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NOVEL SPOXAZOMICINS DERIVED FROM *STREPTOMYCES* SP. RM-14–6 ATTENUATE ETHANOL INDUCED CYTOTOXICITY *IN VITRO*

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THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
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By

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Lexington, Kentucky

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2016

ABSTRACT OF THESIS

NOVEL SPOXAZOMICINS DERIVED FROM *STREPTOMYCES* SP. RM-14−6 ATTENUATE ETHANOL INDUCED CYTOTOXITY IN VITRO

An estimated 13.9% of Americans currently meet criteria for an alcohol use disorder. Ultimately, chronic alcohol use may result in neurological deficits, with up to 85% of alcoholics exhibiting signs of cognitive decline. However, biochemical and behavioral factors contributing to this decline have remained elusive. Our ongoing research program encompasses a multi-tiered screening of a natural product library and validation process to provide novel information about mechanisms underlying these deficits and to identify novel chemical scaffolds to be exploited in the development of pharmacological treatments for alcohol use disorders in a rodent organotypic hippocampal slice culture mode. Experiment 1 sought to establish a 48 h high throughput model for testing novel scaffolds against ethanol (EtOH) toxicity. Experiment 2 tested multiple natural product compounds for their ability to attenuate ethanol-induced cytotoxicity. Results from Experiment 1 revealed EtOH (100 mM) induced significant cytotoxicity at 48 h. Trolox (100 µM), a potent antioxidant, was found to reduce ethanol-induced cytotoxicity in this assay. Experiment 2 revealed two spoxazomicins (**1**, **1-1**) demonstrated potent cytoprotective effects against ethanol toxicity. These findings highlight the potential applications of these novel scaffolds for use in the treatment of alcohol use disorder.

KEYWORDS: Alcohol Use Disorder, Novel Compound Screening, Trolox, Spoxazomicins, Neurodegeneration

Meredith A. Saunders

7/26/2016

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CHAPTER 1: Introduction

Efforts to develop treatment strategies for alcohol use disorder (AUD) have been underway since the Victorian era. However, despite these efforts, AUD still presents itself as a major public health concern, with approximately 13.9 % of the population currently meeting DSM-5 criteria for an AUD (Grant et al., 2015) and costing the United Stated an estimated 223.5 billion annually (Kanny et al. 2013). Alcohol use disorder is a maladaptive pattern of behaviors and symptoms including the inability to reduce alcohol use despite consequences, tolerance, craving, and withdrawal (American Psychiatric Association [DSM-5], 2013). The exact mechanisms underlying AUD have not yet been elucidated (Kril & Halliday, 1999). There are currently 3 FDA approved treatments for AUD: disulfram (Antabuse©), naltrexone (Revia©, Vivitrol©) and acomprosate (Campral©); however, these treatments have demonstrated poor clinical efficacy, revealing the need for additional treatment options (Johnson, 2008; Heinz et al., 2009). Additionally, it has been hypothesized that the poor performance of these treatments may be due to that they target the motivational aspects of drug taking rather than targeting the neurological mechanisms that may underlie the abuse behavior (Koob & Le Moal, 1997). Therefore, more recent findings have led to a shift in drug discovery to identifying potential treatments for AUD that address the neurological damage acquired due to alcohol abuse. Novel, naturally derived compound products, have presented themselves as viable drug discovery targets in recent years due to a wide structural diversity and demonstrated therapeutic properties (for a review see, Harvey, 2008).

Alcohol Use Disorder and Brain Morphology

Ultimately, chronic alcohol use may result in neurological deficits, with up to 85% of alcoholics exhibiting signs of cognitive decline (Parsons & Nixon, 1993). Previous neuropathological necropsy analyses conducted in chronic alcoholics have shown a reduction in brain volume characterized by decreased white matter in the cerebral hemispheres and increased ventricle size (Harper, Kril, & Holloway, 1985), demyelination of the mammillary bodies (Alling & Bostrom, 1980), thinning of the corpus callosum (Harper & Kril, 1988), and decreased cerebellar volume (Sullivan et al., 1998). These post-mortem studies have also been supported and extended upon via the use of modern imaging techniques. Studies conducted in alcoholic individuals through use of magnetic resonance imaging (MRI) and functional magnetic resonance imaging (fMRI) have also revealed abnormalities in brain morphology including, but not limited to, decreased cortical gray matter (Jernigan et al., 1991; Fein et al., 2002; Mann et al., 2001) and white matter (Pfefferbaum et al, 1992); decreased frontal lobe volume (Pfefferbaum et al., 1997); reduction in hippocampal size (Agartz et al., 1999; Bleich et al., 2003); and cerebellar shrinkage (Sullivan et al., 2003). Additionally, studies utilizing single photon emission computed tomography (SPECT) have found decreased regional cerebral blood flow (rCBF) in the frontal lobes of alcoholics when compared to non-alcoholics (Nicolas et al., 1993). It is also important to note that sex and age difference have been observed across these studies, though the patterns have not always been consistent (for a review, see Pfefferbaum & Sullivan, 2005).

Furthermore, this observed neurodegeneration has been correlated with neuropsychological behavioral deficits associated with alcohol use disorder. Cerebellar volume deficits have been shown to be predictive of the executive visuospatial and

balance impairments characteristic of alcohol use disorder (Sullivan, Rosenbloom, & Pfefferbaum 2000; Sullivan et al., 2003). Moreover, fMRI studies have found that alcoholic individuals perform worse than non-alcoholic individuals in terms of their finger tapping output, a measure of automatic processing and cerebellar inefficiency (Parks et al., 2003). Additionally, correlations have been found between performances on neuropsychological tests (i.e. The Wisconsin Card Sorting Task, The Letter Fluency Test, The Stroop Task) and deficits in the superior vermis, cerebellum, frontal lobe, hippocampus, thalami, insula, pons, and brain stem (Sullivan, Rosenbloom, & Pfefferbaum, 2000; Chanraud, 2007). Cardinal behaviors of the alcohol use disorder, such as impaired judgment, blunted affect, reduced motivation, and distractibility, have also been attributed to frontal lobe deficits (for a review, see Sullivan & Pfefferbaun, 2005). Futhermore, decreased rCBF to the frontal brain areas has been associated with lack of inhibition and deficits in short term memory (Noël et al, 2001). As a result of AUD effects on the brain, AUD is associated with Korsakoff's syndrome, Wernicke's encephalopathy, fetal alcohol spectrum disorder (FASD), accidental injury, and accidental death (for a review, see de la Monte & Kril, 2014). Notably, alcohol's effects on brain morphology, as well as its effects on cognition, have been mixed (for a review, see Harper, 1998).

Alcohol & Neurotransmission

There is significant difficulty in identifying a successful treatment for AUD because alcohol has been demonstrated to exert widespread effects on the CNS, interacting with multiple neurotransmitter systems: dopamine (Diana et al., 1999), norepinephrine (Rossetti et al., 1992), acetylcholine (Arendt et al., 1988), serotonin

(Campbell, Kohl, & Mcbride, 1996; Lovinger 1999), glutamate (Samson & Harris, 1992; Fadda & Rossetti, 1998), γ-aminobutyric acid (GABA) (Littleton & Little, 1994), cannabinoid receptors (Blendov, Cravatt, Boehm, Walker, & Harris, 2007), and endogenous opioids (Herz, 1997, Pastor & Aragon, 2006) (for a review see, Myers, Adell, & Leonard, 1995; Nevo & Hamon, 1995; Chastain, 2006; Ward, Lallemand, & Witte, 2009).

