Assessing Phenibut's Inhibition of Glutamate Induced Apoptosis in SHSY5Y cells, an in Vitro Model for Traumatic Brain Injury

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Glutamate is our body's primary excitatory neurotransmitter and plays a critical role in neuronal signaling allowing for perception of the external world and the formation of memories. In excess, glutamate behaves as an excitotoxin that can induce neuronal apoptosis. Evidence exists that during head trauma neuronal cells can be exposed to supraphysiological concentrations of glutamate causing damage or cell death. Both GABA receptor agonists and voltage-gated calcium channel blockers have been shown to have an attenuating effect on glutamatergic excitotoxicity via antagonistic signaling and inhibition of glutamate release. Phenibut, a widely available gabapentinoid, possesses both mechanisms of action, making it an appealing candidate as a therapeutic in the treatment of traumatic brain injury.

In the United States alone, approximately 1.7 million people annually suffer a traumatic brain injury (TBI).[1] Of that number, it is estimated that 2% die due to their injuries.[2] Evidence exists that many of these statistics are skewed less than the actual occurrences given that mild TBIs are often not treated in hospitals.[3] TBIs disproportionately affect children, the elderly, contact sports athletes, and military personnel.[3,4] Those who survive head injury are often left with permanent damage that can

Figure 1 | Representations of the two enantiomeric forms of Phenibut. (**a**) R-Phenibut (**b**) S-Phenibut

result in life-long depression, decreased cognition, decreased self-regulation, and an increased propensity to neurodegenerative diseases and stroke.[5,6] There are two types of damage in TBIs. Primary damage is the mechanical lysing of brain cells due directly to an impact to the head. The damage occurring after the initial trauma is referred to as secondary damage. This happens when cells neighboring the mechanically lysed brain cells are killed by exposure to excessive extracellular glutamate and calcium ions released by the damaged glial cells and neurons.[7,8] Although glutamate is a primary excitatory neurotransmitter in the CNS and is also important for normal neuronal function. When in excess, it behaves as an excitotoxin that can trigger neuronal cell apoptosis. Free glutamate in the extracellular environment binds to N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors, causing calcium influx in neuronal cells.[9,10] In excess, this results in the activation of several calcium-dependent protease pathways leading to mitochondrial dysfunction and ultimately apoptosis.[11-13] Without medical intervention, the secondary damage is difficult to control and will continue to progress for up to several days after the initial trauma. Options for treatment are limited, ranging from therapeutic cooling for mild injury to craniotomy or drug induced-coma for severe injury. Despite evidence for therapeutic agents being beneficial for controlling secondary damage in animal models, there are no therapeutics

approved for TBIs in humans.[14]

Phenibut is a therapeutic that shows efficacy in decreasing TBI secondary damage in rodent model systems.[8,15] Historically it was developed as an anxiolytic in Russia during the 1960s.[16,17] In recent years it has been sold online as a 'nootropic' or cognitive enhancing drug. The R-enantiomer (**Figure 1**) is responsible for the physiological activity of phenibut. It functions in two ways, first, it binds to the $\alpha_2\delta$-1 subunit of voltage-dependent calcium channels, blocking them, it additionally binds as an agonist to GABA receptors.[17,18] These mechanisms are shared with other gabapentinoids which have been found to reduce secondary damage in animal models.[19] Both mechanisms work to counter the excitotoxic effects of excessive glutamate and calcium on brain cells via inhibition of calcium influx, and reduction of neuronal excitation, limiting further glutamate release.[20]

In this study, we sought to investigate phenibut as a therapeutic to attenuate the progression of secondary damage after a TBI. This was done in vitro using SHSY5Y neuroblastoma cells as a model brain after ensuring they expressed the CACN$\alpha_2\delta$-1 subunit that phenibut is a ligand of. Glutamic acid was used as an inducer of neuronal apoptosis. Cell viability was assessed in these cells exposed to glutamic acid, phenibut, or both to see if phenibut was antagonistic to glutamate-in-

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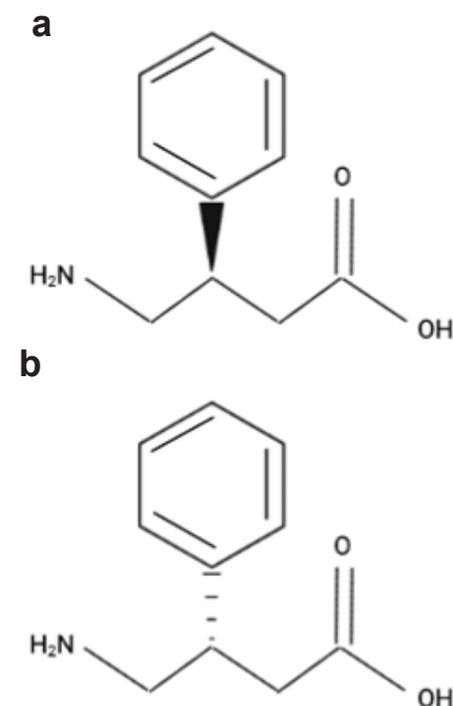
duced apoptosis, or if phenibut possessed toxicity of its own. We found that phenibut appears to be non-toxic at the doses used, and antagonizes apoptosis induced by supraphysiological glutamate exposure.

