56th ANNUAL MIDWEST STUDENT BIOMEDICAL RESEARCH FORUM

Saturday, March 8, 2025

ROOM 3040

8:00 a.m.	O-74	A PHENOTYPIC SCREENING METHOD IDENTIFIES A POTENT ANTI-CANCER PROTAC TARGETING MAP3K1 Presenter: Lelisse Umeta
8:15 a.m.	O-31	POLYMER-ASSISTED MICRORNA DRUG DELIVERY IN ALCOHOL ASSOCIATED LIVER DISEASE TREATMENT Presenter: Marjina Akter Kalpana
*8:30 a.m.	O-75	*GLOBAL ISCHEMIA-INDUCED NEURODEGENERATION AND COGNITIVE DEFICITS ARE ALLEVIATED BY TREM1 INHIBITION.* *Presenter: Rachael Urquhart*
8:45 a.m.	O-54	OVERCOMING RESISTANCE TO TARGETED THERAPIES IN CANCER USING SMALL MOLECULE ACTIVATORS OF PP2A Presenter: Shreya Parmar
9:00 a.m.	O-29	ALTERED VENTILATORY RESPONSES TO HYPOXIA-HYPERCAPNIA IN Kv1.1 KO MICE, A MODEL OF SUDDEN UNEXPECTED DEATH IN EPILEPSY (SUDEP): ROLE OF OREXIN NEURONS Presenter: Shruthi Iyer
9:15 a.m.	O-28	CONTRIBUTION OF SEIZURES AND SYMPATHETIC ACTIVATION ON THE ADAPTIVE IMMUNE RESPONSE OF KCNA1-NULL MICE, A MODEL OF SPONTANEOUS RECURRENT SEIZURES Presenter: Jillian Hinman
9:30 a.m.	O-16	NORTRIPTYLINE: A REPURPOSABLE ADJUVANT TO CHEMOTHERAPY IN GROUP 3 MEDULLOBLASTOMAS THAT TRIGGERS APOPTOSIS BY INDUCING MITOCHONDRIAL DYSFUNCTION Presenter: David Doss
9:45 a.m.		**BREAK**

**No longer Presenting*

A PHENOTYPIC SCREENING METHOD IDENTIFIES A POTENT ANTI-CANCER PROTAC TARGETING MAP3K1

Lelisse Umeta[1], Jayapal Reddy Mallareddy[1], Sarbjit Singh[1], Smitha Kizhake[1], Lidia Boghean[1], Amritha Kizhake[1], Suchita Vishwakarma[2], Katelyn L. O'Neill[1], Lisa Humphrey-Brattain[1], Adrian Black[1], Xu Luo[1], Andrew S. Boghossian[3], Matthew G. Rees[3], Melissa M. Ronan[3], Jennifer A. Roth[3], and Amar Natarajan[1,2].