Pre-clinical data has demonstrated that these ethanol-associated interactions may initiate downstream cascades producing robust CNS insult via disruption in intracellular calcium (Ca^{2+}) homeostasis (Daniell & Harris, 1989; Webb, Walker, & Heaton, 2003; Kouzoukas et al., 2013), moderation of L-type voltage-sensitive calcium channels (Hendricson et al., 2003), increased NMDAR calcium release and cytotoxicity (Prendergast and Mulholland et al., 2012), reduced phosphorylation of the transcription factor CREB (Yang et al., 1998), induction of cytokines (Crews et al., 2006), and disruption of NFκB and MAPK signaling pathways (for a review, see Suk, 2007; Zou & Crews, 2010), and an increase in reactive oxygen species (ROS) and nitric oxide (NO) in the central nervous system (Haorah et al., 2008). Furthermore, ethanol exposure has been shown to directly deplete the endogenous scavenger and antioxidant systems responsible for the regulation of these ROS/RNS (Guerri & Grisolio, 1980; Schlorff, Husain, & Somani, 1999). This imbalance can lead to oxidative stress as well as an increased microglial response (Qin & Crews, 2012), initiating a number of cascades resulting in mitochondrial dysfunction (Ramachandran et al., 2003; Luo, 2014), neuroinflammation (Crews et al., 2006; Qin & Crews, 2012; Marshall, Geil, & Nixon, 2016), and ultimately

the initiation of apoptosis pathways and cell death (Vosler, Brennan, & Chen, 2008; Collins & Neafsey, 2012; Qin & Crews, 2012).

Novel Treatments for Alcohol Use Disorder

While there are currently 3 FDA approved treatments for AUD: disulfram (Antabuse©), naltrexone (Revia©, Vivitrol©) and acomprosate (Campral©), these treatments have demonstrated poor clinical efficacy, with patients reporting 50 percent relapse rates at follow up (Johnson, 2008; Heinz et al., 2009). Moreover, it has been hypothesized that the poor performance of these treatments may be due to their exclusive targeting of the motivational aspects of drug taking and lack of protective effects against the neurological damage induced by alcohol consumption that may underlie the progression to worsening abusive behavior due to impairments at the executive centers of the brain (Koob & Le Moal, 1997). The lack of efficacy of current treatments, as well as evidence suggesting the increased role of neurological mechanisms in the development and worsening of AUD, has highlighted the need for novel pharmacological interventions that address not only the motivational aspects of drug taking, but the neurobiology as well.

Drug research and development. The development of novel drugs is a process that requires multidisciplinary cooperation across multiple scientific fields (e.g. chemists, biologists, psychopharmacologists, etc), and despite remaining a high-risk endeavor, secures an average of capitalized cost of US$802 million annually (DiMasi, Hanson, & Grabowski, 2003). Drug research and development (R&D) requires multiples steps from target screening, which may require the screening of thousands of compounds in the hope

of finding different classes of drugs that work within a desired model, to clinical trials to evaluate drug efficacy in human participants (Kelly, 2009). While many of these R&D projects are initiated, only a fraction succeeds through clinical trials. Futhermore, initiation of a project through drug sale may take an average of 15 years (Levy, 2000). Notably, once a compound is identified, it is called a "hit". A hit may go through to subsequent testing phases in the structural form it was identified in, however, it is also common for the structure of the compound to be modified for changes that are hypothesized to improve the compounds efficacy in the model even more, and then re-test the compound. As model systems do not perfectly predict therapeutic action in clinical trials settings it is important to generate numerous lead compounds, taking into account structural diversity amongst these compounds, before the investment into animal models and clinical trial phases are made within an R&D project (Kelly, 2009).

Natural products. Natural products have been exploited for their medicinal properties as far back as 2600 BCE years in ancient Mesopotamia (Borchardt, 2002). Currently, approximately 60% of drugs on the market are directly or indirectly derived from natural product sources (Newman, 2008). However, in the 1990's the medical field moved away from costly screening of natural-product compounds to high-throughput screening of combinatorial chemistry libraries. Since this shift, very few medications have made it through to the clinical trial phase, resulting in what has been deemed a "development pipeline"; some have blamed this on the lack of diversity and complexity amongst the synthetic libraries (Ji et al., 2009; Newman & Cragg, 2007). However, more recently, knowledge of natural chemical structures has allowed for the synthesis of these structures in the lab setting, relieving the need for constant isolation from natural sources. This has allowed for a reduction in the cost associated with natural-product compound

screening (for a review, see Harvey, 2008). Moreover, chemists have developed strategies to make analogues and derivatives of original compounds, creating new compounds with increased therapeutic potential over their original structures (Sunazuka et al., 2008). These new developments in chemistry have significantly reduced the cost of screening natural-product compound libraries. These developments have given researchers access to compounds that have greater diversity in structure, as well as unique chemical properties, which lend them to be more readily absorbed than synthetic drugs (for a review, see Harvey, 2008). The combination of these two factors has led to a resurgence of interest in the field for the use of natural-product compounds in drug discovery (Galm & Shen, 2007).

Streptomyces. Currently, there are over 100 natural-product-derived compounds currently undergoing clinical trials for a variety of therapeutic applications (e.g. anti-cancer, anti-infective) (for a review see Harvey, 2008). Additionally, most of these natural-product-compounds being tested are derived from plant or microbial sources (for a review, see Harvey, 2008). Increasing amounts of research have highlighted the benefits of compounds derived from extremophile microbes, or microbes found in extreme habitats, due to the unique properties possessed by these molecules which have been developed in order to survive such extreme conditions (for a review, see Wilson & Brimble, 2009). Microbes derived from the *Streptomyces* species, a gram-positive bacterial species predominantly found in soil or decaying vegetation (Chater, 1984), have been used as a source for antibiotics (i.e. streptomycin), antitumor medications (i.e. Blenoxane©) (for a review, see Cragg, Grothaus, & Newman, 2012), and one derivative has even been found to have anti-HIV properties (Kohno et al., 1996). More recently, a compound derived from an extremophile bacteria discovered in the Ruth Mullins coal

mine vents, *Streptomyces* sp. RM-5−8, has also been shown to attenuate ethanol-induced toxicity in the rodent hippocampus *in vitro* (Wang et al., 2015). Unfortunately, the compound identified does not present itself as a viable compound for medication development for AUD due to quantity restrictions of this compound preventing its scaling to larger experiments (personal communication, Dr. Jon Thorson). However, due to these findings, exploration of compounds with similar structures to this *Streptomyces* species may be of interest in AUD research.

Binge Ethanol Treatment

There has been increasing research interest in isolating the neurological mechanisms underlying ethanol withdrawal induced cytotoxicity. However, some neurodegeneration associated with ethanol exposure has not been found to be correlated with instances of withdrawal. Bleich et al. (2003) found that reduction in hippocampal size in alcoholics had no association with the number of withdrawal seizure occurrences, suggesting a mechanism of alcohol-induced neurodegeneration independent of withdrawal.

