day 6 (fluorescent), and (IV) motor neurons (fluorescent) obtained after dissociation of EBs are shown, respectively.

3.3 Investigation of Whether KX2-361 and KX2-391 Have Effects on Cell Viability

3.3.1 Analysis of Whether KX2-361 Has an Effect on Cell Viability Compared to KX2-391 And Controls by MTT Assay

Both small molecules we wanted to test were analyzed in terms of their effects on cell viability at 7 different doses, first by MTT assay using PC12 cells. PC12 cells are extensively used in neuronal studies and have been used in drug toxicity and cell viability testings (Lu et al., 2020). These cells provide cheaper alternatives to our motor neuron assays as the first step of viability tests. In the MTT assay, PC12 cells were seeded at $5x10^4$ cells per well in 96-well plates, and KX2-391 and KX2-361 small molecules were given a wide range of doses (0.1μM, 1μM, 10μM, 20μM, 30μM, 40μM and 50μM). Since these small molecules were dissolved using DMSO, the same amount of DMSO was added to the experimental system as a control (vehicle control). Since the biological half-life of these molecules is 4 hours (Antonarakis et al., 2013), the cells were incubated with the small molecules in a cell culture incubator for 4 hours. After 4 hours, MTT analyzes were performed as stated in Section 2.2. Results from 3 different biological replicates were analyzed using Student's t-test with GraphPad Prism. Both small molecules did not cause statistically significant mortality at 5 of the tested doses (0.1μM, 1μM, 10μM, 20μM, and 30μM), but statistically significant loss of viability appears at the two highest doses (40μM and 50μM; Figure 3.3). However, when we look at the percentages, it is thought that these doses do not cause a severe loss of viability in the cells since

the cell viability is over 80%, even at these high concentrations. In order for these small molecules to be considered as drugs, low doses are preferred, and it was found very positive and essential that they do not cause death at low doses.

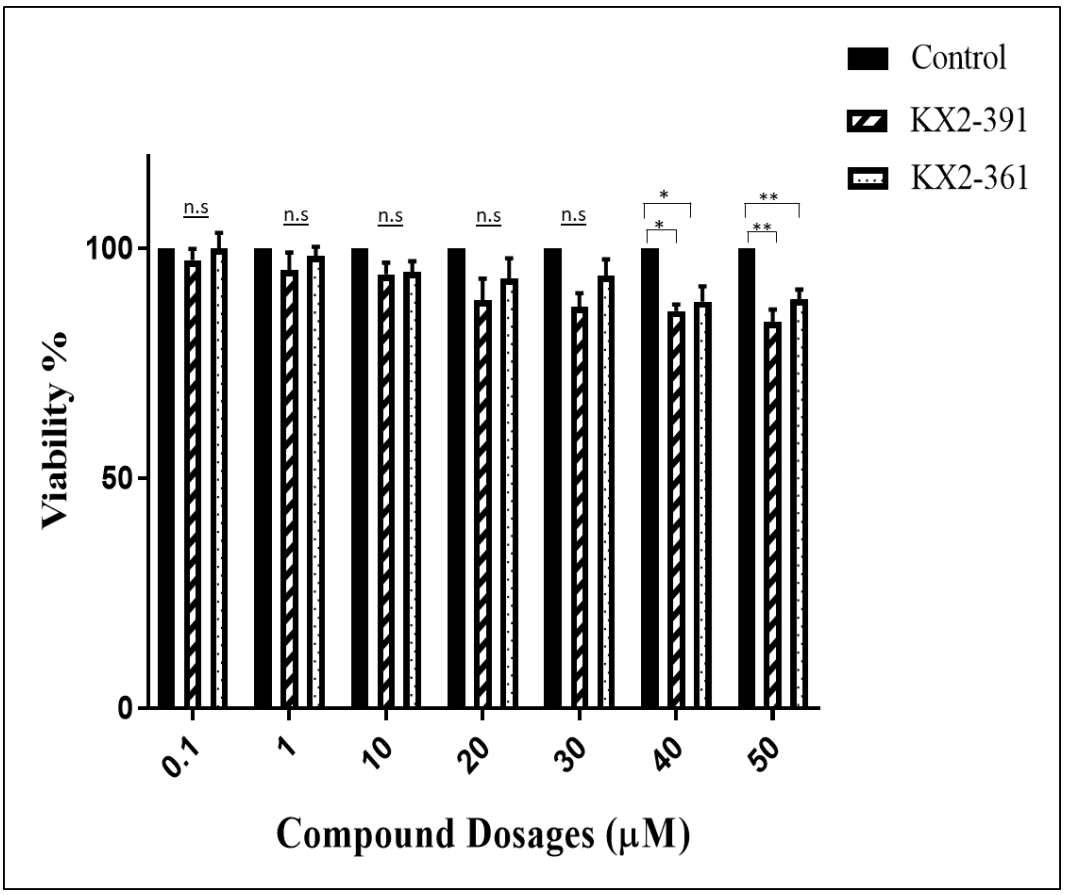

Figure 3.3. MTT analysis of KX2-361 and KX2-391 small molecules to measure their effects on cell viability

*MTT analysis was performed by using PC12 cells that were treated at selected concentrations, 0.1 μM, 1μM, 10 μM, 20 μM, 30 μM, 40 μM, and 50 μM. Results are presented as mean ± SD, and statistical analyses were performed using GraphPad Prism with the student's t-test. All MTT analyses were performed using n=3 biological replicates. *: $P \leq 0.05$; **: $P \leq 0.01$*

3.3.2 Imaging-based Analysis of Whether KX2-361 and KX2-391 Have Effects on Cell Viability on Motor Neurons

As mentioned above, successful differentiation of HBG3 mESCs to motor neurons yields GFP+ motor neurons. Taking advantage of this system, we sought to measure the effect of the compounds on GFP+ neurons using imaging-based quantitative methodologies. For these experiments, motor neurons seeded in 24-well plates were treated with different doses of KX2-391 and KX2-361 small molecules (10μM, 20μM, 30μM, and 40μM) to be used throughout the project, and the possible effects of drugs on cell viability in terms of GFP signal were observed. More specifically, using the FLOID Cell Imaging Station, the GFP signal from the cells was examined under fluorescent light both before compound administration (0H) and at the 4th hour of incubation (4H) with the compound. Since the biological half-life of both compounds is limited to 4 hours, the maximum incubation time of cells with them was determined as 4 hours. GFP signals from cells were analyzed with the CellProfiler program, which was developed by the Broad Institute of MIT and Harvard (Carpenter et al., 2006). It has become a frequently used analysis method in the literature for cell-based analyses (McQuin et al., 2018). According to the findings we obtained as a result of the analyses, both compounds appeared not to show a significant cell death, especially at low doses, despite the increase in dose and incubation period (Figure 3.4). However, statistically significant differences were observed at doses that can be considered high (30μM and 40μM) under the conditions of KX2-361 treatment, but when the changes in cell number are examined, it is clear that there is no big change. It has been evaluated that the GFP+ signal, which appears more as a result of drug treatment at 40μM concentration, may be the error rate of the applied methodology (analysis with CellProfiler). In summary, considering the viability tests of both small molecules, it is evident that low concentrations (10μM and 20μM) did not cause statistically significant GFP+ motor neuron loss.