Materials and Methods

Chemicals
Phenibut HCl was obtained from LiftMode (SKU No. PH-ENIL-C070), and Glutamic acid was from Bulk Supplements (SKU No. LGA100).

Cell culture
SHSY5Y cells were generously donated by the KU Department of Pharmacology and Toxicology. Cells were cultured in with DMEM (Thermo Fisher, Cat. No.11885092) with 10% HyClone Fetal Bovine Serum (Cytiva, Cat. No. SH30071.02).

Western Blot of SHSY5Y cells probing for the Voltage-Gated Calcium Channel (VGCC) CACNα$_2$δ-1 subunit
SHSY5Y cell lysates were prepared using a protocol titled Radio-immunoprecipitation Assay (RIPA) Cell Lysate Preparation by GoldBio.[21] The RIPA buffer contained 10 mM Tris-HCl (pH 8.0) (Corning, Cat. No. 25-053-Cl), 1 mM EDTA (Fisher Scientific, Cat. No. 60-00-4), 1% Triton X-100 (Acros Organics, Cat. No. 9002-93-1), 0.1% Sodium Deoxycholate (Sigma Aldrich, Cat. No. D6750-25G), 0.1% SDS (MP Biomedicals,

Cat. No. 190522), 140 mM NaCl (Fisher Science Education, Cat. No. S25541A), and 1 mM PMSF (Acros Organics, Cat. No. 329-98-6). Protein content in lysates was quantified using a Pierce BCA Protein Assay Kit (Thermo Fisher, Cat. No. 23225) and a Pierce Rapid Gold BCA Protein Assay Kit (Thermo Fisher, Cat. No. A53227) and a SpectraMax M3 Multi-Mode Microplate Reader (Molecular Devices). Samples were prepared with Laemmli SDS-Sample Buffer 6x, Reducing (Bio-World Cat. No. 10570021-2) and electrophoresed through a 10% acrylamide gel. After transferring to a nitrocellulose membrane, the membrane was incubated with 1:1000 CACNα$_2$δ-1 Rabbit Poly Ab (Proteintech, Cat. No. 27453-1-AP), washed, incubated with Ms anti-Rb IgG HRP (EMD Millipore, Cat. No. AP1889P), exposed to ECL chemiluminescent substrate (Thermo Fisher, Cat. No. 34075) and imaged with a LI-COR C-DiGit Blot Scanner.

Cell viability via flow cytometry
SHSY5Y cells were split into 3 populations in a 6-well plate, each receiving 2 mL of complete growth medium. Once roughly 80% confluency was reached, the medium was removed and 2 mL of fresh drug-containing media was applied. Each population received complete media, complete media with 20 mM glutamic acid, or complete media with 20 mM glutamic acid and 1 mM phenibut HCl, respectively. Cells were incubated for 24 hours at 37°C in their respective conditions. Samples
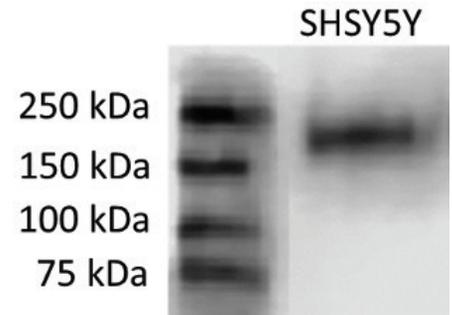
Figure 2 | The band in the SHSY5Y lane corresponds with the CACNα$_2$δ-1 subunit between 150-250 kDa.