The binge ethanol exposure model utilizes short term ethanol administration to isolate potential neuromechanisms involved in ethanol induced cytotoxicity both *in vivo* and *in vitro* without the presentation of an ethanol withdrawal period. Additionally, this model has been shown *in vivo* to produce learning and memory difficulties consistent with observed cognitive deficits in clinical alcohol populations. Additionally, binge and continuous ethanol exposure studies have started to call into question the role of excitotoxicty from ethanol exposure (for a review, see Collins & Neafsey, 2012). This research is increasingly more startling as binge drinking among adolescents is steadily

increasing (Dwyer-Lindgren et al., 2015), and studies have shown adolescent brains to be more sensitive to the effects of binge ethanol exposure *in vivo* (Crews et al., 2000). Obernier, Bouldin, and Crews (2002) found that adult rats experienced necrosis-induced degeneration in their olfactory bulbs after only two days of binge ethanol exposure. Additionally, these deficits extended throughout the entire brain after four days of binge ethanol exposure. Notably, this degeneration was not found to increase following an ethanol-withdrawal period (Obernier, Bouldin & Crews, 2002). Moreover, *in vivo* findings have failed to show significant neuroprotective effects of NMDAR antagonists in binge ethanol models (Neafsey, Mostafa & Collins, 1989; Corso et al., 1998; for a review, see Collins & Neafsey, 2012). In contrast, the use of antioxidants in binge ethanol models have shown neuroprotective effects *in vivo* (Hamelink et al., 2005); these findings support the role of oxidative stress and mitochondrial pathways in ethanol induced neurodegeneration (Hamelink et al., 2005; Collins & Neafsey, 2012). However, Sunkesula, Swain and Babu (2008) did find an up-regulation of NMDAR1, 2B, and 2C subunits in the hippocampus following a 12 week continuous ethanol exposure period in adult rats. Notably, an up-regulation of apoptosis and its associated enzymes was also observed as measured by immunoblots and TUNEL staining, suggesting mitochondrial involvement (Sunkesula, Swain & Babu, 2008). Collectively, these findings suggest that the binge ethanol exposure model allows for the testing of novel pathways of ethanol toxicity and neurodegeneration which may support patterns of neurodegeneration seen in alcohol-dependent humans.

Organotypic Hippocampal Slice Culture.

The organotypic hippocampal slice culture (OHSC) technique allows for the reliable replication of neuronal over-activation in the hippocampus that can be translated to *in vivo* models (Gahwiler, et al., 1997; Noraberg et al., 2005; Reynolds et al., 2015). Moreover, the OHSC technique has been validated in the examination of ethanol neurodegeneration (Collins, Zou, & Neafsey, 1998; Bulter et al., 2013; Reynolds, Berry, Sharrett-Field, & Prendergast, 2015), excitotoxicity (Vornov, Tasker, & Coyle, 1991), neurotoxicity (Newell, Hsu, Papermaster, & Malouf, 1993; Butler et al., 2010), oxidative stress (Wilde et al., 1997), Alzheimer's disease (Kim et al., 2003), Parkinson's disease (Madsen et al., 2003), and stroke (Bonde, Noraberg, & Zimmer, 2002) in the hippocampus. Furthermore, this technique allows for the preservation of neuropil layers and is ideal for the employment of immunohistochemical staining (Noraberg et al., 2005), the quantification of compromised neurons (Noraberg, Kristensen, & Zimmer, 1999), and viewing of localized cell death (Noraberg, Kristensen, & Zimmer, 1999). Additionally, protein can be collected and quantified from OHSC via employment of western blot techniques (Butler et al., 2013).

CHAPTER 2: Method

Organotypic Hippocampal Slice Culture Preparation

Male and female Sprague-Dawley rat pups (i.e., 8 days old) (Harlan Laboratories; Indianapolis, IN) were humanely sacrificed. Whole brains were aseptically removed (after Mulholland et al., 2005) and immediately placed into culture dishes containing chilled dissecting medium composed of Minimum Essential Medium (MEM; Invitrogen, Carlsbad, CA), 25 mM HEPES (Sigma, St. Louis, MO), 21.20 µM Amphotericin B solution (Sigma), and 50 µM streptomycin/penicillin (Invitrogen). Bilateral hippocampi were removed and placed into culture dishes containing chilled culture medium composed of dissecting medium, double distilled water, 36 mM glucose (Fisher, Pittsburg, PA), 25% Hanks' Balanced Salt Solution (HBSS; Invitrogen), 25% (v/v) heat-inactivated horse serum (HIHS; Sigma), 0.05% Amphotericin B solution (Sigma), and 0.10% streptomycin/penicillin (Invitrogen). Excess tissue attached to hippocampi was carefully removed with the use of a stereoscopic microscope. Each hippocampus was then coronally sectioned at 200 µm thicknesses using a McIllwain Tissue Chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, UK) and transferred to a culture dish containing chilled culture medium. Hippocampal slices were selected for inclusion of all three hippocampal regions (CA1, CA3, and dentate gyrus) through examination under a stereoscopic microscope. Following selection, 3-4 slices were plated onto Millicell-CM 0.4 µM biopore membrane inserts sitting in 35-mm 6-well culture plates containing 1 mL of pre-incubated culture medium. This produced 18-24 intact hippocampal slices per 6-well plate. To allow for air exposure, all excess culture medium was carefully removed from the top of each culture well. To allow hippocampal slices to

adhere to the biopore membrane inserts, the tissue was stored in an incubator at 37°C with a gas composition of 5% CO2/95% air for 5 days before experiments were conducted. Care of all animals was carried out in agreement with the University of Kentucky's Institutional Animal Care and Use Committee.

Experiment I: Verification of High Through-Put Screen (HTS) Model

At 5 days in vitro (DIV), after the hippocampal slices have adhered to the biopore membrane, cultures will be transferred at random to 6-well culture plates containing 1mL of culture medium (control) and or 1 mL of culture medium and the addition of 100 mM ethanol (EtOH), a dose that represents 6 times the legal limit in humans. Preliminary studies in our lab have already determined 48 h as the time-point of peak fluorescence of propidium iodide for this model. As a means of validating that the cytotoxicity observed in the current model was due to EtOH exposure, a subset of control and EtOH cultures were also exposed to Trolox (100 µM), a vitamin E analog which has been consistently shown to protect against ethanol induced cytoxicity in binge EtOH models, and thus, is commonly used as a standard in EtOH models (Sripathirathan et al., 2009). In order to prevent diffusion of EtOH from the culture medium, all EtOH treated plates were placed into topless polypropylene containers which contained 50 mL of ddH_2O and 100 mM EtOH. Control groups received similar treatment without the addition of EtOH to the ddH_20. Containers were then placed in sealable 1-gallon freezer bags and filled with compressed gas (5% carbon dioxide/95% air) to mimic the incubator conditions and returned to the incubator for 48 h. Attempts were made to prevent evaporation of EtOH at every step of this procedure; however, prior work has shown that the final starting

concentration of EtOH in the wells may have been reduced by approximately 10% (Prendergast et al. 2004).

Experiment II: Testing of Novel Natural Product-Compounds

Novel compounds (~100), from the natural products repository, including those derived from Streptomyces sp. RM-5-8 (Wang et al., 2015), have been screened *in silico* for blood brain barrier permeability and been provided to our laboratory by the University of Kentucky Center for Pharmaceutical Research and Innovation (CPRI). At 5 days in vitro (DIV), after the hippocampal slices have adhered to the biopore membrane, cultures were transferred at random to 6-well culture plates containing 1mL of culture medium (control) or 1 mL of culture medium with the addition of 100 mM ethanol (EtOH). Additional cultures were also exposed to control or EtOH (100 mM) medium with the addition of a novel compound-product at multiple concentrations (0.01 µM-1 µM) to establish concentration-response relationships. In order to prevent diffusion of EtOH from the culture medium, all EtOH treated plates were placed into topless polypropylene containers containing 50 mL of ddH_2O with the addition of 100 mM EtOH. Control groups received similar treatment without the addition of EtOH to the ddH_20. Containers were then placed in sealable 1-gallon freezer bags and filled with compressed gas (5% carbon dioxide/95% air), to mimic the incubator conditions, and returned to the incubator for 48 h. Attempts were made to prevent evaporation of EtOH at every step of this procedure.