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The identification of Thalidomide, Lenalidomide, and Pomalidomide as Cereblon (CRBN) binders spurred the development of proteolysis-targeting chimeras (PROTACs). PROTACs with CRBN-binding moieties are heterobifunctional molecules that form a ternary complex with CRBN, which recruits the CUL4-DDB1 ubiquitin ligase protein complex and a protein of interest (POI) and targets it for degradation. Despite numerous ongoing efforts, the structure-based design of PROTACs remains elusive. The current approach relies on the synthesis of a PROTAC library and screening the library in the chosen assay format to identify viable hits, which can be time-consuming and resource-intensive, highlighting the need for more efficient screening methods. To address this challenge, we hypothesized that a viability-based phenotypic screening system using HCT116 wild type (WT) POI knock-out (POI-/-) and CRBN knock-out (CRBN-/-) cells would enable rapid identification of PROTACs that display activity in both a POI and CRBN-dependent manner. For this study, we selected MAP3K1, an oncogenic signaling activator, as our POI and conjugated an ATP-competitive MAP3K1 inhibitor with thalidomide using various linkers to generate the library. The library was subjected to a 3-day viability screen using the above WT, MAP3K1$^{-/-}$, and CRBN$^{-/-}$ cell lines. The screen identified 50-008 as a hit that selectively inhibited the viability of the WT cells but not MAP3K1$^{-/-}$ or CRBN$^{-/-}$ cells. 50-008 was chemically validated in a colorectal cancer cell line and organoid model by making single changes that affect either CRBN binding, MAP3K1 binding, or the linker. Follow-up studies showed that 50-008 inhibits cancer cell proliferation, induces apoptosis, and an S-phase cell cycle arrest in a CRBN and MAP3K1-dependent manner. Furthermore, A Profiling Relative Inhibition Simultaneously in Mixtures (PRISM) screen of 50-008 across ~900 cancer cell lines identified 267 sensitive cancer cell lines (IC_{50} < 0.1μM), including luminal B breast cancer cell line T47D and 131 resistant cancer cell lines (IC_{50} > 10 μM). Mechanistic studies in T47D cells demonstrated that 50-008 degrades MAP3K1 and modulates the activation of key downstream effectors, such as ERK and IKKβ. In conclusion, our phenotypic screening system, we identified and validated 50-008 as a potent MAP3K1-targeting PROTAC with selective activity across multiple cancer models. This approach establishes a rapid and reliable pipeline for identifying functional PROTACs while simultaneously validating their cellular specificity and mechanism of action. Our findings not only provide a validated screening platform for future PROTAC development but also demonstrate therapeutic potential in targeting MAP3K1-dependent cancers.

POLYMER-ASSISTED MICRORNA DRUG DELIVERY IN ALCOHOL ASSOCIATED LIVER DISEASE TREATMENT

Marjina Akter Kalpana, David Oupicky (UNMC Omaha, NE)

Background, Significance, Hypothesis: The number of alcohol-induced death have significantly risen over past two decades. According to NCHS data, alcohol associated liver disease (AALD) was the most frequent cause of alcohol induced death with a remarkable increase by 26% during the first year of COVID-19 pandemic. 46% of the total 98,457 liver disease deaths in the US in 2022 involved alcohol which includes but not limited to alcoholic- fatty liver, liver fibrosis or sclerosis, hepatitis, and cirrhosis. Currently, there is no FDA–approved drug except Corticosterioids and Pentoxifyline which can reduce collagen and TNF-α level in acute alcoholic hepatitis but have limitations to improve the long-term survival in patients, that makes a new drug development essential. Interaction between CXCL-12 and chemokine receptor 4 (CXCR4) on activated hepatic stellate cells (HSC) and higher expression of microRNA 155 in Kupffer cells (KC) induces AALD pathogenesis. Many polymer-based nanocarrier systems are explored to deliver genetic drugs into liver in AALD animal models and passed the preclinical trials. But inadequate drug bioavailability remains a challenge to get approval of them for human use. We synthesized a polymeric nanocarrier PAMD from the CXCR4 antagonizing drug AMD3100, modified it with 17%cholesterol, and synthesized PEG-copolymers (5- and 10% PEG conjugated to PAMD-Ch) to develop a microRNA-based therapeutic polyplex with improved stability and bioavailability. We hypothesize that dual inhibition of CXCR4 on HSC and miR155 in KC with this DDS will improve therapeutic outcomes in AALD treatment compared the previously demonstrated single inhibition of CXCR4 on activated HSC or miR155 in KC.

Experimental design: The anti-miR155 encapsulation efficiency by polymer and copolymers were tested with agarose gel retardation assay to determine at what w/w ratio the polymer can fully encapsulate the therapeutic RNA. The percentage of the encapsulation efficiency are measured with RiboGreen assay. To test our hypothesis, we formulated polymer/anti-miR155 polyplexes at different w/w ratios and measured their hydrodynamic size and surface charge (zeta potential) with dynamic light scattering. The colloidal stability test was conducted by incubating the polyplexes into PBS to mimic the aggregation properties of polyplexes in physiological condition. The integrity of polyplexes after body administration are simulated by measuring the FRET signal upon incubating them into human blood plasma. FACS analysis and confocal imaging were performed to analyze the polyplex uptake into macrophages using RAW 264.7 cells where the polymer (or copolymers) and the anti-miR155 were labeled with fluorescent dyes Cy3 and Cy5.5. To test the transfection efficiency of anti-miR155 in macrophages, we performed real time PCR and analyzed its ability to reduce miR 155 gene expression in miR155 overexpressed cell with lipopolysaccharides. Cytotoxic effect of the polyplexes and free polymers are evaluated with cell titer blue assay.