then had their media removed followed by trypsinization with 500 µL of 0.25% Trypsin-EDTA (Thermo Fisher, Cat. No.) and phenol red (Thermo Fisher, Cat. No. 25200056). Trypsinized cells were incubated at 37°C for 5 minutes and then pipetted into 1 mL microcentrifuge tubes containing 500 mL of complete media. Samples were stained with SYTOX" AADvanced™ Dead Cell Stain Kit (Thermo Fisher, Cat. No. S10274) and incubated for 15 minutes in the dark at 25°C prior to being run on an Attune™ NxT Flow Cytometer (Thermo Fisher, Cat. No. A24864). The Sytox serves as a live-dead stain that only stains dead cells. The fluorescent signal of Sytox was analyzed in a histogram after cell debris was gated out.
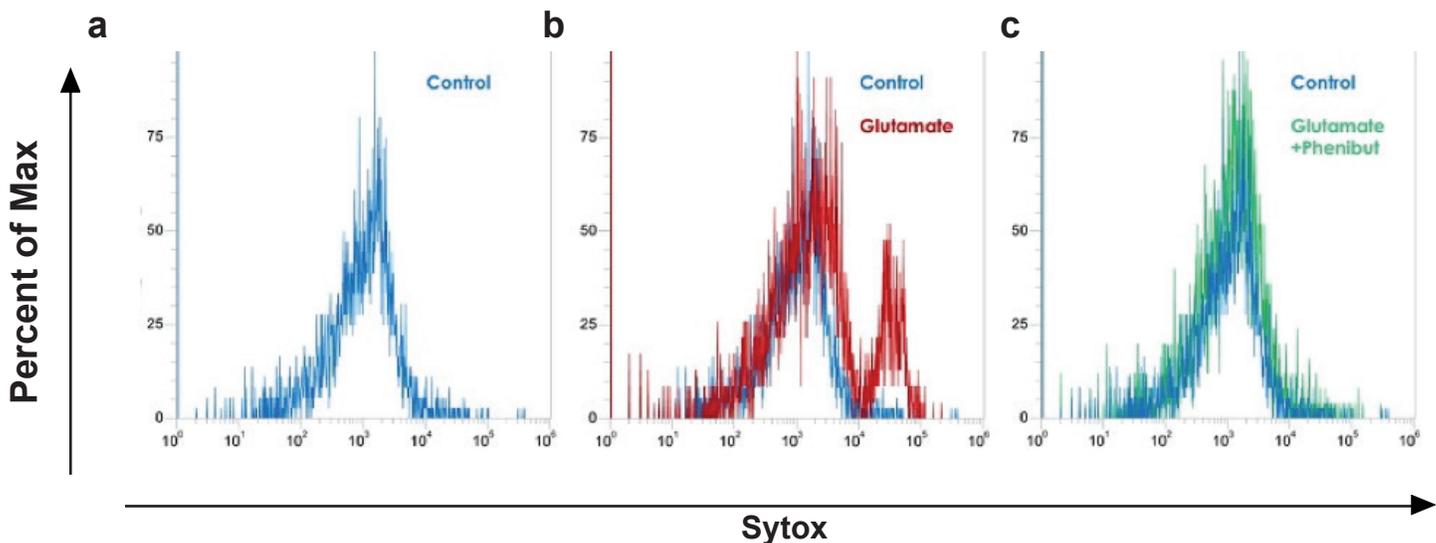
Figure 3 | Sytox staining of SHSY5Y cells. Cells treated with (**a**) control media, (**b**) 20 mM glutamate, or (**c**) 20 mM glutamate + 1 mM phenibut. Peaks centered around 10³ in all samples correspond to live cells that do not take up Sytox dye. The peak centered between 10⁴ and 10⁵ in the 20 mM glutamate sample corresponds to apoptotic cells that have taken up the dye.

Results

Western blot for CACNα₂δ-1 in SHSY5Y cells

Before exploring phenibut's inhibition of glutamate-induced calcium-dependent apoptosis in SHSY5Y cells, it was necessary to determine if SHSY5Y cells express the $\alpha_2\delta$-1 subunit of the N-type VGCC. This was done because phenibut exerts its TBI-relevant therapeutic effects by binding to this target resulting in the closure of the ion channel. Lysates of SHSY5Y cells were electrophoresed through a 10% SDS-PAGE gel, transferred to, and probed on a membrane using rabbit anti-human CACNα₂δ-1 antibody and visualized with mouse anti-rabbit IgG HRP and ECL substrate. The resulting band in the SHSY5Y lane confirms the presence of the 175 kDa CACNα₂δ-1 in SHSY5Y cells (**Figure 2**).

Flow cytometry on glutamate and phenibut treated SHSY5Y cells

To determine whether phenibut has an antagonistic effect on glutamate-induced apoptosis, the effects of glutamate or glutamate + phenibut on cell viability were assessed using flow cytometry. The proportion of dead cells was determined by the uptake of Sytox dye. SHSY5Y cells were treated with 20 mM glutamate, 1mM Phenibut + 20 mM glutamate, or no drug (control), then incubated for 24 hours prior to harvest and staining with Sytox. Control (no glutamate or phenibut) and 20 mM glutamate + 1 mM phenibut have a similar peak centered around 10^3 corresponding to live cells (**Figure 3**). The presence of a second peak centered between 10^4 and 10^5 in the 20 mM glutamate sample indicates approximately 30% of the cells were killed. Taken together, these data suggest that phenibut interrupts the processes leading to glutamate release and acts in a protective manner against the secondary damage manifesting as glutamate-induced apoptosis of SHSY5Y cells.

Discussion

In this study, we confirmed the expression of the $\alpha_2\delta$-1 subunit of the N-type VGCCs in SHSY5Y cells by western blot. This was done because phenibut binds to the $\alpha_2\delta$-1 subunit of the voltage-dependent calcium channels, preventing them from opening and allowing calcium influx into the axon terminal. This inhibits release of glutamate into the synapse. Our cell viability assay results showed that 20 mM glutamate was sufficient to induce approximately 30% cell death after 24 hours. 1 mM phenibut possessed antagonistic effects to glutamate-induced apoptosis in cells exposed to 20 mM glutamate.

In conclusion, phenibut likely has neuroprotective effects in the context of treating the secondary damage of TBIs. Inhibition of glutamate-induced apoptosis suggests phenibut could reduce the progression of neuronal death associated with secondary damage, leading to better treatment outcomes in post-TBI individuals and may be a worthwhile therapeutic for use in the treatment of TBI.

Future Directions

Further exploration into the mechanism by which phenibut inhibits glutamate-induced cytotoxicity should be performed. Differentiation of neuroblastoma cells into mature neurons via Nerve Growth Factor treatment would likely serve as a better model brain.[22] It would be worthwhile to explore the relative abundances of GABA Type A receptors, GABA Type B receptors, N-type VGCCs, and NMDa receptors to establish a baseline of expression. From there, it could be investigated whether phenibut causes a change in expression after long-term exposure as this would correlate with tolerance formation in human and animal models.[23] Derivatives of phenibut are also likely a worthwhile direction to investigate for TBI therapeutics. Fluorophenibut is one such derivative that warrants further exploration. Very little information exists on this drug's pharmacology, making it an interesting and novel avenue for research.[24]

Acknowledgments

Author's Biography

Zach Binkley is an undergraduate in the KU Edwards Biotechnology Program. After graduation, he would like to pursue a career in academia with the goal of acquiring a Ph.D. in Pharmacognosy to explore novel therapeutics found in nature and develop useful derivatives thereof.
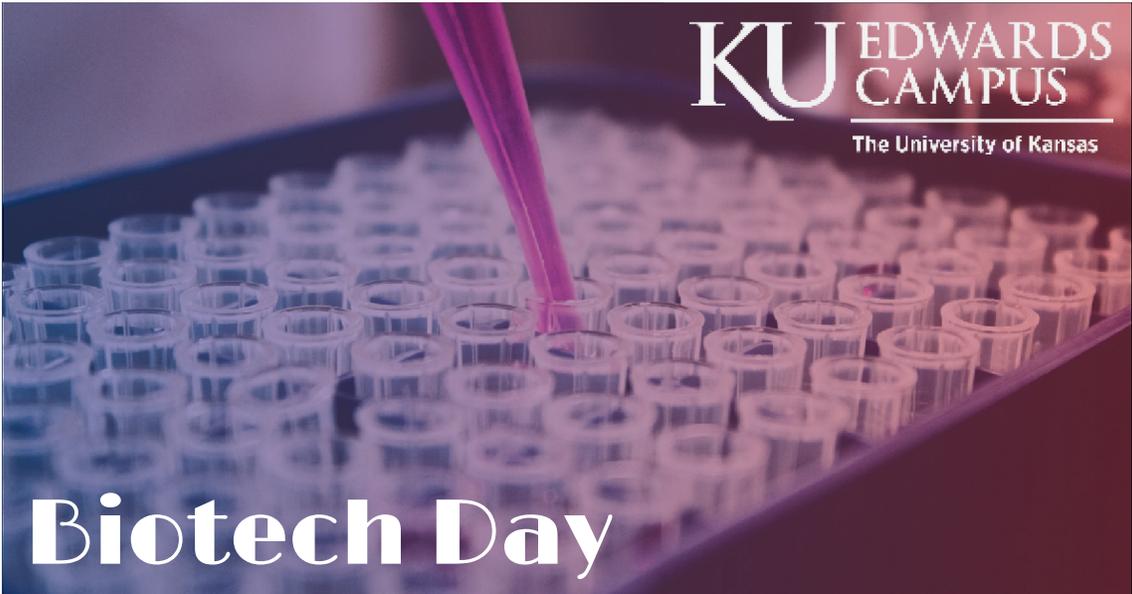
Author Contributions

Z.T.B. contributed to the experimental work, design, and writing of this work; J.F.T. contributed to the editing and design of this work; R.L.L. contributed to the editing and design of this work.

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