Propidium Iodide Staining

During all treatments, cultures also received the addition of propidium iodide (7.48 μM) for cytotoxic assessment. Propidium iodide (PI) allows for the quantification of compromised neurons as it can only enter cells with disrupted plasma membranes, reflecting the presence of necrotic or end-stage apoptotic cells (for a review, see Zimmer et al., 2000). Inside of the cell, PI binds with nucleic acids and produces a red fluorescence in the range of 515-560 nm when excited by light. The more compromised cells that are present, the more intense the fluorescence will be. Measurement of cytoxicity with PI has been well validated, with PI uptake being highly correlated with other markers of cellular viability *in vitro* (Wilkins et al., 2006). At 48 h, PI fluorescence was visualized with SPOT advanced version 4.0.2 software for Windows (W. Nuhsbahm Inc.; McHenry, IL, USA) using a 5x objective with a Leica DMIRB microscope (w. Nuhsbahm Inc.; McHenry, IL, USA) fitted for fluorescence detection (mercury-arc lamp) and connected to a computer via a SPOT 7.2 color mosaic camera (W. Nuhsburg).

Fixation of Cultures

At 7 DIV slices were immediately fixed following imaging by exposure to 1 mL of 10% buffered formalin solution on the top and bottom of each well for 30 minutes. Following this, slices were washed carefully using 1 mL 1 X phosphate buffered saline (PBS) on the top and bottom of each well. This was repeated once, with a plate change between each wash. Teflon inserts containing slices were then transferred to new plates with 1mL 1 X PBS on the bottom of each well and plates were wrapped in foil and placed at 4°C for storage.

Statistical Analysis

Each condition was replicated as many times as resources allowed. The intensity of the PI fluorescence was measured for the entire hippocampal slice with the use of Image J software (National Institutes of Health, Bethesda, MD). A background measurement was taken from the visual field surrounding each slice and subtracted from the whole slice measurement of each slice as a control for background brightness. Measurement of PI fluorescence from each replication was normalized to percentage of control using the following formula: (S -B)/C, where S is the intensity of fluorescence for a given slice, B is the background intensity for that slice, and C is the mean fluorescence for a particular control slices (after Mulholland et al., 2005).

Data generated through the above method was analyzed using a two-factor ANOVA which compared propidium iodide in different treatment groups on EtOH treatment x compound or drug concentration treatment in the hippocampus. When appropriate, post-hoc tests were conducted using Fisher's LSD. The level of significance was set at $p < 0.05$.

CHAPTER 3: Results

Experiment I: Verification of High Through-Put Screen (HTS) Model

Power Analysis. The minimum number of hippocampal slices required was determined by an *a priori* power analysis (Gpower; Faul & Erfelder, 1992). Based on preliminary studies in our lab which found a large effect size in this model (d = 1.33; Cohen, 1988), a total of 10 slices per group would be required in order to detect a significant difference between EtOH (100 mM) and control treatments at 48 h using an independent sample t-test with a power of .80 and $\alpha = 0.05$. These power analyses suggest that the current experiment has ample power for detecting a significant model.

HTS Model. Studies were conducted to examine the effects of 100 mM EtOH on PI-uptake after 48 h. While clinical and *in vitro* findings have occasionally found sex differences among the effects of cytotoxic insult in the hippocampus (Agartz et al., 1999; Li et al., 2005; Prendergast et al., 2000), preliminary studies in our lab have found no significant effect of sex in this model, therefore the sex of the slices were not accounted for in these analyses. Within the hippocampus, an independent sample t-test revealed there was a significant increase in cytotoxicity as measured by PI-uptake in the EtOH (100 mM) treatment condition (M= 142.72, SD= 35.67) when compared to the control condition (M= 100.00, SD= 28.31), $t(16)$= -2.83, p = 0.01.

Trolox. Studies were conducted to examine the effects of Trolox (100 µM), a vitamin E analog which has been consistently shown to protect against ethanol induced cytoxicity, on this 48 h EtOH model. For statistical analyses, a one-factor ANOVA was conducted. Within the hippocampus, a significant effect of treatment was detected

F(3=15.995, $p < 0.001$). Post hoc analyses reveled that within hippocampus, exposure to 100 mM EtOH for 48 h (*M*= 177.30, *SD*= 62.17) resulted in a significant increase in uptake of propidium iodide compared to controls (*M*= 100.00, *SD*= 14.77; $p < 0.001$). Additionally, this uptake was attenuated by co-exposure to 100 µM of the anti-oxidant Trolox (*M*= 81.35, *SD*= 9.83; $p < 0.001$). There were no significant difference between control cultures exposed to Trolox (100 µM) and Trolox-naïve control cultures ($p = 0.15$) (Figure 3.1). Representative images of hippocampal slices labeled with PI are shown in Figure 3.2

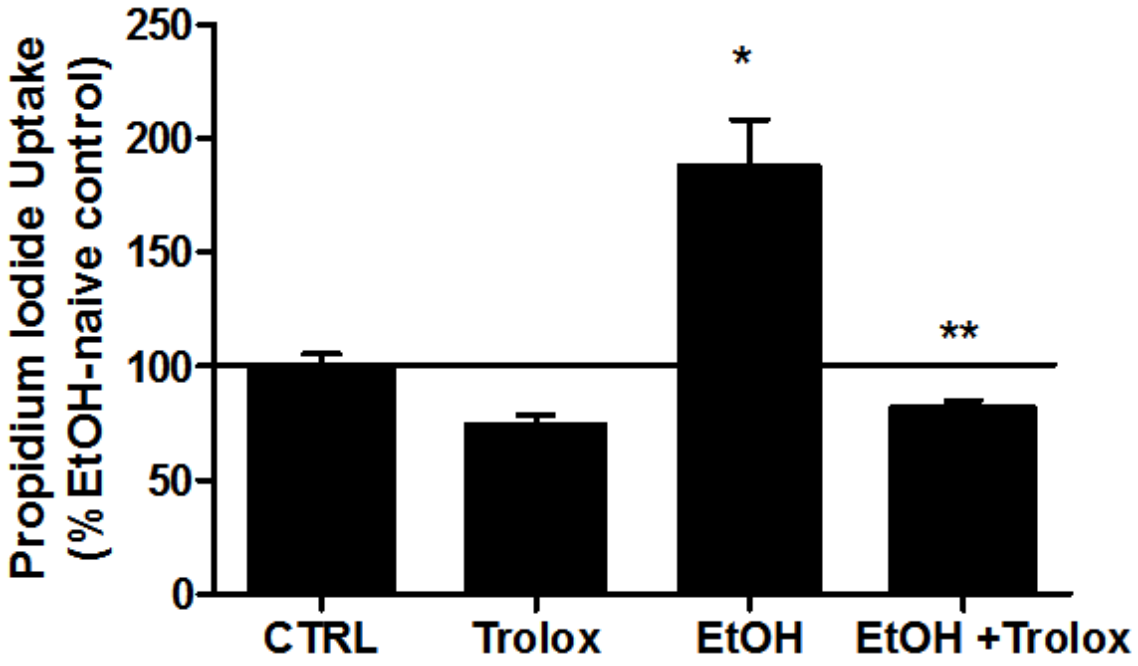

Figure 3.1. Effects of 48 h exposure to EtOH (100 mM) and/or Trolox (100 µM) on propidium iodide uptake. Trolox (100 µM) was found to have no significant effect in EtOH-naïve cultures at 48 h. Exposure to 100 mM EtOH for 48 h resulted in significant increases of propidium iodide uptake compared to control values within hippocampus. This increase was attenuated by the co-exposure to 100 µM of Trolox. * = $p < 0.001$ vs control; ** = $p < 0.001$ vs EtOH.