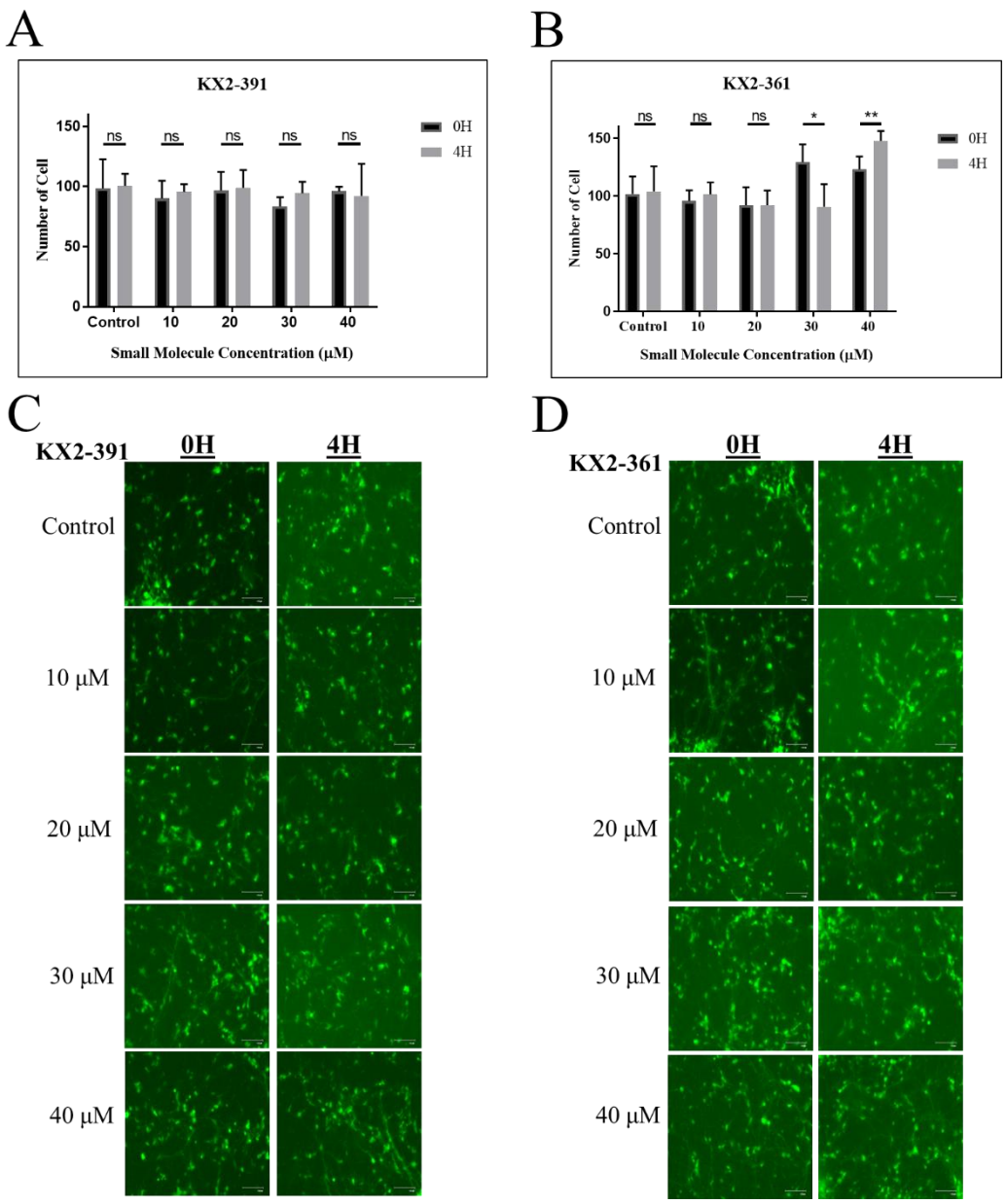

Figure 3.4. Imaging-based analysis of whether KX2-391 and KX2-361 small molecules have any effects on the viability of motor neurons, depending on the GFP signal in cells.

Motor neurons (GFP+) differentiated from HBG3 mouse stem cells were imaged before and 4 hours after treatment with the indicated doses of small molecules and then analyzed with CellProfiler as described in the Methods section. Statistical analyses (A and B) were performed using GraphPad Prism with the student's t-test

*(n=4). *: P ≤ 0.05; **: P ≤ 0.01. C and D panels represents the representative images.*

3.4 Determination of the Inhibitory Profile of KX2-361 Against BoNT/A Light Chain in Pre-Intoxication Conditions in Motor Neurons

One of the essential points in drug development against botulinum neurotoxin poisoning is to understand at what stage the candidate molecules can inhibit the toxin. There are several steps, from entering the cell until it cleaves its target, in which compounds can intervene the toxin (M. Pirazzini et al., 2017). As discussed above, the process that begins with the entry of the toxin into the cell and results in the cleavage of the SNAP-25 protein consists of different processes. To measure whether KX2-361 has any inhibitory effect against BoNT/A, we first utilized pre-intoxication conditions, frequently utilized in the literature as the first step in drug efficacy testing against the toxin. In these experiments, first, the KX2-361 molecule was administered to cells at 10 μM, 20 μM, 30 μM, and 40 μM concentrations and incubated for an hour. Then 10 units/sample of BoNT/A (Masport) were added to the cells, and the plates were incubated in cell culture incubators. Dose selection for the BoNT/A was based on literature (P. Chen et al., 2021; E. Kiris et al., 2015). Following the toxin administration, KX2-361 was added at the same concentrations every 6 hours, taking into account the half-life of the KX2-361 molecule. At the end of 24 hours following BoNT/A administration, cell lysates were collected, proteins isolated, and Western Blot was performed by loading the samples on a 12% SDS-PAGE gel to separate 25 kDa full-length and 24 kDa truncated SNAP-25. Samples were probed with the total SNAP-25 (SMI-81) antibody, which can detect both full-length and truncated SNAP-25, and Western Blot results were imaged on the SynGene Western Imaging Station. β-actin was used as the housekeeping gene. Western Blot results were quantified using the SynGene Western Analysis program and analyzed according to Student's t-test using GraphPad Prism.

According to the findings, approximately 25% cleavage was detected at the end of 24 hours of incubation with 10 units of BoNT/A in only the toxin-treated sample. It was observed that this cleavage rate decreased when the KX2-361 dose was increased, and the cleavage was prevented entirely at the highest dose (Figure 3.5). Experiments were repeated with 4 different biological replicas. According to these findings, it can be suggested that high doses of KX2-361 molecule inhibit BoNT/A. However, based on pre-intoxication experimental conditions, it is impossible to exclude the possibility that the compound might interfere with the toxin uptake into the cell. However, in the case of an actual BoNT/A poisoning, the presence of a molecule that will stop the process after the toxin enters the cell and protect against the toxin is essential because the patients seek medical care once the symptoms begin, that is, BoNT/A starts to cause paralysis by cleaving the SNAP-25 protein at a certain level. For this reason, whether the KX2-361 molecule has protection against the toxin in post-intoxication conditions is also included in our study as follows.