Data and Results: All polymer (or copolymers) fully encapsulate the anti-miR155 at or above w/w ratio 1.5 with an encapsulation efficiency of > 98%. Cholesterol modification of PAMD significantly reduces the hydrodynamic size of polyplexes to <100 nm suitable for entry through liver fenestrae. All four polyplexes PAMD/anti-miR155 (PM), PAMD-Ch/ anti-miR155 (PCM), 5PEG-PAMD-Ch/ anti-miR155 (5PPCM), and 10PEG-PAMD-Ch/ anti-miR155 (10PPCM) at w/w ratios 2 and 4 have positive surface charges aiding endocytosis. Noticeably the charge of the polyplexes containing PEG shows a decreasing trend with increased PEG content. The PCM and PPCM polyplexes have improved colloidal stability suggesting the role of cholesterol in reducing polyplex aggregation in biological system (Fig. 1a). PEG or increased concentration of PEG further improves the stability of polyplexes in PBS. An increased FRET signal in blood plasma is observed for PCM than PM which decreases in the 5- and 10PPCM. However, PEG provides better integrity of the polyplexes demonstrated by less decreasing FRET ratios (Fig. 1b). Cholesterol modification of PAMD significantly improves hydrophobicity and cellular uptake of PCM polyplexes. Whereas PPCM with low zeta potential results into decreased cellular uptake (Fig. 1c). All polymer and copolymers retain microRNA transfection efficiency like the commercially available lipofectamine and significantly reduce the miR155 gene expression in macrophages (Fig. 1d) with minimal cytotoxicity.

Conclusion: In conclusion, we developed the polymeric polyplexes with improved colloidal stability, plasma integrity and transfection efficiency into macrophages. Further studies on CXCR4 antagonism is required to ensure the dual targeting efficacy of our formulated polyplexes and improve the therapeutic outcomes in AALD treatment.

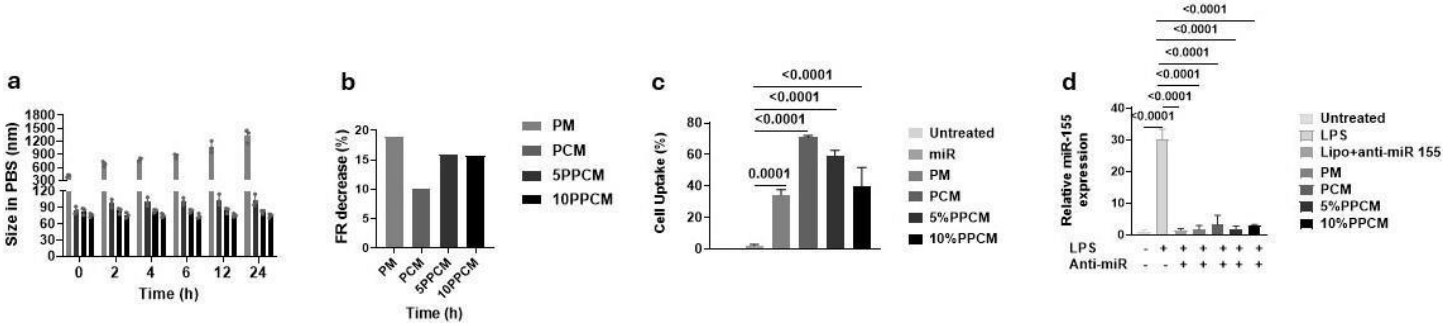

Figure 1. Colloidal stability (a), Blood plasma integrity (b), Uptake by RAW 264.7 (c), and anti-miR155 transfection efficacy (d) of w/w 2 polymeric polyplexes.