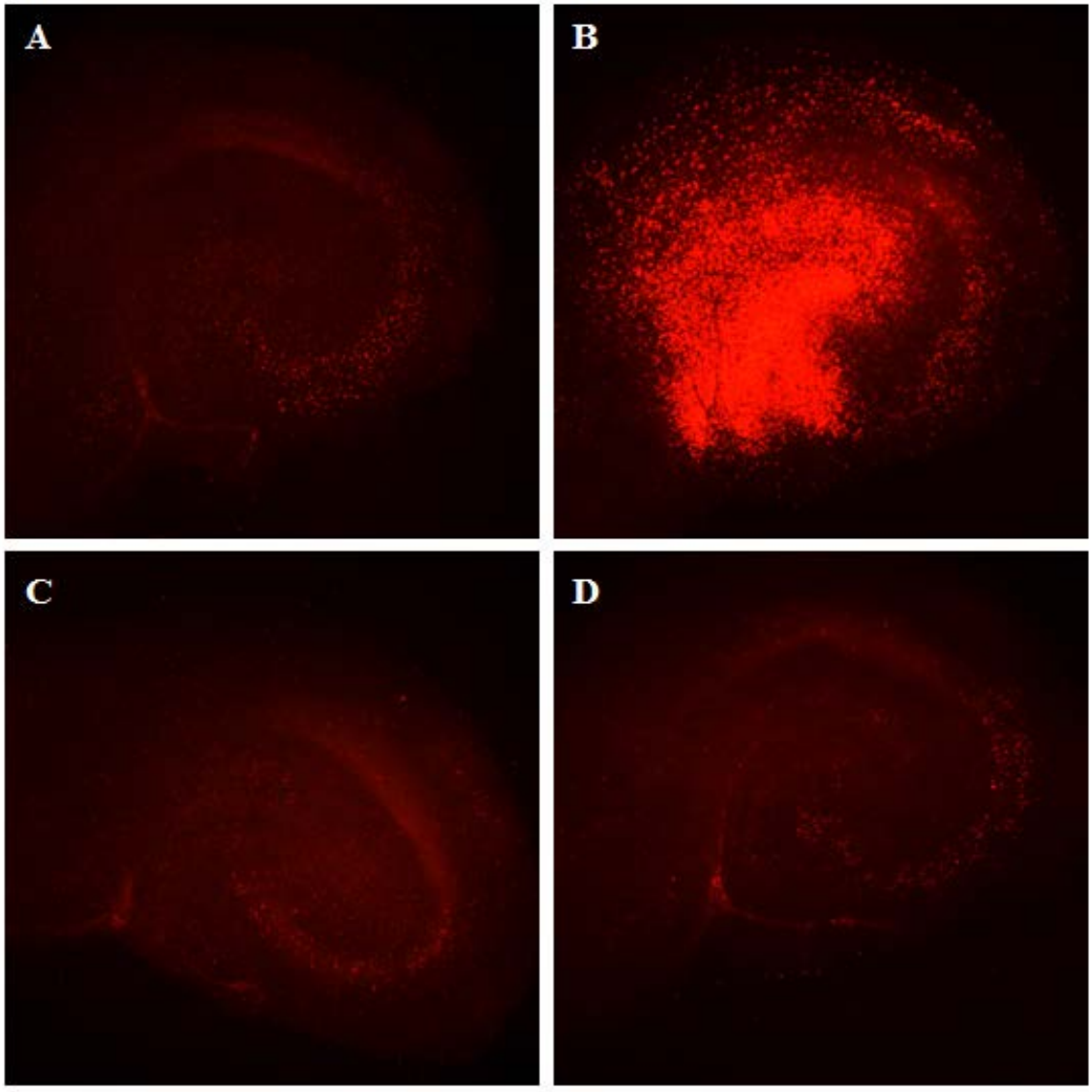

Figure 3.2. Representative images of the effects of 48 h exposure to EtOH (100 mM) and/or Trolox (100 µM) on propidium iodide uptake. (A) control; (B) 100 mM EtOH; (C) 100 µM Trolox control; (D) co-exposure of 100 mM EtOH and 100 µM Trolox.

Experiment II: Testing of Novel Natural Product-Compounds

Studies were conducted to examine the effects of novel compound-products on PI fluorescence after a 48 h co-exposure to EtOH (100 mM) and EtOH-naïve media. For statistical analyses, a one-way ANOVA was conducted to establish a significant model (EtOH [100 mM] vs EtOH- naïve tissue). Additionally, a two-factor ANOVA was conducted (EtOH treatment x compound concentration) to establish any concentration-response relationships in compound treated tissue. A significant effect of treatment between EtOH (100 mM) and EtOH-naïve treated tissue was observed with each replication for all experiments conducted in non-compound treated tissue ($p < 0.05$). In the current experiment, two natural compound-products were identified as significantly attenuating ethanol-induced cytotoxicity (compounds **1**, **1-1**). These compounds have were isolated from extremophile *Streptomyces* sp. RM-14−6 found in the Ruth Mullins coal fire in Perry County, KY, and have been classified as spoxazomicins due to their alkaloid structures resembling those of Spoxazomicins A-C, first identified by Inahashi and colleagues in 2011. A depiction of each compound's unique structure can be seen in Figure 3.3. For compound **1**, a significant effect of EtOH treatment was observed $F(1=16.15, p > 0.001)$. A significant effect of compound concentration was observed in hippocampal cultures exposed to compound **1** $F(3=5.46, p = 0.002)$. Additionally, within the hippocampus, a significant interaction between EtOH treatment x compound concentration was detected in cultures co-exposed to **1** and EtOH (100 mM) $F(3=8.23, p < 0.001)$. Post hoc analyses revealed co-exposure of **1** significantly attenuated EtOH-induced PI-uptake at 0.10 μM ($p < 0.001$) (Figure 3.4; Representative images Figures 3.5). Compound **1** was found to have no significant cytotoxic effects in ethanol-naïve cultures ($p > 0.05$). For compound **1-1**, no significant effect of EtOH treatment was

observed F(1=2.16, $p > 0.05$). Within the hippocampus, a significant effect of compound treatment was observed in cultures co-exposed to **1-1** F(3=3.00, $p = 0.04$). Additionally, within the hippocampus, a significant interaction between EtOH x compound treatment was detected in cultures co-exposed to **1-1** and EtOH (100 mM) F(3=6.21, $p < 0.001$). Post hoc analyses revealed exposure of EtOH-naive cultures to 0.01 μM and 0.10 μM **1-1** resulted in a significant increase of PI-uptake vs control ($p < 0.05$). Co-exposure of **1-1** attenuated EtOH-induced PI-uptake at 0.10 μM and 1.00 μM ($p < 0.05$) (Figure3.6; Representative images Figures 3.7).