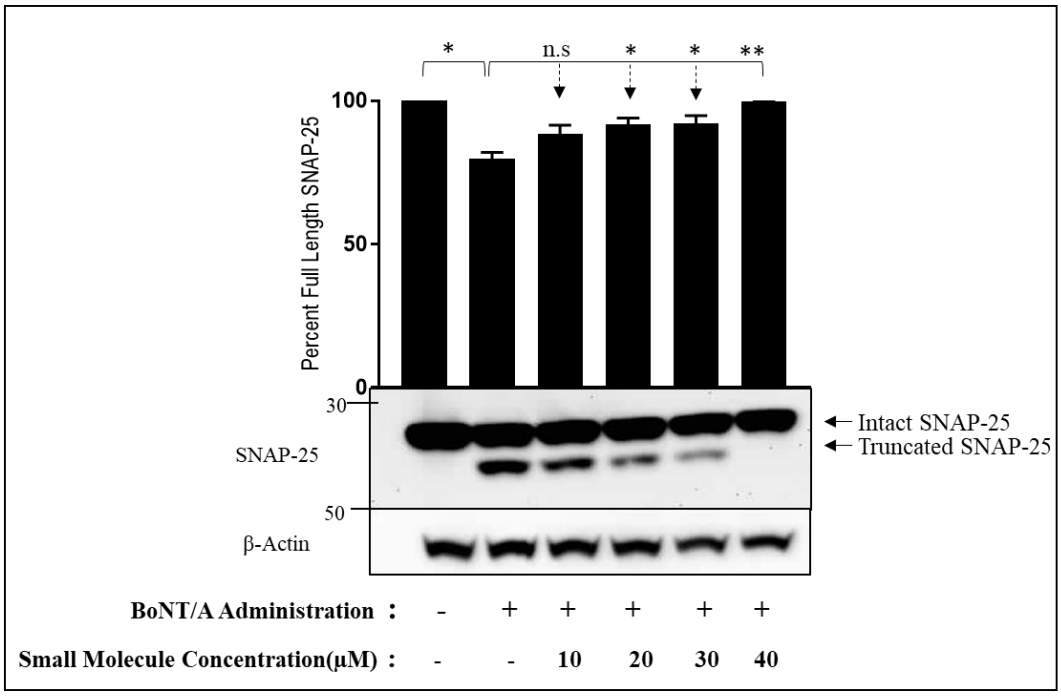

Figure 3.5. Investigation of the pre-intoxication effects of KX2-361 against BoNT/A

*The inhibitory effect of KX2-361 small molecule against BoNT/A in motor neurons differentiated from HBG3 mouse embryonic stem cells in pre-intoxication conditions was analyzed by Western Blot. KX2-361 administration was performed at selected concentrations (10 μM, 20 μM, 30 μM, and 40 μM). BoNT/A administration was performed following the KX2-361 introduction to all samples except positive control. All experiments were carried out with n=4 biological replicates. Western Blot band quantification was performed in SynGene Western Blot Imager Software, and Results are presented as mean ± SD, and statistical analyses were performed using GraphPad Prism with student's t-test. *: $P \leq 0.05$; **: $P \leq 0.01$.*

3.5 Determination of the Protectivity of KX2-361 Against BoNT/A Light Chain in Post-Intoxication Conditions in Motor Neurons

In order to investigate whether KX2-361 has a protective effect against BoNT/A in post-intoxication conditions, two different experimental setups were designed. In the first condition (Figure 3.6.a.), 12.5 units of BoNT/A were added to all samples except the positive control, and then the cells were incubated in a cell culture incubator for 16 hours. Here, we increased the BoNT/A unit dose to challenge the compound further. During this period, BoNT/A is expected to cleave the SNAP-25 protein at a specific rate. After 16 hours of incubation, KX2-361 small molecule at concentrations of 10 μM, 20 μM, 30 μM, and 40 μM was introduced to the samples, excluding positive control and toxin-only samples. KX2-361 was added every 4 hours until the total incubation time with BoNT/A was 24 hours.

In the second experimental design condition (Figure 3.6.b.), BoNT/A was given to the motor neurons as in the first condition, but this time KX2-361 was added every 6 hours of the 24-hour incubation. With this experimental condition, it was desired to investigate whether the earlier administration of the KX2-361 molecule in the stages after the toxin has entered has a more significant effect on the protection. Besides, as mentioned above, after the toxin is taken into the cell, it goes through different processes until it reaches SNAP-25. It was desired to investigate whether

the KX2-361 molecule has an inhibitory effect against these processes by administering KX2-361 with a relatively short time difference following the toxin administration. At the end of the 24-hour incubation, the samples in both conditions were lysed and loaded on 12% SDS-PAGE gel, and a Western Blot was carried out. All experiments were performed with n=4 biological replicates in both conditions.

According to the findings obtained from both conditions, BoNT/A cleaves approximately 40-50% of total SNAP-25 in toxin-only samples. Similar to pre-intoxication conditions, an approximately 20% increase in full-length SNAP-25 was detected in a dose-dependent manner. However, it is quite difficult to mimic the conditions of BoNT poisoning in motor neuron-derived experiments. The determined toxin unit and incubation time can prevent the molecule from reflecting its true potential at selected small molecule concentrations. Likewise, since the uptaken of the toxin is a complex process, it is a great challenge to intervene fully and to obtain the exact post-intoxication conditions. For these reasons, in our study, PC12 cells were transfected with only BoNT/A light chain to obtain SNAP-25 cleavage, which is the last step of poisoning, and the protective effect of the KX2-361 molecule was tested under these conditions.