GLOBAL ISCHEMIA-INDUCED NEURODEGENERATION AND COGNITIVE DEFICITS ARE ALLEVIATED BY TREM1 INHIBITION.

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Global ischemic stroke occurs when blood flow throughout the whole brain is significantly reduced. This occurs most commonly following cardiac arrest when blood flow through both the carotid arteries and vertebral arteries is significantly impaired. Global ischemia promotes selective, delayed neuronal loss of pyramidal cells within the hippocampal CA1, ultimately promoting impaired learning and memory. Current therapies that address cardiac arrest and global ischemia focus on restoring blood flow and cardiac function, but they do not adequately address the long-term effects of global ischemia.

Recently, neuroinflammation has received increased attention for its potential contributions to neurological deficits in global ischemia, however, the molecular mechanisms by which global ischemia induces neuroinflammation are not yet fully delineated, and currently there are no clinically proven pharmacological therapy targeting neuroinflammation for neuroprotection against global ischemic insults. It is thus necessary to gain a deeper understanding of the neuroinflammatory response to global ischemia and develop new therapeutic strategies.

Following global ischemia, there is a significant delay in time between the onset of global ischemia and the development of robust pathology. Although the mechanisms underlying the pathophysiology are as yet unclear, the long delay between insult and neuronal death is consistent with a role for transcriptional changes. Therefore, in this study, we sought to explore how dysregulation of genes might promote the neurodegeneration and cognitive deficit associated with global ischemia. Towards this end, we subjected rats to global ischemia via 4-vessel occlusion (4VO) and performed RNA-seq analysis on the post-ischemic hippocampal CA1. Ingenuity Pathway analysis revealed upregulated gene expression related to immune-response related pathways, specifically neuroinflammation and the triggering receptor expressed on myeloid cells-1 (TREM1). TREM1 is a pivotal myeloid-derived cell surface receptor involved in both initiation and amplification of inflammation. While TREM1's proinflammatory roles are well-documented in systemic conditions such as myocardial ischemia and sepsis, where its inhibition has shown therapeutic benefits, its role in global ischemic stroke remains unknown. To address this, we tested the hypothesis that TREM1-meidated neuroinflammation drives global ischemia pathology, and TREM1 inhibition attenuates global ischemia-induced neurodegeneration and cognitive deficits. To validate RNA-seq and IPA, we utilized RT-qPCR and Western blot analyses and showed that expression of TREM1 and associated inflammatory targets are elevated following global ischemia, which is attenuated following TREM1 inhibition via inhibitory decoy receptor peptide LR12. We further showed that microglial activation, astrogliosis, and blood-brain barrier breakdown are elevated following global ischemia, but this pathology was all prevented following TREM1 inhibition. Lastly, we observed that global ischemia induces significant neurodegeneration in the hippocampal CA1 and results in cognitive deficits, which were prevented by TREM1 inhibition via LR12. Overall, this research establishes the role of TREM1-mediated neuroinflammation in global ischemia pathology and identifies TREM1 as a potential therapeutic target for attenuating global ischemia-induced neurodegeneration and cognitive deficits.

OVERCOMING RESISTANCE TO TARGETED THERAPIES IN CANCER USING SMALL MOLECULE ACTIVATORS OF PP2A

Shreya Parmar, Michelle Lum, Kayla Jonas, Adrian Black, Jennifer Black
(UNMC, Omaha, Nebraska)