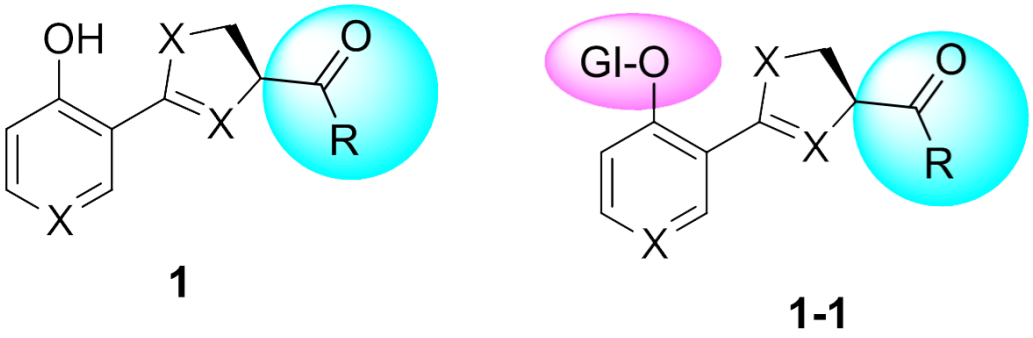

Figure 3.3. Chemical structures of novel compounds isolated from *Streptomyces* sp. RM-14−6.

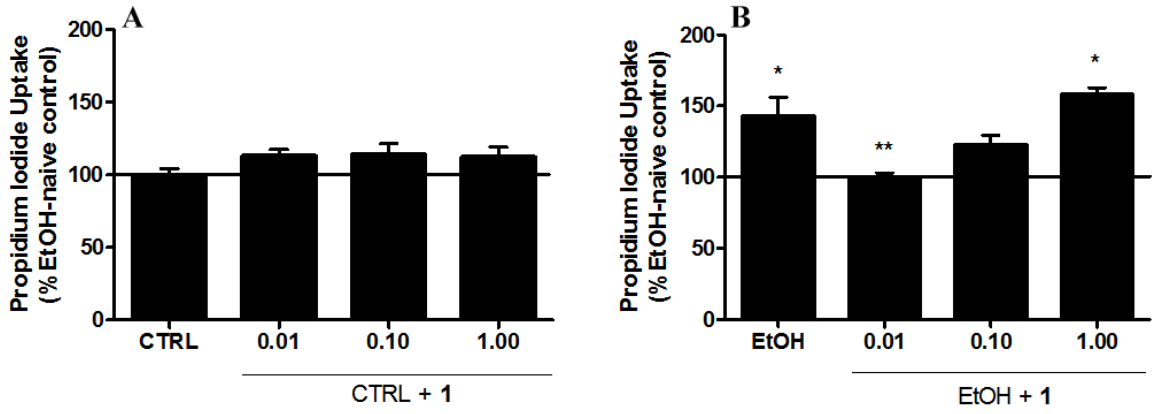

Figure 3.4. Effects compound **1** on propidium iodide (PI) uptake at 48 h in EtOH-naïve (A) and EtOH (100) mM treated (B) organotypic hippocampal slices cultures. Compound **1** demonstrated no cytotoxic effects in EtOH-naïve cultures at 48 h. Exposure to 100 mM EtOH for 48 h resulted in significant increases of PI uptake compared to control values within hippocampus. This increase was attenuated by the co-exposure to 0.01 µM of compound **1**. * = $p < 0.001$ vs control; ** = $p < 0.001$ vs EtOH.

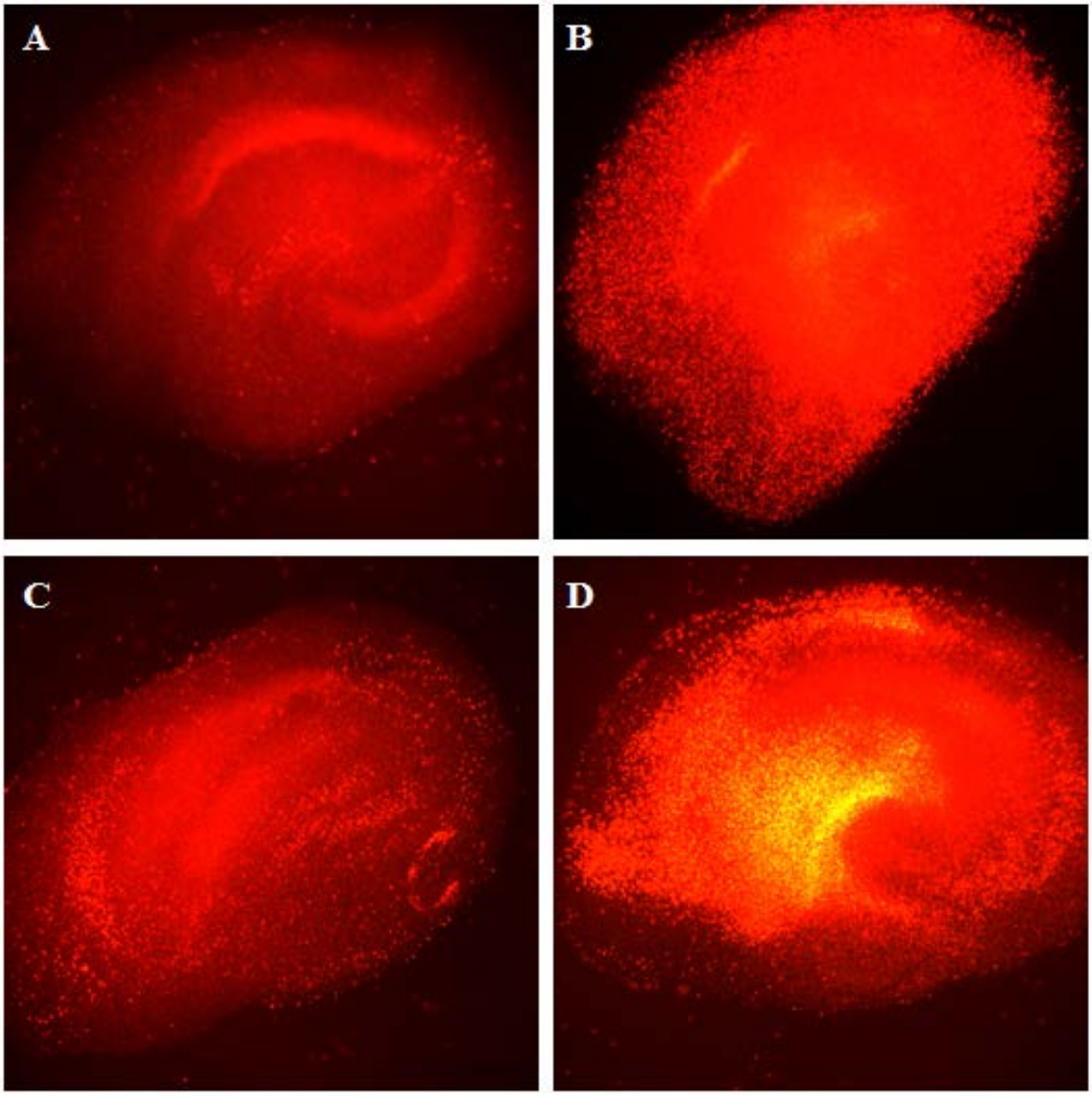

Figure 3.5. Representative images of the effects of 48 h exposure to EtOH (100 mM) and/or compound **1** on propidium iodide uptake. (A) control; (B) 100 mM EtOH; (C) co-exposure of 100 mM EtOH and 0.01 µM **1**; (D) co-exposure of 100 mM EtOH and 1 µM **1**.