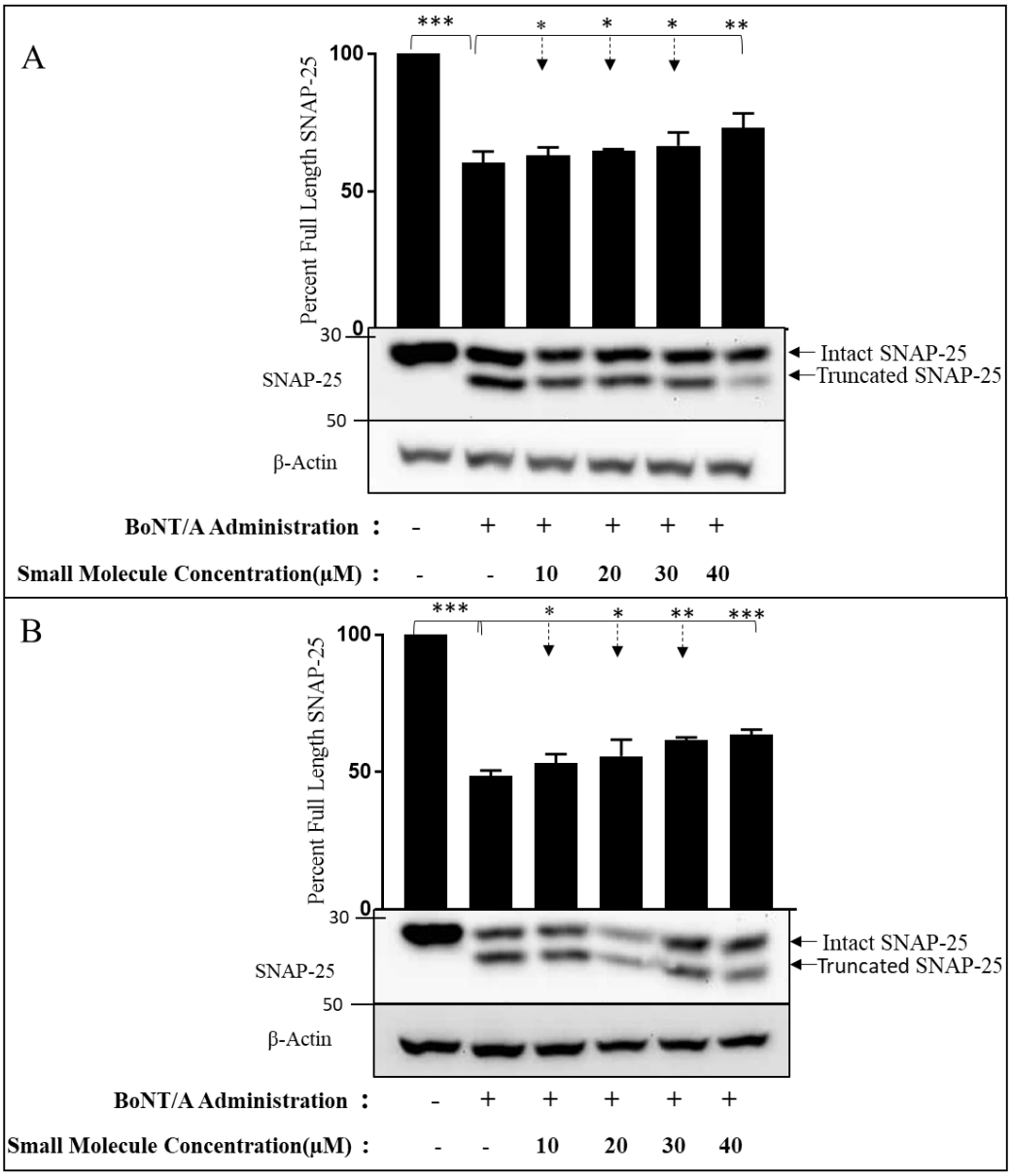

Figure 3.6. Investigation of the protectivity effects of KX2-361 against BoNT/A in post-intoxication conditions

The protective effect of KX2-361 in motor neurons in post-intoxication conditions was analyzed with Western Blot. (A) represents condition 1; BoNT/A administration and incubation for 16H and KX2-361 addition at 16th and 20th hours. (B) represents condition 2; BoNT/A administration and incubation for 6H prior to KX2-361 addition and then small molecule addition every 6H until total incubation time reaches 24H. BoNT/A administration was performed prior to KX2-361

*administration, and for both conditions, 12.5 units/sample BoNT/A was used. KX2-361 molecules were added at indicated concentrations. For both conditions, all experiments were carried out with n=4 biological replicates. Western Blot results were analyzed by using GraphPad with Student's t-test. *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$.*

3.6 Determination of the Comparative Potential of KX2-391 and KX2-361 to Inhibit Botulinum Neurotoxin Serotype A Light Chain in PC12 Cells

The most valuable but challenging part of BoNT drug development studies is the inactivation of the toxin that has already entered the cell. As explained in the introduction, patients generally seek medical help after symptoms appear; at this stage, no drug can neutralize the toxin that has entered the cell (Rao, Sobel, Chatham-Stephens, & Luquez, 2021). We sought to determine whether KX2-361 can inhibit the enzymatic part of the toxin in cells compared to KX2-391. As mentioned above, these cells are used extensively in the field of BoNT and endogenously synthesize the SNAP-25 protein, which is the main target of BoNT/A. Notably, we utilized BoNT/A LC plasmid transfections to intoxicate these cells, which allowed us to measure the inhibitory effect of the compound on already active LC in the cytosol. PC12 cells were cultured as described in section 2.1.3 and transfected with the BoNT/A LC plasmid available in our laboratory. Experimentally, the 24-well plate was seeded with $4x10^5$ cells per well, and transfection was performed using Lipofectamine 2000 to deliver 1 μg of BoNT/A LC plasmid to each well. After the transfection mixture was given to the cells, the cells were incubated for 24 hours in the cell culture incubator. At the 16th and 20th hours of the incubation, some of the wells were treated with KX2-391 or KX2-361, as indicated in Figure 3.7, at doses of 10, 20, 30, and 40 μM, and the experiments were carried out as 3 different biological replicas at different times. Cells were lysed using NP-40 lysis solution after 24 hours. SNAP-25 truncation was analyzed by Western Blot.

According to our results, considering the dose-dependent increase in the percentage of full-length SNAP-25 protein, it was evaluated that KX2-361 appeared to inhibit SNAP-25 cleavage in a dose-dependent manner (Fig. 3.7). While KX2-391 inhibited the toxin at low doses, its ability to neutralize the toxin was lost as the dose increased (Fig. 3.7). This interesting result raises a question that can be studied jointly with Medical Chemist researchers in further studies. We think that it is crucial that the KX2-361 small molecule, which is the main focus of the study and has been shown to penetrate BBB, stop the LC-mediated SNAP-25 cleavage in a dose-dependent manner. This result suggests that BoNT/A enzymatic activity can be inhibited in the presence of KX2-361.

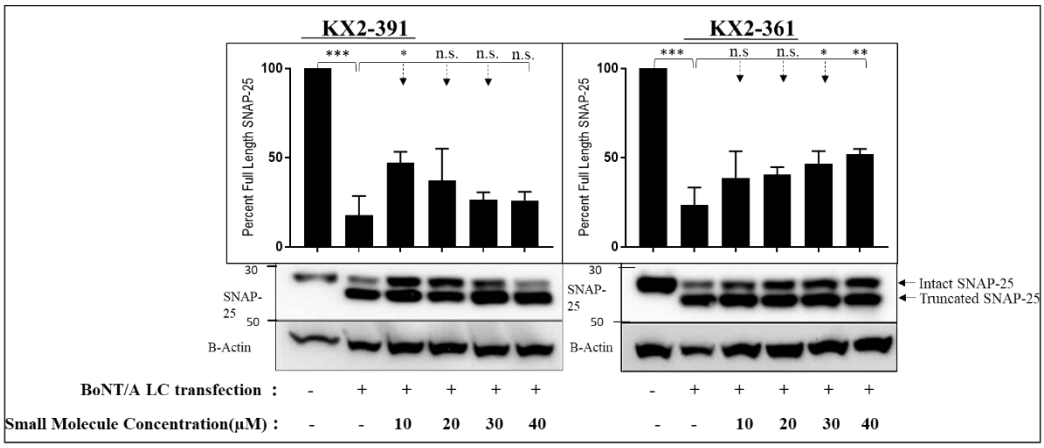

Figure 3.7. Investigation of the post-intoxication effects of KX2-361 compared to KX2-391 in PC12 Cells

*BoNT/A LC transfected PC12 cells were treated with either KX2-391 or KX2-361 at indicated doses. Samples were loaded on 12% SDS-PAGE gel to visualize both 25kDa full-length SNAP-25 and 24kDa truncated SNAP-25. All Western Blots were performed with n=3 biological replicates, and as the loading control, β-Actin was used. Western Blot results were analyzed by using GraphPad with Student's t-test. *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$.*

3.7 Determination of Changes in Phosphorylation Profile of Src Kinase in the Presence of KX2-361 in BoNT/A Intoxication Conditions

Activation of Src kinase in the cell is a process dependent on the phosphorylation of Src (Ohnishi et al., 2011). Since it has been suggested in previous studies that Src can phosphorylate the BoNT/A light chain (Ibanez et al., 2004), whether KX2-361 small molecule, an Src kinase inhibitor, causes a change in Src phosphorylation during BoNT/A intoxication was investigated. Previous studies have reported that phosphorylation in the tyrosine 418 region activates Src (Amata, Maffei, & Pons, 2014). In light of this information, in our study, phosphorylation levels and total Src levels were investigated using phospho-Src (Y418) post-intoxication conditions. B-actin and the neuron-specific marker BIII Tubulin were used as loading controls (Fig. 3.8). Each experiment was repeated with 4 biological replicates. According to our results, an increase in phospho-Src (Y418) level was detected following BoNT/A administration, while total Src levels were similar among samples, and this increase in phospho-Src appeared to be decreasing in a dose-dependent manner following the addition of KX2-361. These results are interesting however requires further investigations under various conditions. Importantly, different formulations of BoNT should be tested under both pre- and post-intoxication conditions. Future more comprehensive work will provide a better picture in terms of BoNT and/or KX2-361 mediated phosphorylation changes of Src kinases.