Background, Significance, Hypothesis: Targeted anticancer therapies such as inhibitors of mTOR or the ERK pathway rely on inhibition of eIF4E-dependent protein synthesis for tumor suppression. Hyperactive protein synthesis is a hallmark of cancer, with key roles in reprogramming the proteome to fuel tumor development, progression, and therapy resistance. eIF4E-dependent translation involves assembly of the eIF4F translation initiation complex, which consists of the mRNA cap-binding protein, eIF4E, the molecular scaffold, eIF4G, and the RNA helicase, eIF4A. The translational and oncogenic functions of eIF4F are negatively regulated by the translation gatekeeper protein, 4E-BP1, which inhibits eIF4F assembly and suppresses protein synthesis. Various targeted therapies are dependent on 4E-BP1 for tumor suppression; thus, 4E-BP1 inactivation is a major mechanism of resistance to these therapies. Tumors circumvent the translation suppressive effects of 4E-BP1 by transcriptional repression of 4E-BP1 expression or by functionally inactivating 4E-BP1 via hyperphosphorylation. We have found that both expression and activity of 4E-BP1 can be restored in tumor cells using novel small molecule activators of PP2A (SMAPs) that selectively stabilize and activate PP2A heterotrimers containing B56 (a, b or ε) regulatory subunits. Selective activation of B56α-, δ and/or ε-containing PP2A holoenzymes results in PP2A-dependent transcriptional induction of 4E-BP1 expression and activation of the translation-repressive function of 4E-BP1 by hypophosphorylation. Based on our findings, we hypothesized that a combination of SMAP compounds with ERK or mTOR inhibitors will potentiate tumor suppression and overcome drug resistance by increasing the levels of active 4E-BP1. The objectives of this study were (1) to compare the effects of ERK or mTOR inhibitors (SCH772984 or INK128) with those of SMAP compounds (e.g., DT-061) on 4E-BP1 expression and phosphorylation, and (2) to investigate the ability of combinations of ERK or mTOR inhibitors with DT-061 to restore 4E-BP1 function and thus overcome drug resistance to these targeted therapies.

Experimental design: The ability of SMAPs (lead compounds DT-061 and EV-440), ERK inhibitors (e.g., SCH772984), and mTOR inhibitors (e.g., INK128) to modulate 4E-BP1 expression and phosphorylation was compared using Western blot analysis and Reverse Transcription-real time Polymerase Chain Reaction assays (RT-qPCR). Similar approaches were used to explore the effects of drug combinations on 4E-BP1. The analysis was performed using pancreatic (BxPC3 and Capan1) and colorectal (SW-620) cancer cell lines.

Data and Results: While SMAPs DT-061 and EV-440 upregulate 4E-BP1 mRNA and protein in a variety of tumor types and promote the accumulation of active, translation repressive 4E-BP1 by hypophosphorylation, ERK inhibition alone (with SCH772984) does not induce expression or dephosphorylation of 4E-BP1. Notably, SMAPs maintain their ability to promote the accumulation of active 4E-BP1 in the presence of ERK inhibitors. In contrast to ERK inhibition, mTOR inhibitors hypophosphorylate/activate 4E-BP1 and upregulate 4E-BP1 levels through stabilization of the protein, with no effect on 4E-BP1 mRNA levels. However, the effect of mTOR inhibitors on 4E-BP1 expression is transient. In contrast, combination of mTOR inhibitors (INK128) and DT-061 results in sustained induction and activation of 4E-BP1. Thus, SMAPs retain their ability to induce high levels of active 4E-BP1 when combined with ERK or mTOR inhibitors. Together, our findings indicate that SMAPs hold promise for enhanced tumor suppression and for overcoming resistance to agents that require active 4E-BP1 for their antitumor activity.

Conclusion: Tumors cells disable 4E-BP1 and therefore develop resistance to targeted therapies such as ERK or mTOR inhibitors thar require activity of 4E-BP1 for their antitumor effects. SMAPs promote robust, PP2A-dependent upregulation and activation of 4E-BP1 by inducing its transcription and dephosphorylation. Importantly, SMAPs retain this activity when combined with ERK and mTOR inhibitors. Thus, combining SMAPs with ERK or mTOR inhibitors represents a promising therapeutic approach for tumor management and for overcoming resistance to these targeted agents.

Title: ALTERED VENTILATORY RESPONSES TO HYPOXIA-HYPERCAPNIA IN Kv1.1 KO MICE, A MODEL OF SUDDEN UNEXPECTED DEATH IN EPILEPSY (SUDEP): ROLE OF OREXIN NEURONS

Author: Shruthi Iyer*, Jillian Hinman, Parisa Rafei, Stephanie Matthews, Timothy Simeone, Kristina Simeone.