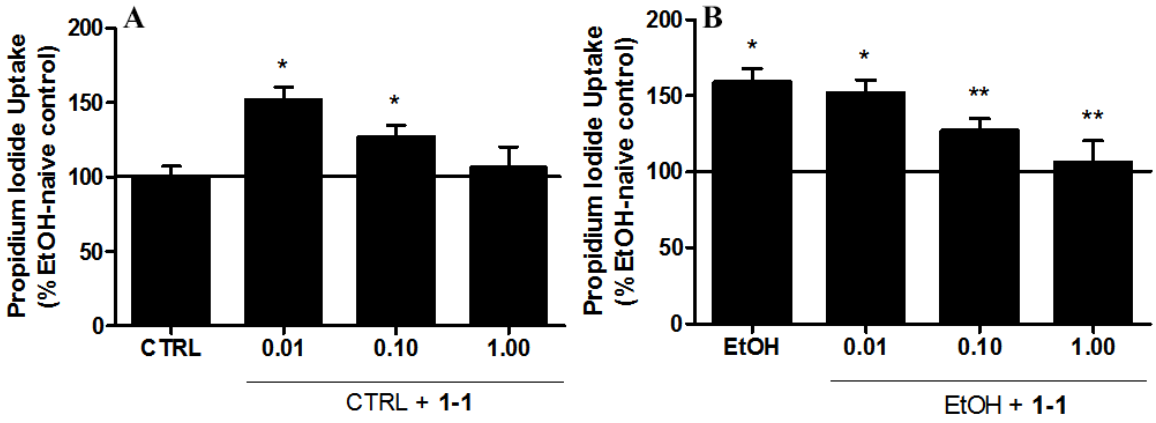

Figure 3.6. Effects compound **1-1** on propidium iodide (PI) uptake at 48 h in EtOH-naïve (A) and EtOH (100) mM treated (B) organotypic hippocampal slices cultures. In EtOH-naïve cultures, 0.01 µM and 0.10µM of **1-1** resulted in significant increases in PI-uptake at 48 h. Exposure to 100 mM EtOH for 48 h resulted in significant increases of PI uptake compared to control values within hippocampus. This increase was attenuated by the co-exposure to 0.10 and 0.01 µM of compound **1-1**. * = $p < 0.001$ vs control; ** = $p < 0.05$ vs EtOH.

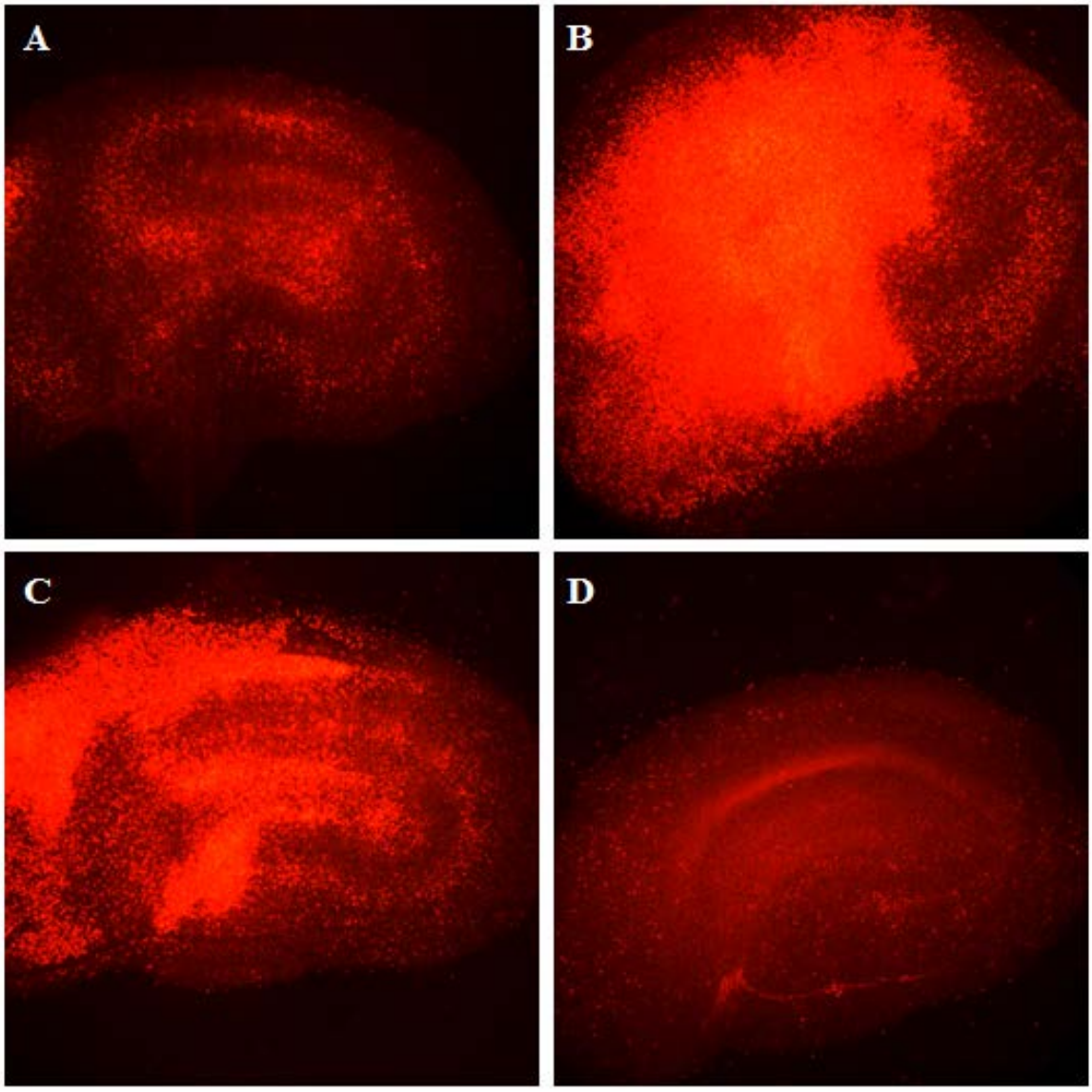

Figure 3.7. Representative images of the effects of 48 h exposure to EtOH (100 mM) and/or compound **1-1** on propidium iodide uptake. (A) control; (B) 100 mM EtOH; (C) co-exposure of 100 mM EtOH and 0.10 µM **1-1**; (D) co-exposure of 100 mM EtOH and 1 µM **1-1**.

CHAPTER 4: Discussion

Both clinical and pre-clinical findings have demonstrated that ethanol consumption is associated with neurobiological deficits, and it has been hypothesized that the development of these deficits may serve to drive the development of AUD or worsen its existing condition. While the exact mechanisms which leads to these neurological deficits has not yet been elucidated, Mello and Mendelson (1972) have demonstrated that alcoholics exhibit a pattern of alcohol consumption which promotes repeated withdrawals, and withdrawal from alcohol has been shown to have cytotoxic effects *in vivo* and *in vitro* due to effects on calcium dysregulation and HPA axis dysfunction (for a review, see Prendergast and Mulholland, 2012). Additionally, chronic exposure may also contribute to these deficits via oxidative stress, neuroinflammation, and downstream cascades associated with mitochondrial dysfunctions (Ramachandran et al., 2003; Crews et al., 2006; Luo, 2014). While treatments are currently available for AUD, they do not address the issue of neurological dysfunction, which may serve to explain their poor efficacy in the clinical setting. Additionally, while *in vivo* and *in vitro* studies have been conducted and confirmed the relationship between ethanol consumption and neurological damage (Pfefferbaum & Sullivan, 2005; Crews et al., 2006; Qin & Crew, 2012; Prendergast & Mulholland, 2012), none have been able to propose a treatment option.