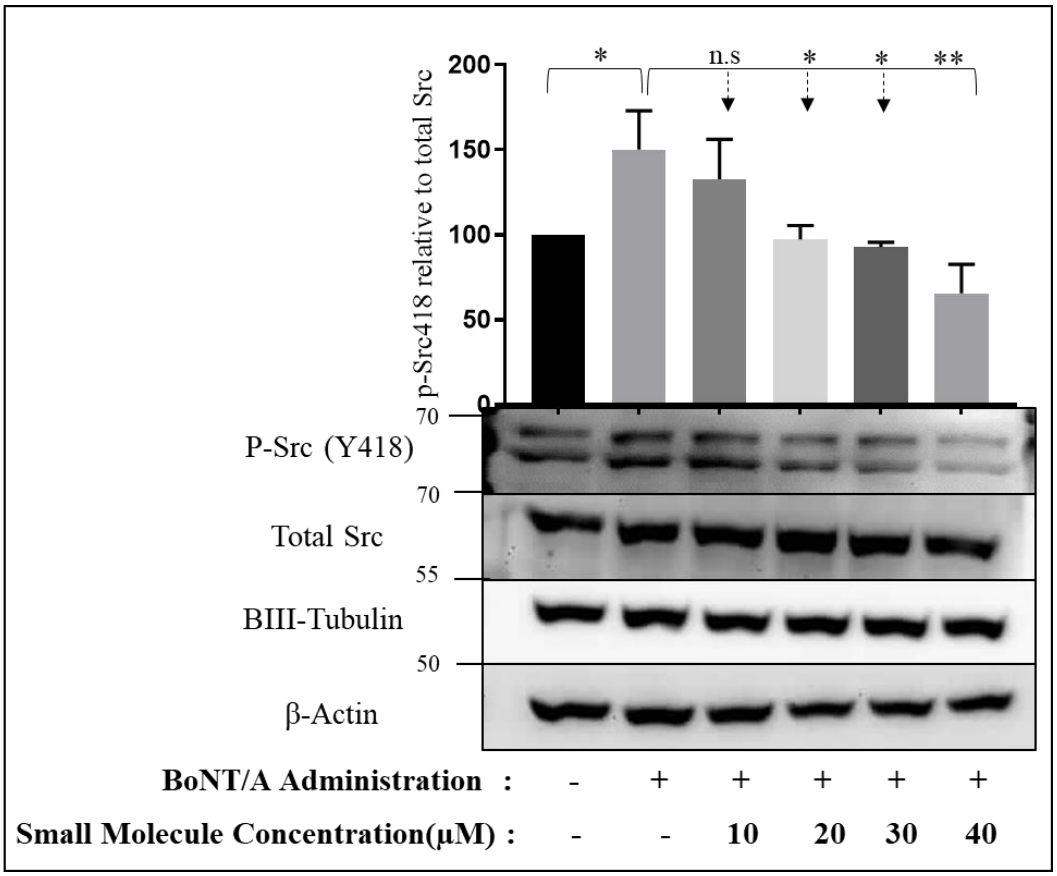

Figure 3.8. Investigation of the phosphorylation profile of Src with respect to KX2-361 administration

In motor neurons derived from HBG3 mouse embryonic stem cells, phosphorylation levels of Src were examined. As a loading control, β-Actin and neuron-specific marker βIII Tubulin were used. All Western Blots were conducted with n=4 biological replicates and imaged by SynGene Western Blot Imaging Station.

CHAPTER 4

DISCUSSION

Botulinum neurotoxins, the most potent neurotoxins known, causes botulism, which causes paralysis and even death (Rossetto et al., 2014). The primary targets of BoNTs are peripheral cholinergic nerve endings and the autonomic nervous system (Rummel, 2015). In BoNT intoxication involving the autonomic nervous system, death occurs due to the inability of the diaphragm muscle to contract (Dhaked, Singh, Singh, & Gupta, 2010). BoNTs cleave the SNARE complex elements, which are their target proteins, causing inhibition of acetylcholine release to muscle cells. In particular, BoNT serotype A, which leads to the highest number of human botulism cases compared to other serotypes, can cleave a tiny percentage of the total SNAP-25 in motor neurons, and such an effect is sufficient to result in complete inhibition of acetylcholine release alone and thus complete inhibition of muscle movement (M. Pirazzini et al., 2017). The result is paralysis, and death occurs if the patient does not receive medical support.

No therapeutic option exists to treat BoNT/A poisoning after the toxin enters the cell (Sobel, 2005). It is too late to prevent BoNT from entering the cell in real case scenarios, as patients apply to hospitals for medical help after symptoms appear, which means some toxins are already internalized. Patients need to receive respiratory support and meticulous care to survive this poisoning (Rossetto et al., 2020). However, considering the long half-life of BoNT/A, it may take up to 6 months for the toxin to be cleared from the system naturally (Sobel, 2005). Considering this situation, it may not be possible for every patient struggling with botulism to access mechanical respiratory support. Although the most common type of transmission is through food poisoning, it is known that BoNT/A has also been

used as a bioterror agent in history (Arnon et al., 2001). In such a situation where mass poisonings can occur, it does not seem possible to provide respiratory support to a large number of patients. The recent Covid-19 pandemic has also shown that many countries have failed to provide long-term respiratory support to a large number of patients. Similarly, in BoNT/A poisoning, which can affect respiration and lead to death, it is clear that hospitals cannot cope with this intensity and that every patient cannot receive the medical support they need, especially in case of mass poisonings. Antibodies have been developed against botulinum neurotoxin, but no drug or treatment alternative can stop the toxin's mechanism after it enters the cell (Dembek et al., 2007). Existing antibodies can only inhibit circulating BoNTs. However, as mentioned above, patients only seek medical support when symptoms begin, and it is usually too late for antibody treatment. Besides its dangerous nature, botulinum neurotoxin is a crucial treatment alternative in many diseases (S. Chen, 2012). Its high specificity toward its target and long half-life makes it an alternative treatment for many conditions, from dystonia to cosmetic use (M. Pirazzini et al., 2017). However, this widespread use of BoNT brings with it the risk of poisoning that may arise from misuse and overuse. In summary, it is essential to develop drugs against BoNT intoxication that can especially inhibit the toxin inside the neurons.

There have been various approaches to discovering and developing BoNT/A inhibitors, including direct enzymatic inhibitors that can neutralize the LC (Lin, Olson, Eubanks, & Janda, 2019). Although this approach makes sense, no significant progress has been made in terms of developing compounds for clinical trials. Another approach would be the modulation of neuronal processes to directly or indirectly affect LC activity in cells. One example is the utilization of small molecules to regulate the ubiquitin-proteasome system to enhance the degradation of the LC (Sen, Kota, Panchal, Bavari, & Kiris, 2021). There has been growing interest in understanding molecular players involved in BoNT/A intoxication and/or recovery. Studies conducted in this direction argue that Src kinases may be involved in BoNT/A toxicity (Ibanez et al., 2004). Although Src kinases are often associated with cancer, they play an essential role in developing and maintaining the nervous

system (Ohnishi et al., 2011). Src kinases, predominantly expressed in motor neurons, are involved in neurotransmitter release and axonal growth. It has been suggested that Src phosphorylates the BoNT light chain in BoNT intoxication (Ibanez et al., 2004). In previous studies of our group, a set of small molecules targeting Src family kinases was screened, which led to the discovery of several small molecules with inhibitory activity against BoNT (E. Kiris et al., 2015). Among these molecules, KX2-391 (Tirbanibulin) showed protective effects against BoNT/A in *in vitro* experiments but failed to show similar results in *in vivo* studies. It has been suggested that the failure of KX2-391 in *in vivo* tests may be due to its inability to cross the blood-brain barrier. Importantly, later on, 10 different derivatives of the KX2-391 molecule were generated, and among these derivatives, KX2-361 is specifically designed to cross the blood-brain barrier (Smolinski et al., 2018). Although KX2-361 has been studied in several conditions, its potential effects as BoNT/A inhibitor have not been studied.