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Significance: Sudden unexpected death in epilepsy (SUDEP) is a cause of mortality in epilepsy, affecting 1 in 1000 patients each year. The retrospective MORTEMUS study reported that right before SUDEP, patients experienced generalized tonic-clonic seizure followed by repeated apnea. These events promoted intermittent hypoxic and hypercapnic (H-H) fluctuations in blood gases, an inability to recover from which, resulted in a prolonged terminal apnea and death. Thus, recovery from repeated and prolonged HH changes is critical for survival, and maybe impaired in SUDEP. The exact mechanisms underlying this failure in SUDEP are unknown. To bridge this gap in knowledge, we developed novel preclinical in vivo tests to mimic the HH changes prior to SUDEP and study the mechanisms upstream of recovery.

Background: Recovery from severe or repeated HH is mediated by hypothalamic orexin neurons that detect blood gas instability and trigger adaptive ventilation by increasing the output of key respiratory brainstem nuclei**.** Alternatively, some brainstem respiratory nuclei including the locus coeruleus (LC), can directly detect the HH changes and mediate adaptive ventilatory changes. The homeostatic orexinergic-LC chemosensing network is critical for recovery from repeated/prolonged HH. Failure at one or many levels of this chemosensing system maybe upstream of impaired ventilatory changes and blood gas instability in SUDEP. We have previously reported that the Kv1.1 KO (KO) mice, a model of SUDEP, have increased seizures, apnea, chronic intermittent hypoxia, all indicative of increased blood gas instability. Blocking orexin receptors reduced apneas, and increased longevity in these mice. This suggests an increased orexinergic influence on ventilatory responses in this SUDEP model. Further, the LC nuclei could also be adding to the impaired ventilation in this model.

Hypothesis: A) Kv1.1 KO mice have increased activation of LC ventilatory center and impaired *in vivo* ventilatory responses to repeated HH challenges. B) Orexinergic system contributes to the impaired HH ventilatory response in the KO mice. Pharmacological blockade of orexin receptors will improve HH ventilatory responses in the KO mice.

Experimental Design: Wildtype (WT) and KO mice at high-risk for SUDEP (~P47) were placed in a whole-body plethysmography chamber and subjected to one of the two gas challenges; either (a) repeated triple HH (3mins; 85% N_2, 9% CO_2, 6% O_2,) test, or (c) a prolonged HH test (5mins; 85% N_2, 9% CO_2, 6% O_2). In the first cohort, following the repeated HH test, WT and KO brains were fixed with 4% PFA and cFos activation in their brainstem LC nuclei was assessed using tyrosine hydroxylase as a marker for this region. In the second cohort, mice were pretreated with orexin receptor antagonist TCS1102 (100mg/kg, ip) and subjected to either prolonged or repeated HH test. Endpoints included survival, tidal volume, minute ventilation and peak expiratory flow.

Results: In response to repeated HH, ventilatory response in high-risk KO included elevated tidal volume, peak expiratory flow and minute ventilation, compared to WT controls. These respiratory parameters increase in proportion to oxygen debt and are exaggerated in the KO mice. Further, with repeated challenges, the KO mice fail to maintain these ventilatory responses. 75% of the KO mice succumbed to a prolonged HH test and died while all the WT controls survived the test. Following the repeated HH, there was a higher percentage of cFos positive chemosensing neurons in the LC, indicating that this brainstem region maybe upstream of the elevated ventilatory responses to HH. We also assessed the contribution of orexinergic system that regulates the LC and is upstream of HH ventilatory responses. Acute blockade of orexin receptors stabilized tidal volume, minute ventilation, duty cycle and peak expiratory flow of KO mice to levels resembling those of WT control. Further, orexinergic blockade enabled a 100% of the KO mice to survive the prolonged HH challenge.