The hypothesis for the current studies was that ethanol would induce significant neurological cytotoxity during a 48 h binge period. Additionally, multiple novel natural product compounds were screened with the hope that a novel pharmacotherapy may be identified which would attenuate this increased cytotoxity in hippocampal slices. The results of the current study are discussed below.

Experiment I: Verification of High Through-Put Screen (HTS) Model

The current study sought to develop a HTS rodent organotypic hippocampal slice culture model in order to test novel natural compound-products for their ability to attenuate ethanol-induced cytotoxity. Methods were adapted from Mulholland et al. (2005). In this study a 100 mM treatment of ethanol, a concentration approximating 460 mg/dl, or nearly six times the legal limit for driving, produced a mean 45% increase in ethanol induced cytotoxity, as measured by propidium iodide (PI) uptake, in the hippocampus. While the current 100 mM ethanol concentration, correlating to a BAC of ~400 mg/dl, used was high, it is still clinically relevant. A case study of 117 alcoholic patients has found BACs ranging from 29 to 577 mg/dl upon hospital admittance, with many of the patients still walking and cognitive with BACs over 300 mg/dl (Adachi et al., 1991). While the results of the current model differ with the more chronic model findings of Mulholland et al. (2005), which found no effect of EtOH (50 mM) on PI-uptake at 10 days *in vitro*, these results were still consistent with our hypothesis for the current model. Past unpublished data from our lab had led us to believe that the prior published model could be abbreviated with adjustments to the water bath, increasing the EtOH concentration in the bath, therefore reducing diffusion of EtOH from the media in the slice culture wells. This adjustment may allow for concentrations of ethanol within the media to remain more consistent over the experimental timeline (Prendergast et al., 2004). While these findings may imply that ethanol does have effects on its own that have not been seen before in past organotypic hippocampal slice culture studies where only propidium iodide was used as a marker, these findings are consistent with findings of Obernier, Bouldin, and Crews (2002), where significant neurodegeneration was demonstrated after a 2-day binge *in vivo*. Additionally, findings that the ethanol toxicity

within the current model was completely attenuated by the addition of 100 µM Trolox, a potent antioxidant commonly used as a standard of comparison in ethanol models (Sripathirathan et al., 2009), suggest the current model may be inducing generation of ROS, and thus dysfunction of downstream cascades via oxidative stress and/or neuroinflammation (Radesäter et al., 2003; Hamelink et al., 2005; Crews et al., 2006; Sunkesula, Swain & Babu, 2008; Collins & Neafsey, 2012). Studies to confirm ethanol's exact mechanism of action within this novel model are still needed.

Experiment II: Testing of Novel Product-Compounds

The present studies identified 2 natural product-compounds (**1**, **1-1**) that significantly attenuated ethanol (100 mM) induced toxicity as measured by propidium iodide uptake at 48 h. Additionally, both of these compounds were within the same product family, spoxazomicin. The only currently identified spoxazomicins, Spoxazomicins A-C, have been found to have anti-parasitic properties (Inahashi et al., 2011), although efforts for exploration of additional therapeutic targets remains unclear. Notably, compound **1-1** only demonstrated cytoprotective effects at the 1µM concentration, and also demonstrated cytoxicity in control cultures at 0.01 µM and 0.10 µM concentration. Concentration-response curves of this pattern suggest that this compound may only exert its attenuation effects due to multiple mechanisms of action, or other-wise stated non-specific effects. Clinically, this would translate to large doses of the drug being required for treatment as well as a higher likely-hood of adverse side effects. Due to this, compound **1-1** does not present itself as a lead compound, despite its significant attenuation of ethanol induced-cytotoxicity. In R&D research the average hit

rate is around 2% (Kelly, 2009), thus, the identification of 2 lead compounds (**1, 1-1**) in the current study is a high hit rate.

Limitations & Future Directions

While the basic methodologies within the current studies were modeled after the methods used by Mulholland et al. (2005), they were not identical. In attempts to reduce the experimental treatment time from 10 days to 48 h, the water bath surrounding the culture plates during the incubation period was doubled from the 50 mM EtOH concentration used in the past study to 100 mM. The determination of this concentration was not done based on any previous research or methods but rather on an experimental guess. It is not yet known what effects (if any) this change in method may have had on the present results. However, this information would be useful before the current 48 h method is used in any future research.

Additionally, within R&D research, it is standard to identify a target before proceeding into lead optimization phase, here the target is simply cell death- quite crude, more specific targets within the model (such as calcium dysregulation, oxidative stress, etc) may need to be explored/isolated to gain better knowledge when a hit is obtained. Additionally, while PI has been correlated with other markers of neuron and glia in slice culture (Wilkins et al., 2006); analysis of this marker is time dependent, giving the opportunity for user error. While more time consuming, employment of more specific and reliable markers, such as the neuron-specific marker NeuN (Kim, Adelstein, & Kawamoto, 2009), may help elucidate mechanism, as well as serve to better verify

findings of cell death, before advancing a compound into the more costly lead optimization phase.

Additionally, it has been discussed in prior reviews that HTS may have disadvantages in successfully advancing compounds through clinical trials due to the lack of structural diversity identified in these screens (Kelly, 2009). When a hit is identified in a HTS, effort is shifted to examining compounds within the same class to find better leads, ultimately resulting in simpler model classification (Lam, 2001). When advancing into the clinical trial phase of R&D, most new leads have been discovered through random screening, where all compounds in a compound library of interest are tested for a specific biological activity, and the active compounds are then selected for more advanced model testing (Lam, 2001). This approach ultimately results in a greater diversity of hits, and reduces the risk of investing time into development of a compound where activity in the model system may not translate to the clinical setting. While the current study has developed leads into multiple compounds, they are all structurally similar and in the same compound family. Future studies, while still utilizing the currently developed HTS model, may benefit from a more randomly guided approach.

Conclusions

The HTS organotypic hippocampal slice model discussed here presents a novel method by which new treatments aimed at AUD may be screened. Additionally, significant attenuation of ethanol-induced cytotoxicity withinin this model by the anitioxidant Trolox may suggest ethanol exerts its degenerative effects in binge models via oxidative stress. However, further studies, employing additional markers are needed

to confirm ethanol's effects within the current HTS model and the specific pathways that may be involved in order to further drive lead identification studies.

Furthermore, novel natural products derived from *Streptomyces* sp. RM-14-6 were found to protect against ethanol-induced cytotoxicity *in vitro* in the current 48 h HTS. The specific biochemical effects of these scaffolds have yet to be characterized; however, the previous Trolox findings may support the potential anti-oxidant properties of these compounds. However, it should be noted that distinct patterns of cytoprotection among the scaffolds, despite their similar chemical structures, suggest the likely presence of other mechanisms which remain to be elucidated. Future research should be conducted to characterize the mechanism by which the 2 lead compounds identified here (**1, 1-1**) exert their cytoprotective effects in order to further explore the potential of these two novel natural-compound products as potential treatment options in reducing injury caused by ethanol and similar toxins.

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