This thesis is based on understanding whether the small molecule KX2-361 has an inhibitory role against BoNT/A intoxication. Since BoNT/A explicitly targets motor neurons, it was deemed appropriate to use them for the study. For this purpose, it was first aimed to obtain motor neurons from HBG3 mouse embryonic stem cells. Feeder cell layers are needed to culture healthy and undifferentiated stem cells before differentiation (Amit & Itskovitz-Eldor, 2009). For this reason, MEF cells mitotically inactivated with Mitomycin C were used in our study. HBG3 mouse stem cell line cultured with MEF cells was successfully differentiated into motor neurons as a result of the differentiation process. The HBG3 mouse stem cell line is derived from HB9::GFP transgenic mice (Kiris et al., 2011). These mice have the eGFP gene linked to the HB9 gene. Since HB9 is a motor neuron marker, towards the end of the differentiation process, the HB9 gene begins to be expressed. Depending on the expression of this gene, GFP expression is also observed in cells. When neurons are obtained at the end of the differentiation protocol, the GFP signal can determine motor neuron success (Wichterle & Peljto, 2008). Overall, the utility of the motor

neuron system in this study was essential as these cells are the physiological target of the toxin.

Cell viability assays are frequently used in drug development studies. In our study, an MTT assay was carried out to examine the viability of cells under the effect of KX2-391 and KX2-361. Several concentrations were tested on PC12 cells. As a result, at lower concentrations, none of the compounds leads to any significant decrease in cell viability. For the KX2-361 compound, at the highest two concentrations, a significant decrease in viability was observed; however, even at these concentrations, it was found that the rate of cell viability was above %80. This is an important finding since the low toxicity of candidate molecules against BoNT/A intoxication is an essential property. Low doses are preferred for these small molecules to be considered as drugs, and it is crucial that they have no toxic effects at low doses.

Since this study is motor neuron oriented, it is important to consider whether these compounds cause any cell death in motor neurons at selected concentrations according to the MTT assay. Imaging-based analysis was performed on whether KX2-391 and KX2-361 compounds have any effect on cell viability. By utilizing the eGFP expression in motor neurons, cell numbers and cellular integrity were observed and analyzed via CellProfiler based on GFP-signaling motor neurons. Our results show that both KX2-391 and KX2-361 compounds do not have any effect on cell viability, which is critical to ensure that these compounds, which will be tested under BoNT/A intoxication conditions, do not cause additional cell death.

In this study, we attempted to understand at what stage KX2-361 stops BoNT/A in the process, from the first encounter with motor neurons to cleaving SNAP-25. This is highly important as there is a need for compounds that can be utilized post-intoxication. We used different experimental setups to understand the compound's mechanisms of action. First, we investigated whether KX2-361 had a protective effect in pre-intoxication conditions. According to the results, while approximately 25% cleavage of full-length SNAP-25 was observed in toxin-only conditions, this

cleavage was almost completely inhibited with respect to KX2-361 administration in a dose-dependent manner. Initial data represents that KX2-361 has a protective effect against BoNT/A in pre-intoxication conditions; however, in real cases, patients usually seek medical care after symptoms appear, which means BoNT/A has already uptaken into motor neurons.

Since the most critical point in botulism conditions is to inhibit the toxin after it enters the cell, the protective effect of the KX2-361 molecule in post-intoxication conditions was also investigated. In this experimental setup, 2 different conditions were examined, and we tried to understand at what stage the KX2-361 molecule was effective during the intracellular travel of the toxin. In both conditions, there was an approximately 40-50% cleavage in total SNAP-25 in the toxin-only sample, while an increase in the percentage of full-length SNAP-25 was observed due to the KX2-361 dose increase. Although KX2-361 showed a noticeable protective effect in both conditions, the conditions did not show a significant difference compared to each other.

Overall, our data show that KX2-361 has a protective effect against BoNT/A. Still, the process from the uptaking of the toxin to the cleavage of its target protein is complex. Hence, PC12 cells were transfected with BoNT/A LC so that it could be examined whether the KX2-361 compound directly interfere with the enzymatic part of BoNT/A. As a result, there is a significant increase in the percentage of full-length SNAP-25 depending on the dose increase in cells given KX2-361, indicating that KX2-361 can directly interfere with BoNT/A LC activity. A similar result was obtained for KX2-391, but KX2-391 was more effective at lower doses. In both conditions, our findings might be significant because a drug candidate that can directly interfere with the enzymatic activity of BoNT/A is groundbreaking in terms of being a treatment option in post-intoxication conditions.

Finally, Src phosphorylation was studied to examine if Src phosphorylation changes under KX2-361 treatment conditions after BoNT/A intoxication. It appeared that treatment with BoNT/A increased the phosphorylation of the activating region of Src

Y418 (Y416 in chicken)(Koga et al., 2006), but this phosphorylation level decreased upon KX2-361 treatments. This finding may indicate that the KX2-361 compound may change Src activity via phosphorylation, leading to changes in the activity of BoNT/A. However, future work is required to confirm this data, especially with different formulations of BoNT/A (other than Masport utilized in this study). Additionally, various experimental conditions, including both pre- and post-intoxication conditions, should be included in such future work.

Our study shows that KX2-361 has an effect against BoNT/A; however, the action mechanism is still not understood. KX2-361 can inhibit BoNT/A in at least three potential ways: i) it has been shown that Src phosphorylates BoNT/A LC (Ibanez et al., 2004), and KX2-361 may affect BoNT/A LC enzymatic activity through phosphorylation by Src. ii) KX2-361 may affect BoNT/A LC degradation through the intracellular ubiquitin-proteosome system, or iii) KX2-361 may affect other currently unknown cellular mechanisms critical for BoNT/A intoxication and indirectly affect the biological effects of the toxin. It is plausible that KX2-361 may change the phosphorylation of the BoNT/A LC by Src; therefore, its interacting proteins may also change, which may be crucial for BoNT/A LC stability and activity. It is well-established that specific proteins directly interact with BoNT/A LC, including ubiquitin proteosome pathway members (Tsai et al., 2017; Tsai et al., 2010). Therefore, as part of this project, we sought to utilize the TurboID approach to examine BoNT/A LC interacting partners with and without KX2-361 treatment and generate the plasmids (described in Appendix D). However, due to budget issues could not move forward. In future studies, utilizing this approach to better understand the effects of KX2-361 on BoNT/A LC activity will be essential.