Conclusions: The ventilatory chemoresponses indicate that the KO mice are sensitive to smallest blood gas fluctuations and must compensate more to maintain homeostatic O_2 levels. Blocking orexin receptors improved recovery from severe/repeated HH changes in these mice. Thus, maladaptive orexin chemosensing and an amplified activation of brainstem LC ventilatory nuclei could be contributing to the central chemoreception dysfunction in preclinical SUDEP.

TITLE: CONTRIBUTION OF SEIZURES AND SYMPATHETIC ACTIVATION ON THE ADAPTIVE IMMUNE RESPONSE OF *KCNA1*-NULL MICE, A MODEL OF SPONTANEOUS RECURRENT SEIZURES

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Creighton University School of Medicine, Department of Pharmacology and Neuroscience, Omaha, NE, USA

Abstract:

Background: Approximately one-third of the epilepsy patient population that experience generalized seizures is refractory to available anti-seizure medications. Chronic untreated epilepsy is known to induce inflammatory responses in the brain. Neuroinflammation promotes neurodegeneration after seizures and may contribute to both the generation and exacerbation of seizures. Seizures can spread to autonomic centers and stimulate or directly modify autonomic control of peripheral organs. Most generalized seizures activate the sympathetic system and preliminary data indicate a strong peripheral immune response in *Kcna1*-null mice that have been experiencing a high frequency of generalized seizures. **Significance of Problem:** Chronic peripheral inflammation occurs in epilepsy; however, the contribution of seizures and the sympathetic system is unknown. **Hypothesis:** Seizures increase splenic CD8+ T cells via activation of the sympathetic system, which then infiltrate the brain. **Experimental Design:** We utilized the *Kcna1*-null mouse model of epilepsy which experience spontaneous recurrent seizures. To test the impact of spontaneous recurrent seizures, we pharmacologically attenuated seizures with Phenobarbital (30 mg/kg) in *Kcna1*-null mice for 5 days. To test the contribution of the sympathetic system we treated mice with Butoxamine (5 mg/kg), a blood-brain barrier impermeable beta-2 adrenergic receptor antagonist, for 5 days in both *Kcna1*-null and wild-type mice. After both treatments, brain and spleen tissue was extracted and prepared for flow cytometry to measure CD4+ and CD8+ lymphocytes. **Results:** We were able to separate lymphocytes from the brain and spleen of wild-type and *Kcna1*-null mice. Previous data from our lab indicated *Kcna1*-null mice have slightly increased CD4+ and CD8+ lymphocytes cells in the brain. When we blocked seizures with Phenobarbital and the sympathetic system with Butoxamine, brain CD4+ and CD8+ lymphocytes decreased in *Kcna1*-null mice. CD4+ lymphocytes also decreased in the spleen of *Kcna1*-null mice with Phenobarbital and Butoxamine, but only Butoxamine decreased CD8+ lymphocytes in the spleen. **Conclusions:** This preclinical data may indicate that blocking seizures and sympathetic activation decreases the adaptive immune response in the brain of *Kcna1*-null mice. This provides evidence that chronic epilepsy may be linked to changes in the adaptive immune response.

NORTRIPTYLINE: A REPURPOSABLE ADJUVANT TO CHEMOTHERAPY IN GROUP 3 MEDULLOBLASTOMAS THAT TRIGGERS APOPTOSIS BY INDUCING MITOCHONDRIAL DYSFUNCTION

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Background: Nebraska has the 7th highest incidence of pediatric brain tumors in the US. Medulloblastomas (MB) are the most common malignant pediatric brain tumors and are divided into four primary subgroups. Group 3 (G3) MB are the most aggressive. Despite ongoing efforts to improve outcomes, patients with G3MB tumors have 5-year overall survival rates of <50%, compared to 70-95% for the other three subgroups. Current standard of care for G3MB consists of maximal safe surgical resection with subsequent craniospinal irradiation and systemic chemotherapy consisting of cisplatin, cyclophosphamide, vincristine, and lomustine.

Significance: Despite molecular differences between subgroups, this treatment regimen is applied to all patients, resulting in a widened survivorship gap for patients with the most aggressive tumors belonging to group 3. Limitations to mainstay therapies due to drug-induced toxicities further plague G3MB patients with high recurrences. Thus, G3MB patients suffer the most dismal outcomes, underscoring a dire need to develop novel strategies to treat these tumors. Targeted therapies have shown promise in WNT and SHH subgroups but show limited progress for G3MBs. To address this therapeutic and survivorship gap for G3MB patients, we pursued a novel *a priori* approach to drug discovery that relies on patient-derived subgroup-specific RNA-sequencing data.

Hypothesis: Using this approach, we isolated nortriptyline (NT) as a top candidate. We hypothesize that nortriptyline is an effective, anti-neoplastic agent that may be repurposed for the treatment of G3MB and acts to induce apoptosis through a mitochondrial-dependent mechanism.

Experimental Design: We analyzed G3MB RNA-seq data (local cohort: GSE148389, n=47; external: GSE164677, n=67) against the LINCS database to identify FDA-approved drugs capable of reversing G3MB expression profiles to a more normal state. Compounds were filtered for oral bioavailability, blood-brain barrier penetration, and FDA approval. Upon determining the top candidates *in silico*, we performed functional assays to quantify the drugs' effects on cytotoxicity, clonogenicity, wound healing, migration and invasion, and apoptosis *in vitro* in the G3 MB cell lines HDMB03 and D425. Following confirmation of functional efficacy, we proceeded to characterize the mechanism of action of our top candidate compound through RNA sequencing. Putative mechanisms identified through RNA sequencing were functionally validated through Seahorse and flow cytometry. Synergy with a standards of care drug, cisplatin, was quantified using the SynergyFinder platform.

Results: One hundred and six candidate compounds passed preliminary filtering thresholds and qualified for further testing. We screened the list of qualifying compounds and identified thirteen core compounds with favorable safety profiles in a pediatric population. The cytotoxic IC_{50} of each compound was determined through an MTT assay. Of these compounds, three classes of drugs consistently ranked among the top candidates: selective serotonin reuptake inhibitors (SSRIs), statins, and tricyclic antidepressants (TCAs). Results from MTT assays demonstrated 48h IC_{50} values in the low micromolar range for our top compound of interest, NT (7.0μM). NT abrogated colony formation during *in vitro* clonogenic assays at its IC_{50} value. Additionally, NT demonstrated a dose- and time-dependent reduction in cell migration, through a 2D wound healing ("scratch") assay, and in medullosphere formation, through a 3D tumor spheroid assay. Flow cytometry of cells stained with Annexin V and propidium iodide demonstrated a dose- and time-dependent increase in apoptotic cells relative to control when treated with NT. To support these data, induction of apoptosis was confirmed by probing for key markers of intrinsic apoptosis on Western blot; namely, cleaved caspase 3, cleaved PARP, and BCL-2. RNA-sequencing of HDMB03 cells treated with NT revealed suppression of pathways related to mitochondrial metabolism alongside upregulation of lipid metabolism. NT suppressed oxidative phosphorylation (Seahorse assay); no compensatory increase in glycolysis was observed. NT also induced mitochondrial stress by increasing mitochondrial superoxide generation (as measured by MitoSOX fluorescence) and disrupted the mitochondrial membrane potential (as measured by TMRM staining). Finally, NT synergized with cisplatin, a mainstay of G3MB chemotherapy, to enhance cell death in HDMB03 cells.

Conclusions: Taken together, our pipeline has successfully identified several lead compounds that are cytotoxic to G3MB cells *in vitro*. NT showed the most consistent anti-neoplastic effects, as well as synergy with standard of care chemotherapy. Mechanistically, NT induced apoptosis, partly by triggering mitochondrial dysfunction resulting in elevated oxidative stress and loss of membrane integrity. Overall, this pipeline provides a useful model for identifying repurposable FDA-approved drugs, with the potential to enhance current chemotherapies, allowing for reduced dosing without losing overall cancer-specific cytotoxicity.