CHAPTER 5

CONCLUSION AND FUTURE STUDIES

This work has established a scientific basis and knowledge that KX2-361 is vital for further studies to test as a candidate small molecule for anti-BoNT/A activity. We showed that KX2-361 did not affect PC12 cell viability at tested lower doses measured by MTT assays. Additionally, KX2-31 did not affect motor neuron viability significantly, at lower doses, based on fluorescence-based imaging assays. Importantly, KX2-361 provided a dose-dependent protection against BoNT/A in mouse ES-derived motor neurons in both pre-and post-intoxication conditions. Furthermore, KX2-361 protected PC12 cells transfected with BoNT/A LC, meaning that the compound was able to inhibit the active enzymatic component in cells. We also showed that KX2-361 could affect Src activity similarly to its parental compound by modulating its phosphorylation levels. Although the protective effect we observe is not very high, and the compound concentrations we tested are not in the desired nanomolar ranges, this study provides a significant base for further studies to collaborate with medicinal chemists to develop of structural analogs of KX2-361 and optimize it for better efficacy in low doses. In addition to its *in vitro* success in drug development, its effect and success in *in vivo* studies are also of great importance. For this reason, the investigation of the protective effect of KX2-361 against BoNT/A intoxication in *in vivo* studies is one of the important part of our future directions. Nonetheless, only a few compounds have been reported in the literature with post-intoxication activity against BoNT/A despite extensive research efforts, and our study demonstrates KX2-361, known to penetrate BBB, as a potentially valuable lead to further studies.

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APPENDICES

A. CELL CULTURE MEDIUM COMPOSITIONS

MEF Growth Medium: Contains 88% DMEM High Glucose (Gibco, 11965092), 10% Fetal Bovine Serum (FBS) (Gibco, 10270106), 1% Pen/Strep (Gibco, 15140122) and 1% GlutaMAX (Gibco, 35050061).

Mouse Embryonic Stem Cell Growth Medium (mESC Medium): Contains 82% DMEM High Glucose (Gibco, 11965092), 15% FBS, 1% Pen/Strep, 1% Non-Essencial Amino Acid (NEAA), 1% GlutaMAX, 1000 unit/ml Leukemia Inhibitory Factor (LIF), 0.1 mM β-Mercaptoethanol.

Differentiation Medium (ADFNK Medium): 1:1 Advanced DMEM/F12 and Neurobasal medium, 1% Pen/Strep, 1% GlutaMAX, 10% Knockout Serum Replacement (KSR) and 0.1 mM β-Mercaptoethanol.

Motor Neuron Medium (ADFNB): 1:1 Neurobasal medium and Advanced DMEM/F12, 1% Pen/Strep, 1% GlutaMAX and 2% B27 serum-free supplement.

PC12 Growth Medium: Contains %73 DMEM High glucose, 15% Heat inactivated horse serum, 10% FBS, 1% Pen/Strep, 1% Glutamax

B. PLASMID MAPS

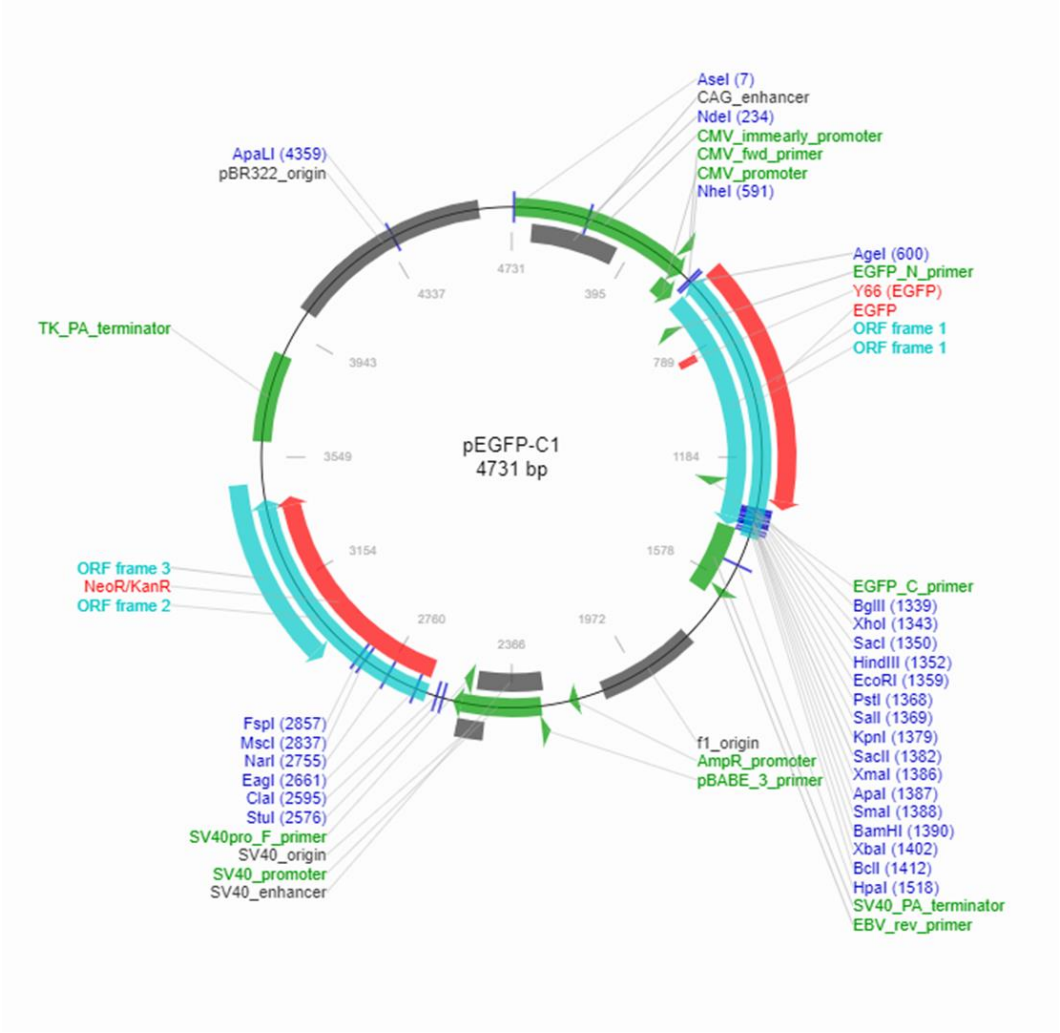


Figure B.1. Plasmid map of pEGFP-C1 plasmid

C. BUFFER CONTENTS

6X LAEMMLI SAMPLE BUFFER

12%	SDS
60%	Glycerol
0.012%	Bromophenol Blue
0.375 M	Tris-HCl, pH= 6.8
30%	β-Merccaptoethanol

10X TBS pH =7.6

24 g	Trizma Base
88 g	NaCl
dH_2O	up to 1 L

1X TBS-T

100 mL	10X TBS
900 mL	dH_2O
1 mL	Tween20

5% TBS-T-SKIMMILK SOLUTION

2.5 g	Skimmed Milk Powder
1X TBS-T	50 mL

MILD STRIPPING BUFFER

15 g	Glycine
1 g	SDS
10 mL	Tween20
1 L	deionized water, pH = 2.2

10X RUNNING BUFFER

30 g	Trizma Base
144.1 g	Glycine
10 g	SDS
1 L	dH_2O, pH=8.3

1X RUNNING BUFFER

100 mL	10X Running Buffer
900 mL	dH_2O

10X TRANSFER BUFFER

30.3 g	Trizma Base
144.1 g	Glycine
1 L	dH_2O, pH=8.3

1X TRANSFER BUFFER

100 mL	10X Transfer Buffer
700 mL	dH_2O
200 mL	Methanol

D. PRODUCTION OF TURBOID RECOMBINANT PLASMID CONTAINING BOTULINUM NEUROTOXIN SEROTYPE A ENZYMATIC FRAGMENT

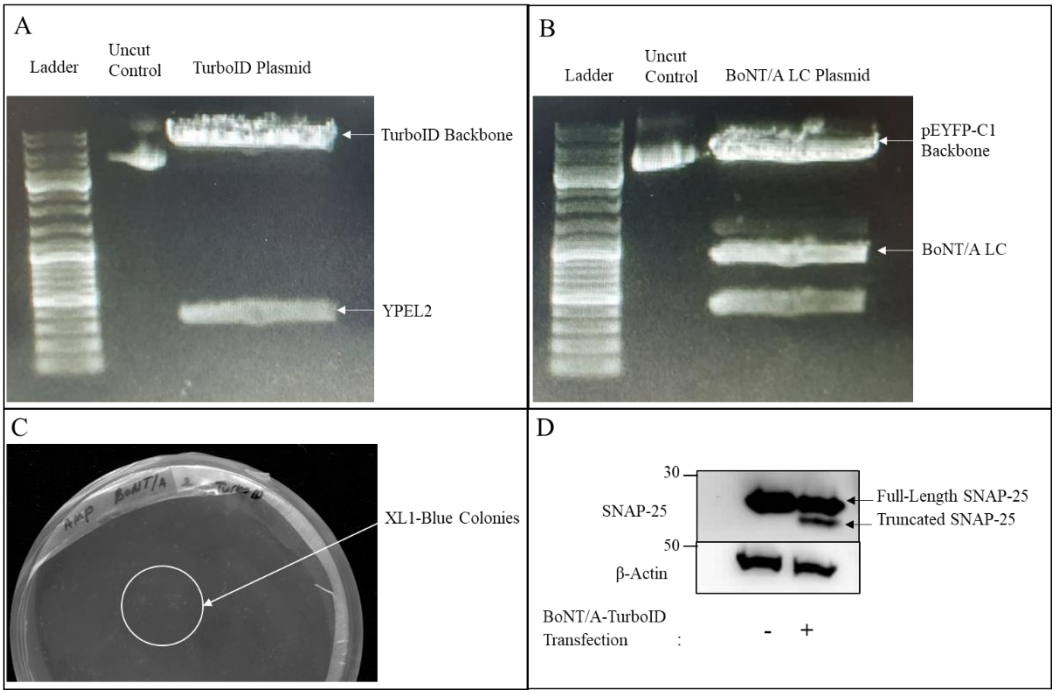
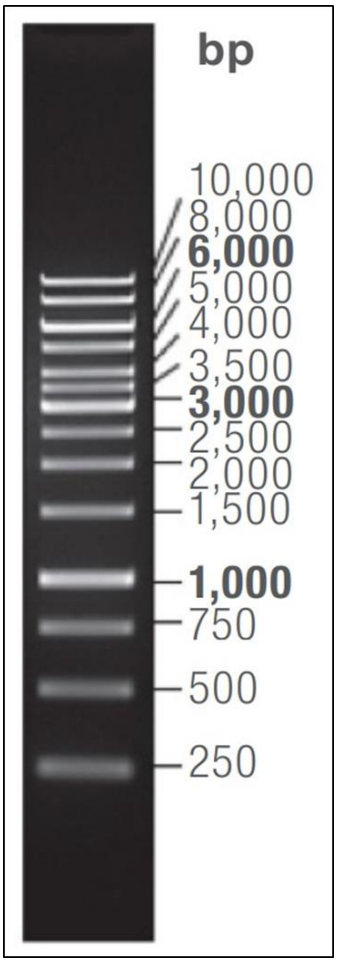


Figure D.2. Production steps of TurboID Recombinant Plasmid containing BoNT/A LC fragment

Cloning was performed to produce a TurboID recombinant plasmid containing the BoNT/A LC fragment. TurboID backbone plasmid was a kind gift from Prof. Dr. Mesut Muyan (Middle East Technical University, Turkey). The YFP-tagged LCA (BoNT/A light chain, BoNT/A LC) is present on the pE-YFPC1 plasmid, which is part of the BoNT/A light chain plasmid and the YFP-tagged LCA has been cloned by using Xho I and BamHI sites. For cloning processes, (A) TurboID plasmid was cleaved from its Xho I and BamHI sites to provide sticky ends at restriction sites that allow LCA binding. The cleaved plasmid was loaded into an agarose gel. TurboID backbone was isolated from agarose gel, and plasmid purification was performed. (B) pEYFP-C1 backbone containing LCA was restricted at Xho I and BamHI restriction sites and loaded into an agarose gel. LCA fragment was purified from gel

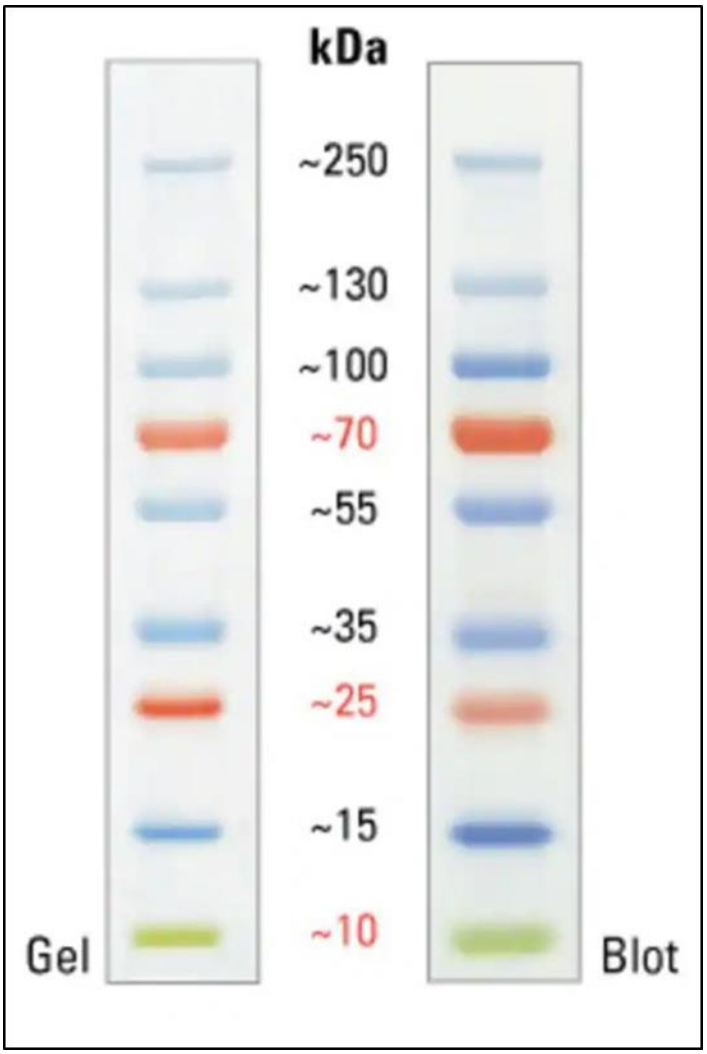
and ligated with linearized TurboID backbone. (C) The obtained recombinant plasmid was transformed into XL1B competent cells and plated on ampicillin-containing agar plates. Since TurboID backbone has ampicillin resistance gene, obtained colonies were expected to contain TurboID backbone. (D) PC12 cells were transfected with this plasmid to assess the functionality of TurboID recombinant plasmid containing LCA. Western Blot results show that the newly obtained recombinant plasmid had functional LCA since it cleaved full-length SNAP-25. However, transfection efficiency appeared to be low, so further optimizations are planned for future directions.

E. DNA LADDER



Thermo Scientific™ GeneRuler 1 kb DNA Ladder (SM0311)

F. PROTEIN LADDER



Thermo Scientific™ PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa