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DISCOVERY OF NATURAL PRODUCT ANALOGS AGAINST ETHANOL-INDUCED CYTOTOXICITY IN HIPPOCAMPAL SLICE CULTURES

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Arts and Sciences at the University of Kentucky

By

Meredith Amelia Saunders-Mattingly

Lexington, Kentucky

Director: Dr. Mark A. Prendergast, Professor of Psychology

Lexington, Kentucky

2018

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ABSTRACT OF DISSERTATION

DISCOVERY OF NATURAL PRODUCT ANALOGS AGAINST ETHANOL-INDUCED CYTOTOXICITY IN HIPPOCAMPAL SLICE CULTURES

An estimated 13.9% of Americans currently meet criteria for an alcohol (ethanol; EtOH) use disorder (AUD). While there are 4 medications approved by the Food and Drug Administration (FDA) to treat AUD, these treatments have demonstrated poor clinical efficacy. Our ongoing research program encompasses a multi-tiered screening of a natural product library and validation process to provide novel information about the mechanisms underlying EtOH-induced changes in neurobiology and to identify novel chemical scaffolds to be exploited in the development of pharmacological treatments for AUD in a rodent organotypic hippocampal slice culture model. Initial screens of several natural product compounds identified 3 compounds which attenuate 48 h EtOH-induced cytotoxicity in vitro. As analogs of natural products can be developed to have enhanced therapeutic potential over parental structures, Study 1 sought to extend on prior findings via the screening of several natural product analogs for their ability to attenuate EtOH-induced cytoxicity. Nine natural produce analogs demonstrated potent cytoprotective effects against EtOH-induced toxicity at 48 h. Several reports suggest EtOH-induced neurotoxicity may be secondary to the induction of persistent neuroimmune activation, and isoflavonoids have been shown to have effects on neuroimmune signaling. Thus, Study 2 compared the effects of compound 9b, an isoflavonoid analog identified in Study 1, to daidzein (DZ), a prototypical isoflavonoid, in the same 48 h model, with the addition of a neuroimmune component. Specifically, culture media was collected to assess for the release of the neuroimmune mediators HMGB1, TNF-α, IL-6, and IL-10 via ELISA. Compound 9b and DZ protected against EtOH-induced cytotoxicity at 48 h. EtOH exposure significantly increased secretion of HMGB1 and IL-6 into culture media at 48h. Compound 9b and DZ attenuated these increases at all concentrations tested. These results suggest potential neuroimmune modulating properties of isoflavonoids which may contribute to their neuroprotective effects against EtOH in vitro. These findings highlight the potential applications DZ and the novel isoflavonoid analog 9b for use in the treatment of AUD.

KEYWORDS: Alcohol Use Disorder, Novel Compound Screening, Isoflavonoid, Neuroimmune Signaling, Neurodegeneration

Meredith A. Saunders-Mattingly

7/03/2018

Date

DISCOVERY OF NATURAL PRODUCT ANALOGS AGAINST ETHANOL-INDUCED CYTOTOXICITY IN HIPPOCAMPAL SLICE CULTURES

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CHAPTER ONE: Introduction to Alcohol Use Disorder

1.1. Background and Significance

1.1.1. Prevalence and Definition

An estimated 13.9% of American adults currently meet Diagnostic and Statistical Manual of Mental Disorders-Fifth Edition (*DSM-5)* criteria for an alcohol use disorder (AUD) (Grant et al, 2015). This statistic is particularly worrisome as it indicates a 49.4% increase in current AUD status since 2001-2002 (2001-2002 and 2012-2013 corresponding *DSM-IV* rates 8.5% and 12.7%, respectively) (Grant et al., 2017). AUD is defined by *DSM-5* as a "maladaptive pattern of alcohol use, leading to clinically significant impairment or distress". Specifically, AUD is defined by 11 diagnostic criteria including: drinking in a higher volume or longer than intended, spending significant time drinking or recovering after a drinking episode, preoccupation with thoughts of alcohol use, inability to reduce alcohol use, neglect or reduction in important activities as a result of use, or continuation of use, despite physical, psychological, or family problems induced or exacerbated due to alcohol, craving, tolerance, and withdrawal (American Psychiatric Association [*DSM-5*], 2013). While these criteria are similar to the previous *DSM-IV* criteria, the *DSM-5* eliminates use of separate categories for alcohol abuse and dependence diagnoses, and instead allows for a spectrum of severity within the AUD diagnosis: mild (i.e., the presence of 2-3 symptoms) moderate (i.e., the presence of 4-5 symptoms), and severe (i.e., the presence of 6 or more symptoms) (American Psychiatric Association [*DSM-5*], 2013).

Increases in *DSM-IV* AUD during the past decade may partially reflect a 29.9% increase in high risk, or heavy, alcohol consumption during the same period (from 9.7%

to 12.6%) (Grant et al., 2017). Specifically, according to the 2014 National Survey on Drug Use and Health (NSDUH), approximately 16 million Americans can currently be characterized as heavy drinkers, 1.3 million of which are adolescents (Center for Behavioral Health Statistics and Quality, 2015). In this particular study, heavy drinking was defined as having at least 5 binge drinking episodes in the past month (with a "binge" being further defined as 5 or more drinks/occasion) (Center for Behavioral Health Statistics and Quality, 2015). Notably, this does not reflect the National Institute on Alcohol Abuse and Alcoholism's (NIAAA) revised definition of a binge drinking, which is simply any point at which a person's blood alcohol concentration (BAC) exceeds 0.08% (the legal limit in the United States) during a drinking session, regardless of alcohol units consumed (National Institute on Alcohol Abuse and Alcoholism, 2004). Definitions aside, this increasing trend in heavy drinking is concerning, as binge drinking frequency has been correlated with an increased risk of AUD (Dawson & Archer, 1993). This risk is ever higher if alcohol drinking is initiated during adolescence (Grant & Dawson, 1997; Nixon & Mclain, 2010; Crews, Vetreno, Broadwater, & Robinson, 2016). Excessive alcohol use (including binge, underage, and pregnant alcohol consumption) also contributes to a significant economic burden, costing the United States approximately $249 billion in 2010 (Sacks, Gonzales, Bouchery, Tomedi, & Brewer, 2015). This figure includes costs due to health care claims, criminal activity, property damage, fetal alcohol syndrome, loss of productivity, and mortality.

Notably, among persons 12 years of age and older needing treatment for AUD, there was a self-indicated desire for effective pharmacotherapy-based treatment options in 2013 (Substance Abuse and Mental Health Services Administration (SAMHSA, 2014).

Unfortunately, while there are currently 4 medications approved by the US Food and Drug Administration (FDA) to treat AUD: disulfiram (Antabuse©), naltrexone (Revia©, Vivitrol©), and acamprosate (Campral©), these treatments have proven inadequate (50-80% reporting a relapse to alcohol use within 1 year) (Johnson, 2008; Moos and Moos, 2006; Heinz et al., 2009). The lack of efficacy of these pharmacotherapies may be because they work at preventing future alcohol consumption by lowering its value to the user (for example, taking disulfiram will make someone feel sick who consumes alcohol), and not by targeting the underlying neurodegeneration (Koob & Le Moal, 1997; Van Skike et al., 2016). Thus, the development of novel pharmaceutical interventions is still needed to address this gap in care.

1.1.2. Alcohol and Neurodegeneration

Prolonged, excessive alcohol consumption can ultimately lead to significant brain damage. Neuropathological analyses of post-mortem alcoholic brains have revealed trends of localized atrophy, showing decreased white matter volume in the cerebral hemispheres (Harper, Kril, & Holloway, 1985), decreased cerebellar volume (Sullivan, Mathalon, Lim, Marsh, & Pfefferbaum, 1998), increased ventricle size (Harper, Kril, & Holloway, 1985), demyelination of the mammillary bodies (Alling & Bostrom, 1980), and thinning of the corpus callosum (Harper & Kril, 1985). In kind, studies conducted using magnetic resonance imaging (MRI), functional magnetic resonance imaging (fMRI), or voxel-based morphometry (VBM) have also revealed abnormalities in brain morphology of alcoholics, including decreased cortical gray matter (Jernigan, Schafer, Butters, & Cermak, 1991; Fein et al., 2002; Mann et al., 2001; Mechtcheriakov et al.,

2006) and white matter (Pfefferbaum et al., 1992; Mechtcheriakov et al., 2006); decreased frontal lobe volume (Pfefferbaum, Sullivan, Mathalon, & Lim, 1997); reduction in hippocampal size (Sullivan, Marsh, Mathalon, Lim, & Pfefferbaum, 1995; Agartz, Momenan, Rawlings, Kerich, & Hommer, 1999; Bleich et al., 2003; Beresford et al., 2006; Mechtcheriakov et al., 2006; Wilson, Bair, Thomas, & Iacono, 2017); and cerebellar shrinkage (Sullivan, Rosenbloom, Serventi, Deshmukh, & Pfefferbaum, 2003; Mechtcheriakov et al., 2006). However, while the above studies have focused on the adult brain, it is worth noting that the adolescent brain may be more vulnerable to alcohol's effects, as research has shown that several brain areas (e.g. prefrontal cortex, nucleus accumbens, hippocampus) are still developing and undergoing substantial changes during adolescence (Lenroot & Giedd, 2006; Bava & Tapert, 2010; Nixon & McClain, 2010; Arain et al., 2013). Nagel, Schweinsburg, Phan and Tapert (2005) found that adolescents with AUD have smaller left hippocampal volumes than non-drinking controls. However, other MRI studies suggest that adolescent-onset AUD may be required for the hippocampal volume loss observed in adulthood (De Bellis et al. 2000; Nagel et al., 2005; Ozsoy, Durak, & Esel, 2013). Furthermore, while there has been difficulty in delineating the exact association between alcohol and reduced brain volumes, a recent study by Wilson and colleagues (2018) used adolescent female twin-pairs (ages 11-24) to reveal the greater alcohol-using twin had smaller hippocampal volumes than their co-twin, suggesting a direct alcohol-exposure related effect on reductions in hippocampal volume. In addition to these hippocampal effects, MRI studies also show adolescent binge drinking may be predictive of cerebellar, thalamus, putamen, and brain stem volume loss (Pfefferbaum et al., 1992; Fein et al., 2013; Lisdahl, Thayer,

Squeglia, McQueeny, & Tapert, 2013). Notably, there have been sex and age differences reported across both adult and adolescent studies, but these patterns have not been consistent (for a review, see Sullivan & Pfefferbaum, 2005; Ewing, Sakhardande, & Blakemore, 2014).

Ultimately, chronic alcohol use results in cognitive deficits, with up to 85% of alcoholics exhibiting signs of cognitive decline (Parsons & Nixon, 1993). Notably, associations between the performance on neuropsychological tests (e.g., 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, have been shown (Sullivan, Rosenbloom, & Pfefferbaum, 2000; Chanraud, et al., 2007; Ozoy et al., 2013). Cardinal behaviors of AUD, such as impaired judgment, blunted affect, reduced motivation, and distractibility, have been attributed to frontal lobe deficits (for a review, see Sullivan & Pfefferbaun, 2005). Cerebellar volume deficits have been shown to be predictive of executive visuospatial and balance impairments (Sullivan et al., 2000; Sullivan et al., 2003). Additionally, 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). Studies utilizing single photon emission computed tomography (SPECT) have shown less regional cerebral blood flow (rCBF) in the frontal lobes of alcoholics when compared to non-alcoholics (Nicolas et al., 1993); furthermore, this has been associated with lack of inhibition and deficits in short term memory (Noël et al, 2001). In adults, the duration and amount of alcohol consumed is associated with the severity of neurobehavioral deficits (Parsons & Leber, 1981; Basso & Bornstein, 2000; Dwivedi, Chatterjee, & Singh, 2017).

Secondary disease states related to alcohol consumption, such as Korsakoff's syndrome, Wernicke's encephalopathy, and fetal alcohol spectrum disorder (FASD), can also contribute to alcohol's neurobehavioral effects (for a review, see de la Monte & Kril, 2014).

1.1.3. Mechanisms of Alcohol-Induced Neurotoxicity

There is significant difficulty in developing a successful treatment for AUD, as alcohol (referred interchangeably as "ethanol" [EtOH] in preclinical studies) does not act at one specific target, but rather exerts widespread effects on the CNS, interacting with multiple neurotransmitter systems, ion channels, and signaling pathways (for a full review see, Nevo & Hamon, 2004; Chastain, 2006; Ward, Lallemand, & Witte, 2009). While the exact neurochemical processes leading to EtOH's neurodegenerative effects have remained elusive, extensive research has demonstrated a role for oxidative stress (Ramachandrin et al., 2003; Hamelink, Hampson, Wink, Eiden, & Eskay, 2005; Collins & Neafsey, 2012), pro-inflammatory neuroimmune signaling (Blanco, Valles, Pascual, & Guerri, 2005; Fernandez-Lizarbe, Pascual, and Guerri, 2009; Alfonso-Loeches, Pascual-Lucas, Blanco, Sanchez-Vera, & Guerri, 2010; Pascual, Baliño, Alfonso-Loeches, Aragón, & Guerri, 2011; Crews et al., 2015), and excitotoxicity (Lovinger, 1993; Davis & Wu, 2001; Nagy & Laszlo, 2002; Prendergast et al., 2004) in the promotion of EtOH-induced neurotoxicity during different phases of dependence. Specifically, oxidative stress and pro-inflammatory neuroimmune signaling have been shown to contribute to neurotoxicity observed during periods of EtOH exposure (Ramachandrin et al., 2003; Hamelink et al., 2005; Collins & Neafsey, 2012; Crews et al., 2015). In contrast,

excitotoxicity is associated with the increased neurotoxicity observed during EtOH-withdrawal (EWD) (Davidson, Shanley, & Wilce, 1995; Harris et al., 2003; Prendergast et al., 2004). However, overlap between these mechanisms does exists (for a review, see Fadda & Rossetti, 1998; Crews et al., 2015), suggesting there are likely several common targets which could be exploited for drug development. The below sections will briefly discuss these mechanisms of EtOH-induced neurotoxicity, as well as discuss possible interactions.

1.1.3.1. Oxidative Stress. Oxidative stress-induced mitochondrial damage is implicated in the development of several neurodegenerative diseases (Barnham, Masters, & Bush, 2004; Lin & Beal, 2006), and occurs when the generation of reactive oxygen species (ROS) exceeds the endogenous antioxidant control mechanisms (Battino, Bullon, Wilson, & Newman, 1999). EtOH can potentiate ROS levels through multiple routes, including oxidation in the brain via metabolism in the brain by CYP 2E1 (Zimatkin, Pronko, Vasiliou, Gonzalez, & Deitrich, 2006); induction of NADPH/xanthine oxidase (NOX/XOX) and nitric oxide synthase (NOS) in neurons and microglia (Haorah, Schall, Ramirez, & Persidsky, 2008; Qin & Crews, 2012); and depletion of the endogenous scavenger and antioxidant systems responsible for the regulation of these ROS (Guerri & Grisolio, 1980; Schlorff, Husain, & Somani, 1999). Chronic EtOH exposure has been shown to lead to increases in ROS and reactive nitrogen species (RNS), inducing oxidative stress (Nordmann, Ribière, & Rouach, 1992; Haorah, Schall, Ramirez, & Persidsky, 2008). In addition to oxidative stress, this imbalance can initiate a number of cascades, including mitochondrial dysfunction. Ramachandrin and colleagues (2003)

found that EtOH-induced oxidative stress directly initiated mitochondrial apoptosis pathways in cultured fetal cortical rat neurons. Obernier, Bouldin, and Crews (2002) found that adult rats experienced neurodegeneration in their olfactory bulbs after only 2 days of binge EtOH exposure, and these deficits extended throughout additional brain regions, including the hippocampus, after 4 days of binge EtOH exposure. Furthermore, the neurodegeneration was not observed to be exacerbated by an EWD period (Obernier et al., 2002), suggesting a mechanism of neurotoxicity independent of excitotoxicity. Additional studies have demonstrated that the potent antioxidants, butylated hydroxytoluene (BHT) and α-tocopherol, can block binge EtOH-induced neurodegeneration in the rat hippocampus and entorhinal cortex *in vivo* (Hamelink et al., 2005). In kind, our lab has found that the potent antioxidant, Trolox, blocks cytotoxicity produced by a 48 h binge-like EtOH exposure in OHSC (Saunders, 2016). These findings support the role of oxidative stress and mitochondrial pathways in EtOH-induced neurotoxicity.

1.1.3.2. Neuroimmune Signaling. While glial activation (both microglia and astrocytes) plays an important neuroprotective role in response to harmful stimuli (Cherry, Olschowka, & O'Banion, 2014), sustained neuroimmune responses contribute to the progression of neurodegenerative disease (Giovannini et al., 2003; Hunot & Hirsche, 2003; Frank-Cannon, Alto, McAlpine, & Tansey, 2009), brain injury (Schmidt, Infanger, Heyde, Ertel, & Stahel, 2004; Cherry, Olschowka, & O'Banion, 2014), and psychiatric disorders (Lucas, Rothwell, & Gibson, 2006). Microglia are the primary mediators of the brain's innate immune systems (Rivest, 2009), and are commonly categorized as

classic/proinflammatory (M1) or alternate/anti-inflammatory (M2) based on their observable morphology (Colton, 2009; Michelucci, Heurtaux, Grandbarbe, Morga, & Heuschling, 2009). Notably, these classifications are considered somewhat oversimplified, as intermediate phenotypes do exist (Hu et al., 2015; Jha, Lee, & Suk, 2016; Orihuela, McPherson, & Harry, 2016; Ransohoff, 2016). Post-mortem alcoholic brains show increased microglial activation and astrogliosis (He & Crews, 2008). Increased markers for the pro-inflammatory transcription factor nuclear factor kappa-light-chain-enhancers of activated B cells (NF-κB) (Liu & Malik, 2006; Ökvist et al., 2007) and upregulated neuroimmune gene expression have also been observed (He & Crews, 2008).

In pre-clinical studies, activation of a second pro-inflammatory transcription factor, activator protein 1 (AP-1), has also been observed in response to EtOH (Fried, Kotarsky, & Alling, 2001; Blanco, et al., 2005). Both NF-κB and AP-1 are known to increase the expression of cytokines (TNF-a, IL-1β, IL-6, MCP-1), and these effects are associated with inflammation and cell death (for a review, see Blanco & Guerri, 2006). Notably, the regulation of the immune response by EtOH is complex, depending on the dose, duration of EtOH treatment (binge vs. chronic), and model. A single (acute) binge EtOH exposure *in vitro* and *in vivo* has been shown to suppress the innate immune response peripherally in mice and in macrophage cultures, as measured a reduction in the expression of TNF-α, IL-1β, and IL-6 mRNA in response to lipopolysaccharide (LPS), an endotoxin used to induce inflammation (Pruett, Schwab, Zheng, & Fan, 2004; Goral, Choudhry, & Kovacs, 2004). Moreover, Suryanarayanan and colleagues (2016) have shown that IL-10, an anti-inflammatory cytokine, is upregulated in the rat hippocampus

as soon as 1 h following the oral administration of a single intoxication dose of EtOH. However, while induction NF-κB DNA binding is required for expression of TNF-α, IL-1β, and IL-6, studies suggest that acute binge EtOH exposure does not inhibit NF-κB activity *in vivo* (Ward et al., 1996; Pruett & Fan, 2009) or *in vitro* (Davis & Syapin, 2004; Blanco et al., 2005). In kind, Doremus-Fitzwater, Gano, Paniccia, and Deak (2015) have shown that acute EtOH decreases mRNA expression for IL-1 and TNFα, IL-6 mRNA is actually upregulated, especially in the hippocampus of adult rats. Notably, mRNA expression is only predictive of outcome protein expression ~40% of the time (Kendrick, 2014). A study by Marshall, Geil, and Nixon (2016) suggests this acute activation may lead to primed (i.e., more susceptible) microglia, as a subsequent binge EtOH treatment revealed both a potentiated classically activated microglial response and increased release of TNF-α in the rat hippocampus. This finding is consistent with chronic *in vitro* and *in vivo* EtOH exposure models which show an increase in NF-κB and AP-1-mediated transcription of pro-inflammatory genes in astrocytes, microglia, and slice culture (Ward et al., 1996; Vallés, Blanco, Pascual, & Guerri, 2004). Notably, the previous studies have focused on single cell models or the hippocampus, however, interactions between several neurotransmitters (e.g., serotonin, dopamine, GABA) and cytokines (e.g., IL-1β, IL-6, TNF-α) have also been observed in brain regions associated with EtOH reward, including the amygdala, lateral hypothalamus, and nucleus accumbens (Brebner, Hayley, Zacharko, Merali, & Anisman, 2000; Knapp et al., 2011). While the direct relationship between EtOH-induced cytokine release and neurodegeneration is not well characterized, LPS-evoked TNF-α has been shown to cause cytotoxicity in dopamine cells (Harms et al., 2012). Interestingly, astroglia and microglia of mice deficient in, or with knock-out of,

toll-like receptor 4 (TLR4), the canonical mechanism of NF-κB activation (Hoesel & Schmid, 2013), were protected against EtOH-induced apoptotic cell death (Blanco et al., 2005; Fernandez-Lizarbe, et al., 2009; Alfonso-Loeches et al., 2010; Pascual et al., 2011). The Integrative Neuroscience Initiative on Alcoholism (INIA-Neuroimmune) consortium has recently presented a series of studies demonstrating no significant impact of the TLR4 on EtOH drinking behavior in models of CIE exposure; notably, mice and rats received NAcc-specific knock-out (KO) of the TLR4 in these studies (Harris et al., 2017). However, Pascual and colleagues (2011) have shown that TLR4 KO mice exhibit less EtOH-induced cognitive and motor impairments than wild type mice in a model of chronic EtOH drinking and subsequent EWD (Pascual et al., 2011). Additionally, amygdala-specific TLR4 inhibition in alcohol preferring (P) rats has been shown to reduce EtOH self-administration (Liu et al., 2011).

However, Alfonso-Loeches and colleagues (2010) found that activation of NF-κB in response to chronic EtOH exposure was associated with both increased levels of IL-1β, TNF-α, IL-6, and several oxidases (e.g., COX-2 and iNOS) in the cerebral cortex of female wide-type mice. This increase in COX-2 and iNOS by NF-κB could lead to oxidative stress through the generation of ROS (Sun et al., 2001). As ROS can directly activate NF-κB, oxidative stress may contribute to the persistent innate immune response observed following chronic EtOH consumption (Qin & Crews, 2012). These findings already suggest significant interactions with other mechanisms in the promotion of EtOH-induced neurotoxicity. In support, Crews and colleagues (2006) found that treatment with BHT, a potent antioxidant, during binge EtOH exposure reduced NF-κB activation and COX-2 expression in the hippocampus of adult male Sprague-Dawley rats.

Furthermore, this reduction was associated with a reversal in binge EtOH-induced brain damage (Crews et al., 2006). More recently, 2 additional antioxidants, EUK-134 and Trolox, were shown to protect EtOH-treated microglia from oxidative stress and apoptosis-induced cell death (Boyadjieva & Sarkar, 2013). These studies support a neuroimmune mechanism in EtOH-induced neurotoxicity. Additionally, they suggest that interactions may exist between oxidative stress and neuroimmune signaling to promote this neurotoxicity.

1.1.3.3. Excitotoxicity. Although both acute and chronic EtOH exposure alter the expression and/or function of many receptor subtypes, withdrawal from chronic EtOH is known to be particularly detrimental, resulting in severe symptoms in both humans and animals including, anxiety, hypoactivity, agitation, insomnia, tremor, seizures, delirium, and in some cases, death (Grant Valverius, Hudspith, & Tabakoff, 1990; Davidson et al., 1995; Trevisan, Boutros, Petrakis, & Krystal, 1998). The ionotrophic N-methyl-D-aspartate receptor (NMDAR), a glutamate receptor involved in controlling synaptic plasticity and learning, and may be involved in promoting the cognitive deficits observed following chronic alcohol use (Hoffman & Tabakoff, 1994). NMDAR are heteromeric in structure, consisting of three different subunits (NR1, NR2, and NR3), with mammalian cells consisting of at least one NR1 subunit and one NR2 (A-D) subunit (for a review, see Paoletti & Neyton, 2007). Notably, the NR2A and NR2B subunits have been shown to be the most polyamine (and glutamate)-sensitive subunits, and thus, are the mostly widely studied in regards to alcohol's effects on the brain (Mirshahi & Woodward, 1995). Acute EtOH exposure reduces excitatory glutamatergic synaptic transmission by inhibiting

NMDAR function, which alters intracellular calcium (Ca^{2+}) levels and modifies downstream cell-signaling cascades, such as phosphorylation (Lovinger, 1993; Tsai, 1998; Davis & Wu, 2001; Ron, 2004). As a result, chronic EtOH administration results in a compensatory upregulated expression in NMDARs with an EtOH-sensitive NR1/NR2B subunit conformation (Lovinger, 1993; Tsai, 1998; Wirkner et al., 1999; Davis & Wu, 2001; Nagy & Laszlo, 2002; Ron, 2004). Antagonism of the NMDAR during chronic EtOH exposure is known to reduce EWD-induced neuronal hyperexcitability *in vitro* (Thomas, Monaghan, & Morrisett, 1998). Additionally, our lab has previously shown that excitotoxicity during EWD from continuous EtOH exposure is dependent on this increase in NR1/NR2B subunits in OHSC (Harris et al., 2003; Prendergast et al., 2004). Using a chronic intermittent EtOH (CIE) treatment regimen, characterized by cycles of binge-like exposure and EWD, we have also found that co-exposure to the NMDAR antagonist APV during periods of EWD prevents hippocampal cell death (Reynolds, Berry, Sharrett-Field, & Prendergast, 2015). In rodents, altered glutamatergic activity has been shown to lead to a prolonged upregulation of sensitized NMDARs (Rani & Ticku, 2006), resulting in increased withdrawal severity (Veatch & Becker, 2002), alcohol-seeking (Griffin, Lopez, Yanke, Middaugh, & Becker, 2009), and relapse to EtOH (Wang et al., 2010). Antagonism of the NMDAR during chronic EtOH exposure has been shown to reduce EWD-induced seizure activity in rats (Morrisett et al., 1990). However, this reduction in seizure activity through blockade of the NMDAR does not affect EtOH self-administration (Bienkowski et al., 2001).

EWD-induced excitotoxicity is also associated with alterations in intracellular Ca^{2+} signaling (Prendergast et al., 2004; Lau & Tymianski, 2010), and EWD-induced

Ca^{2+} influx can be inhibited by NMDAR antagonists in OHSC (Mayer et al., 2002). This influx of Ca^{2+} ultimately leads to dysregulated calcium homeostasis, and this imbalance can initiate a number of cascades, including mitochondrial dysfunction function, oxidative stress, and cell death (as discussed above in the Oxidative Stress section). However, mitochondrial dysfunction and oxidative stress have also been shown to activate the protease, calpain, which can directly upregulate EtOH-sensitive NMDA NR2B subunits (Norberg et al., 2008). Sunkesula, Swain and Babu (2002) found an up-regulation of NMDA NR1, 2B, and 2C subunits in the hippocampus following a 12 week continuous EtOH exposure period in adult rats. Notably, an up-regulation of proteases such as calpain and caspase were also observed, suggesting mitochondrial involvement (Sunkesula et al., 2002). In support, data which are currently in preparation from our lab, indicate that antagonism of calpain completely prevents EWD-induced loss of synaptic vesicle protein, an indicator of axon integrity and neurotoxicity (Saunders et al., *in preparation*).

TLR4 signaling, through upregulation of the TLR4 agonist high-mobility group box 1 (HMGB1), has also been shown to potentiate EtOH-induced CA^{2+} influx and excitotoxicity (Viviani et al., 2003; Vezzani, Maroso, Balosso, Sanchez, & Bartfai, 2011; Balosso, Liu, Bianchi, & Vezzani, 2014). Furthermore, greater HMGB1 release has been shown in response to EWD (Whitman, Knapp, Werner, Crews, & Breese, 2013), and studies have found increased HMGB1 to be associated with hippocampal excitability and seizures in a model of epilepsy (Maroso et al., 2011). Furthermore, upregulations in HMGB1 were shown to be persistent following hippocampal excitability (Maroso et al., 2011), suggesting a feed-forward cycle may exist between neuroimmune responses and

excitotoxicity. In kind, chronic EtOH exposure has been shown to result in TNF-α-mediated inhibition of glial glutamate transporters, thus preventing uptake of glutamate from the synapse, and potentiating EWD-induced excitotoxicity in hippocampal-entorhinal (HEC) slice cultures (Zou & Crews, 2005; 2010).

Collectively, these studies suggest that EtOH exposure results in neuroadaptations that interact to promote excitotoxicity during EWD. Furthermore, it suggests that excitotoxicity may be secondary to oxidative stress and pro-inflammatory neuroimmune signaling in response to EtOH. Therefore, therapeutic strategies which address mechanisms of toxicity during EtOH exposure could be effective at ameliorating the toxicity of both phases of dependence.

1.2. The Current Project

Collectively, this review demonstrates that chronic, excessive alcohol consumption predicts worse neurological outcome in the clinical AUD population. Unfortunately, the current pharmacotherapies for AUD work at preventing future alcohol consumption by lowering its value to the user, and not by targeting the underlying neurodegeneration (Koob & Le Moal, 1997; Van Skike et al., 2016). Thus, the development of novel pharmaceutical interventions is still needed to address this gap in care. Pharmacotherapies which address events that alter neuroplasticity, such as neurodegeneration and neuroinflammation, may improve treatment outcomes for AUD (Koob & Le Moal, 1997). Further, as reward and cognitive circuits overlap, there are likely several common targets which could be exploited for drug development (Adinoff, 2004). Natural product compounds have presented themselves as appealing drug

discovery targets in recent years, having greater structural diversity and increased therapeutic success compared to synthetic compounds (for a review see, Harvey, 2008). Moreover, development of synthetic natural product analogs allows for molecular modifications which can: 1) enhance desired bioactivity, 2) add mechanisms of action, and/or 3) improve solubility and metabolism (Kerns & Di, 2003). Despite this, natural products and their analogs remain an untapped resource in drug discovery and development for AUD. The purpose of the present dissertation is to identify novel chemical scaffolds which may be exploited in the development of pharmacological treatments for AUD. Thus, Study 1 presents the discovery of several natural product analogs which attenuate EtOH-induced cytotoxicity *in vitro*. Study 2 extends these findings by discussing the role of neuroimmune signaling in the development of EtOH-induced neurotoxicity and evaluating the effects of 1 lead candidate compound, identified in Study 1, on neuroimmune mediators released into culture media during the same EtOH-exposure model described in Study 1.

CHAPTER TWO: Study One: Effect of Novel Natural Product Analogs on Binge Ethanol-Induced Cytotoxicity *In Vitro*

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2.1. Introduction

The development of novel drugs is a high-risk endeavor which requires multidisciplinary collaboration across several scientific fields (e.g. chemists, biologists, psychopharmacologists) and procures an average cost of $2.6 billion per new market drug in the United States (DiMasi, Hanson, & Grabowski, 2003). Drug research and development (R&D) requires multiple steps from target screening, to clinical trials, to the evaluation of drug efficacy in human participants (Kelly, 2009). While many of these R&D projects are initiated, only a fraction succeeds through clinical trials, which may require the screening of thousands of compounds in the hope of finding different classes of drugs that work within a desired model (Kelly, 2009). Furthermore, initiation of a project through drug sale may take an average of 15 years following the identification of a viable compound candidate (called a "hit") (Levy, 2000). A hit may go through to subsequent testing phases in the structural form it was identified; however, it is also common for the structure of the compound to be modified for changes which may further improve its efficacy in the model. This newly modified compound may then be re-evaluated for efficacy in all testing phases. Once a hit compound is advanced into further testing phases, it may be referred to as a "lead" compound. As model systems do not

perfectly predict therapeutic action in the clinical trial setting, 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).

Specifically, to gain FDA approval, a hit compound must be evaluated in, and successfully advance through Absorption, Distribution, Metabolism, Excretion (ADME) and Toxicology safety tests, preclinical testing, and four clinical trial phases. Rules, such as Lipinski's Rule of Five, have been developed which help in ADME consideration and improve the predictability of preclinical and clinical trial outcomes. Lipinski's rule is used to assess a chemical compound's "drug-likeness" and whether it possesses pharmacological or biological activity that is predictive of an orally active drug in humans. A compound is more likely to have poor absorption and permeability when: (1) there are more than 5 hydrogen bond donors, (2) the molecular mass is greater than 500 daltons, (3) there are more than 10 hydrogen bond acceptors, (4) the calculated log P is greater than 5, and (5) natural product compounds, as well as any compounds which are substrates for biological transport systems, are exempt from these rules (Lipinski, Lombardo, Dominy, & Feeney, 2001). This suggests an advantage for natural product compounds, over synthetic compounds, going into the screening process (Newman, 2008).

Natural products have been exploited for their medicinal properties as far back as 2600 BCE in ancient Mesopotamia (Borchardt, 2002). Currently, approximately 60% of drugs on the market are directly or indirectly derived from natural product sources (Newman, 2008). Notably, in the 1990's, the medical field shifted away from the

screening of natural product compounds to high-throughput screening (HTS) of combinatorial chemistry libraries due lower costs. Since this shift, fewer medications have successfully gained FDA approval, resulting in what has been deemed a “development pipeline”. Many have pointed out a lack of diversity and complexity amongst combinatorial chemistry libraries (Ji, Li, & Zhang, 2009; Newman & Cragg, 2007). Natural product compound libraries present with increased diversity over synthetic libraries as natural product compounds are simply spatially more diverse than synthetic compounds in respect to the number of chiral structures, rings, bridges, and functional groups in the molecule (Henkel, Brunne, Müller, & Reichel, 1999). Chirality is particularly interesting as this is when a compound has 4 different groups around a central atom (assuming carbon) and presents an asymmetric structure; the unique structure of these molecules could be particularly beneficial in CNS drug R & D as they may provide a superior fit to receptors and other target proteins due to these different functional groups coming off the central atom (Mentel, Blankenfeldt, & Breinbauer, 2009). Additionally, natural products tend to be highly soluble in aqueous media, a feature not seen with many synthetic compounds, and which is desirable in drug formulations as it predicts absorption (Krishnaiah, 2010).

However, more recent knowledge of natural chemical structures has allowed for the synthesis of natural structures at the bench top, relieving the need for constant isolation from natural sources. These new developments in chemistry have significantly reduced the cost of screening natural product compound libraries (for a review, see Harvey, 2008). Moreover, chemists have developed strategies to create compound analogs and derivatives (i.e., compounds which are similar to, but differ from the parental

compound by at least a single element group) with an increased therapeutic potential over their original structures (Sunazuka, Hirose, & Omura, 2008). Specifically, the development of natural product analogs allows for additional molecular modifications which can: 1) enhance desired bioactivity, 2) add mechanisms of action, and/or 3) improve solubility and metabolism (Kerns & Di, 2003). 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 purely synthetic compounds (for a review, see Harvey, 2008). The combination of these two factors has revived interest in the use of natural product compounds and their synthetic analogs in drug discovery (Galm & Shen, 2007).

2.2. Experimental Rationale

Natural product compounds represent an untapped resource within AUD drug discovery, as the more complex chiral structures of these compounds may provide a superior fit to receptors and other target proteins (Mentel et al., 2009). As discussed in Chapter 1, addressing the underlying neurobiological deficits induced by AUD may be an essential feature of an effective pharmacotherapy. Notably, HTS techniques employed during drug R & D are typically employed in single cell models where the target of interest is known. However, cells and tissues are physiologically connected, and these interactions are important to the progression of many disease states (Giacomotto & Ségalat, 2010). For these reasons, HTS techniques are not applicable in the early stages of new drug development for diseases in which these interactions are known. In AUD,

neuron-glia interactions are critical in the progression of AUD neuropathology (for a review, see Crews et al., 2015). The organotypic hippocampal slice culture (OHSC) technique allows for the reliable replication of neuronal over-activation in the hippocampus that can be translated *in vivo* (Gahwiler, Capogna, Debanne, McKinney, & Thompson, 1997; Noraberg et al., 2005; Sharrett-Field, Butler, Berry, Reynolds, & Prendergast, 2013; Reynolds, Williams, Saunders, & Prendergast, 2015; Reynolds, Saunders, & Prendergast, 2016), and has been validated in the examination of EtOH-induced neurodegeneration (Bulter et al., 2013; Reynolds et al., 2015a; Reynolds et al., 2016). Specifically, this model preserves hippocampal neuropil layers, and thus, 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). Furthermore, the OHSC technique has been used to measure EtOH-induced neuroimmune signaling (Moon et al., 2014; Lutz, Carter, Fields, Barron, & Littleton, 2015a,b), and important neuron-glia interactions are preserved in this model (Benediktsson, Schachtele, Green, & Dailey, 2005; Haber, Vautrin, Fry, & Murai, 2009; Dailey & Waite, 1999). Moreover, beyond being validated as a model for the examination EtOH-induced changes in the brain, deficits observed in the hippocampal brain region of alcoholics is thought to contribute to neurobehavioral consequences (Sullivan, Rosenbloom, & Pfefferbaum, 2000; Chanraud, et al., 2007; Pitel et al., 2007; Ozoy et al., 2013), including deficits in reward sensitivity (Makris et al., 2008) and enhancement of drug-context associations which could facilitate relapse and maintenance of alcohol use (Müller, 2013; Kutlu &

Gould, 2016). Collectively, this suggests the OHSC model is an ideal model for the screening of novel compound candidates in the early drug development stages for AUD.

In collaboration with scientists at the University of Kentucky Center for Pharmaceutical Research and Innovation (CPRI), our lab has previously screened several compounds from the CPRI natural products repository for their ability to attenuate 48 h EtOH (100 mM)-induced increases in propidium iodide (PI) uptake in a rat OHSC model (Wang et al., 2015; Shaaban et al., 2017; Zhang et al., 2017). PI allows for the quantification of cytotoxicity as it can only enter cells with disrupted plasma membranes, reflecting the presence of necrotic or end-stage apoptotic cells within the primary neuronal and glial cell layers of hippocampal cultures (for a review, see Zimmer, Kristensen, Jakobsen, & Noraberg, 2000). Prior work has shown a 48 h binge EtOH exposure is sufficient to produce necrosis-induced degeneration in the olfactory bulbs of adult rats *in vivo* (Obernier et al., 2002), and that *in vivo* binge EtOH exposure in adolescent rats produces hippocampal-dependent learning and memory deficit in adulthood similar to those observed in clinical AUD populations (Vetreno & Crews, 2015). Terfestatin B, a natural product compound isolated from *Streptomyces* sp. RM-5-8 (Wang et al., 2015), and both spoxamicin D and oxachelin C, natural product compounds isolated from *Streptomyces* sp. RM-14-6 (Shaaban et al., 2017), were found to have cytoprotective effects in our model. As the creation of natural product compound analogs can result in compounds with enhanced-desired characteristics (Kerns & Di, 2003), the purpose of the present study was to screen novel analogs of previously identified natural product compounds (from both our lab and others) for their ability to attenuate binge-like EtOH-induced hippocampal cytotoxicity *in vitro*. The hypothesis of the current study is

that analogs of natural products previously shown to attenuate EtOH-induced neurodegeneration or neurobehavioral deficits will attenuate EtOH-induced cytotoxicity with enhanced efficacy over their parental compound structures.

2.3. Method

2.3.1. 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 thickness 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 method produces 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.

2.3.2. EtOH and Compound Analog Treatment

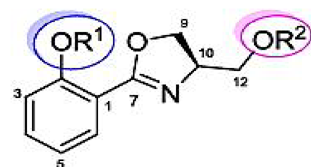
Natural product analog compounds (~20) from the natural and synthetic products repository, including those derived from *Streptomyces* sp. RM-5-8 (Wang et al., 2015) and *Streptomyces* sp. RM-14-6 (Shaaban et al., 2017; Zhang et al., 2017), have been provided to our laboratory by the University of Kentucky Center for Pharmaceutical Research and Innovation (CPRI). Compound analogs were pre-screened for *in silico* blood brain barrier (BBB) permeability, and only compounds found to have a predicted probability value > 0.90 (according to the admetSAR model; Cheng et al., 2012), were provided by the CPRI. The activity of compound parental structures in various EtOH treatment models is presented in Table 2.1. Where allowed, a description of each compound analog's unique structure can be seen in Figure 2.1. The parent and analog structures for compounds **1**, **6**, **7**, **10**, and **11** were withheld at the request of the CPRI to preserve intellectual property (IP). Compound analogs were delivered with their structures blinded via arbitrary compound naming. At 5 days *in vitro* (DIV), after the

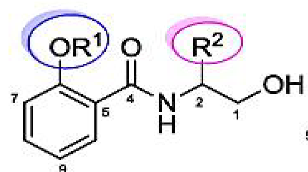
hippocampal slices had adhered to the biopore membrane, cultures were transferred at random to 6-well culture plates containing 1 mL of culture medium with 0.01% dimethyl sulfoxide (DMSO vehicle control [CTRL]; Fisher) or 1 mL of culture medium with the addition of 0.01% DMSO and 100 mM EtOH (a concentration that represents 6x the legal limit in humans [480 mg/dL]). While this concentration is high, a case study of 117 alcoholic patients found BACs ranging from 29 to 577 mg/dL upon hospital admittance, with many of these patients still walking and cognizant with BACs over 300 mg/dl (Adachi et al., 1991). Additional cultures were exposed to CTRL or EtOH (100 mM) medium with the addition of a product analog at multiple concentrations (0.01-1.0 μM) to establish concentration-response relationships. Compounds were provided by the CPRI in 10 mM DMSO stock solutions which were diluted in culture medium to a final DMSO concentration of 0.01%. All culture medium also received the addition of propidium iodide (PI; 7.48 μM) during the 48 h exposure (discussed below in *Measurement of Cytotoxicity via Propidium Iodide Staining*). 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. CTRL groups received similar treatment, without the addition of EtOH. Containers were then placed into 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 suggests ~10% reduction in the final starting concentration of EtOH (Prendergast et al., 2004). This 48 h treatment regimen is shown in Figure 2.2.

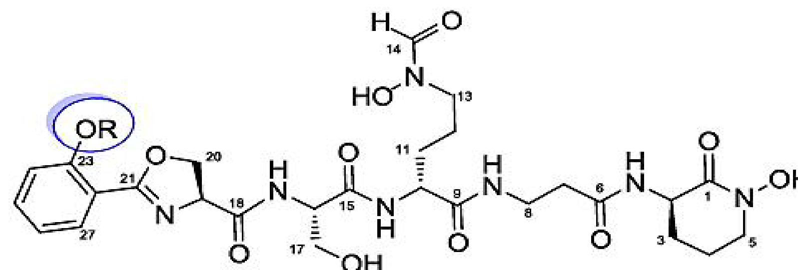
Table 2.1. Activity of compound parental structures in various ethanol treatment models.

Compound	Parent Structure	Population	Method	Effect Observed	References
1	withheld	–	–	–	–
2a 2b 2c	Spoxazomicin C*	SD	OHSC	Inactive	Shaaban et al., 2017
3	*N*-salicyloyl-2-aminopropane-1,3-diol*	–	excluded initially	–	Zhang et al., 2017
4a 4b	(2R)-*N*-salicyloyl-2-aminopropan-1-ol*	–	excluded initially	–	Zhang et al., 2017
5a 5b	Oxachelin†	SD	OHSC	Inactive	Shaaban et al., 2017
6	withheld	–	–	–	–
7	withheld	–	–	–	–
8a 8b	Terfestatin B	SD	OHSC	↓ binge EtOH-induced cytotoxicity	Wang et al., 2015
9a	Isoflavonoid	SD	OHSC	Rhamnetin ↓ EWD cytotoxicity	Lutz et al., 2015a
9b		SD	EtOH (7%, w/v) continuous	50 and 150 mg/kg ip puerarin ↓ EWD	Overstreet et al., 2003
9c		P-rats	EtOH (10% w/v) 2-bottle, free-choice	Daidzin, daidzein, and puerarin ↓ EtOH intake	Lin & Li, 1998
9d		P-rats	EtOH (15% w/v) 2-bottle, free-choice	0.5 g/kg kudzu root ↓ EtOH intake, ↓ EWD	Benlhabib et al., 2004
9e		Heavy Drinkers	Pilot and randomized between-subject, double-blind, placebo-controlled study	750-1200 mg daily isoflavone ↓ EtOH intake	Lukas et al., 2005; 2013; Penetar et al., 2012
9f		Male Binge Drinkers	Double-blind, placebo-controlled, between subjects design	A single 520 mg isoflavone ↓ EtOH binge intake	Penetar et al., 2015
10	withheld	–	–	–	–
11	withheld	–	–	–	–

Note: *Spoxazomicin D related (cytoprotective against EtOH in OHSC; Shaaban et al., 2017); † Oxachelin C related (cytoprotective against EtOH in OHSC; Shaaban et al., 2017); SD = Sprague Dawley strain of rat; EWD = EtOH-withdrawal. Studies presented utilized isoflavonoid compounds isolated from various food or plant sources.



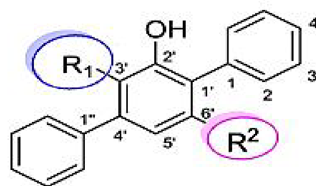


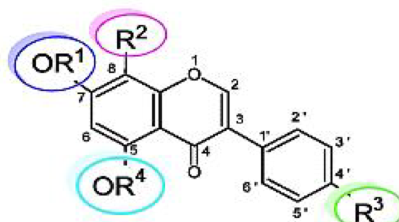


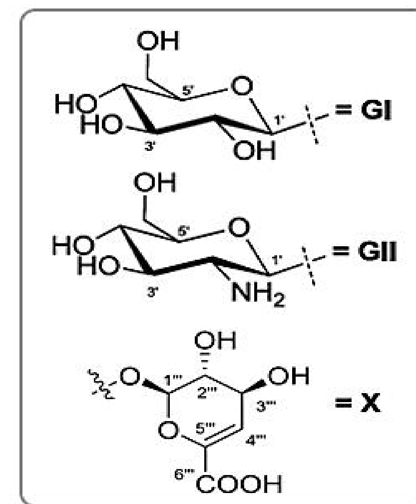
R1=R2=H; Spoxazomicin C
2a: R1=GI ,R2=H
2b: R1=GI ,R2=H
2c: R1=GI ,R2=H

R1=H, R2= CH_2OH; N-salicyloyl-2-aminopropane-1,3-diol
3: *synthesis N-salicyloyl-2-aminopropane-1,3-diol
R1=H, R2=α-CH_3; (2R)-N-salicyloyl-2-amopropan-1-ol
4a: R1=H, R2=α-CH_3R
4b: R1=H, R2=α-CH_3S

R=H; Oxachelin
5a: R=GI
5b: R=GII







R1=OCH, R2=X; Terfestatin B
8a: R1=OCH, R2=OH
8b: R1=OCH, R2=Ome

R1=R2=H, R3=OH ,R4=H; Basic Isoflavonoid
9a: R1=H, R2=$O(CH_2CH_2)_2NH$, R3=Cl, R4=Ome
9b: R1=H, R2=$(CH_2)_5NH$, R3=F, R4=Ome
9c: R1=H, R2=H, R3=F, R4=OH
9d: R1=H, R2=H, R3=F, R4=Ome
9e: R1=H, R2=$(CH_2)_5NH$, R3=H, R4=Ome
9f: R1=H, R2=$O(CH_2CH_2)_2NH$, R3=F, R4=Ome

Figure 2.1. Adapted from Zhang et al., 2017. Chemical structures of novel compounds analogs with structural modifications from parental compounds indicated. *synthesis compounds are not purified from natural source but rather the total synthesis of the natural product is enabled thought organic reactions.

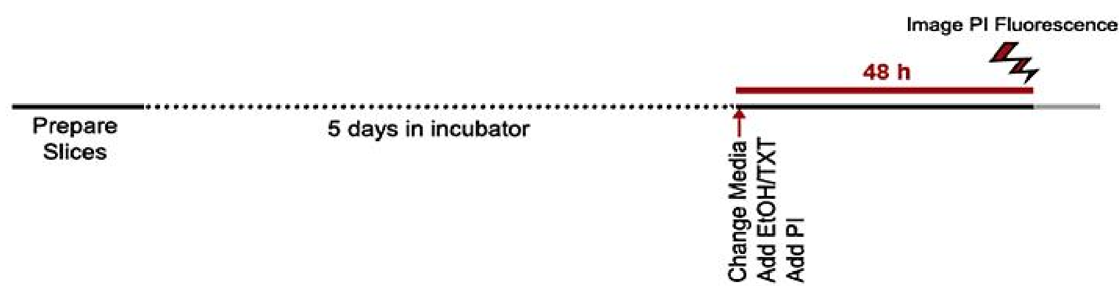


Figure 2.2. Diagram showing the timeline of experimental procedures and treatments.

2.3.3. Measurement of Cytotoxicity via Propidium Iodide Staining

In all experiments, culture medium received the addition of propidium iodide (PI; 7.48 μM) during the 48 h exposure. PI allows for the quantification of cell death 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). Once 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 (e.g., NeuN, GFAP, BrdU) *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). Densitometry using Image J software (National Institutes of Health, Bethesda, MD) was used to measure the intensity of the PI fluorescence within the entire hippocampal slice. A background measurement of fluorescence was taken from the visual field surrounding each slice and was subsequently subtracted from the measurement of each slice before analysis. To control for the variability between each screen, measurement of PI fluorescence from each experiment 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 slice (after Mulholland et al., 2005).

2.3.4. Statistical Analysis

Statistical analyses were conducted to assess the effect of each compound analog against EtOH-induced cytotoxicity. As each compound was provided in limited quantity by the CPRI, each cytotoxicity screen was performed only 1 time, consistent with our prior work (Wang et al., 2015; Shaaban et al, 2017). Notably, a post-hoc power analysis, based on the results of our natural product screens (effect size = 2.49; Cohen, 1988), suggest 8 slices per group is sufficient to detect a significant difference between EtOH (100 mM) and CTRL PI uptake at 48 h using an independent sample t-test with 80% power, $\alpha = 0.05$ (2-tailed test; Gpower; Faul & Erfelder, 1992) (Shaaban et al., 2017). As screens were performed over several months, compounds were screened using tissue obtained from different rat litters. Data from each screen were converted into percent control values (described in Statistical Analysis), yielding a total of 8-9 slices per treatment group (EtOH treatment × compound concentration). Data generated from the above method was analyzed using a 2-factor ANOVA (EtOH treatment × compound concentration), conducted in Prism 5.0 (Graph Pad Software Inc., San Diego, CA, USA). The effect of sex was not assessed as prior studies using this model have not identified any significant sex differences (Saunders, 2016). When appropriate, post-hoc tests were conducted using Tukey. The level of significance was set at $p < 0.05$.

2.4. Results

Studies were conducted to examine novel analog compounds (0.01-1.0 μM) for their ability to protect against EtOH (100mM)-induced cytotoxicity in OHSC, as determined by uptake of PI at 48 h. The effects of analogs on EtOH-naïve treated

hippocampal explants were also examined. For statistical analyses, a 2-factor ANOVA was conducted (EtOH treatment x compound concentration) to establish any concentration-response relationships in compound treated tissue. Consistent with previous data (Wang et al., 2015; Shaaban et al., 2016), Tukey posttests revealed mean increases of PI uptake of approximately 160% of EtOH-naïve controls for EtOH-treated tissue, in every experiment ($p < 0.05$). Following the analog screen, 9 compound analogs were identified which significantly attenuated EtOH-induced cytotoxicity (compounds **2a**, **4a**, **5a**, **6**, **7**, **9b**, **9d**, **10**, **11**). Two-way ANOVA results for each of these compounds are summarized in Table 2.2. A significant EtOH treatment x compound concentration interaction was revealed for all compounds ($p < 0.05$), except compound **11** ($p = 0.07$). This suggests that for most compounds, the effect of EtOH treatment on cytotoxicity is dependent on the concentration of the compound analog. Post hoc analyses revealed co-exposure of **2a** significantly attenuated EtOH-induced PI-uptake at 0.01 and 0.10 μM ($p < 0.05$). Compound **2a** had no significant cytotoxic-effects in CTRL cultures (Figure 2.3). Compound **4a** co-exposure significantly attenuated EtOH-induced PI-uptake at all concentrations tested (0.01-1.0 μM) ($p < 0.05$). However, **4a** was found to have significant cytotoxic-effects in CTRL cultures at 1 μM ($p < 0.05$) (Figure 2.4). Compound **5a** co-exposure also significantly attenuated EtOH-induced PI-uptake at all at all concentrations tested (0.01-1.0 μM) ($p < 0.05$). Compound **5a** had no significant cytotoxic-effects in CTRL cultures (Figure 2.5). Compound **6** co-exposure significantly attenuated EtOH-induced PI-uptake at 0.01 μM ($p < 0.05$), and was cytotoxic in CTRL cultures at both other concentrations (0.10 and 1.0 μM) (Figure 2.6). Compound **7** co-exposure significantly attenuated EtOH-induced PI-uptake only at the highest (1.0 μM)

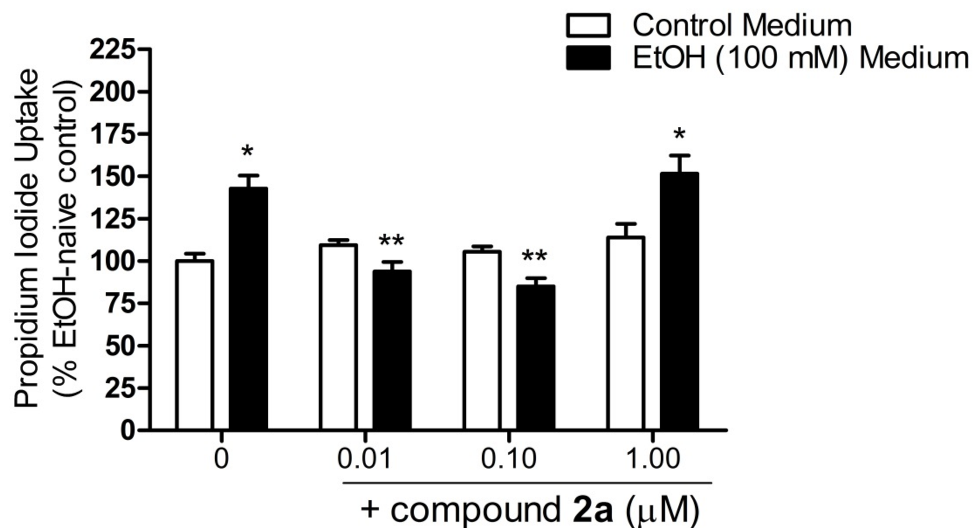
concentration tested ($p < 0.05$). In contrast, **7** was cytotoxic at all concentrations except the lowest tested (0.01 μM) in CTRL cultures (Figure 2.7). Compound **9b** co-exposure significantly attenuated EtOH-induced PI-uptake at the 0.01 and 0.1 μM concentrations ($p < 0.05$). Additionally, **9b** was found to have significant cytotoxic-effects in CTRL cultures at 1 μM ($p < 0.05$) (Figure 2.8). Compound **9d** co-exposure significantly attenuated EtOH-induced PI-uptake at all concentrations tested (0.01- 0.1 μM) ($p < 0.05$), and had no cytotoxic-effects in CTRL cultures (Figure 2.9). Compound **10** co-exposure significantly attenuated EtOH-induced PI-uptake at all concentrations tested (0.01-1.0 μM) ($p < 0.05$). In CTRL cultures, co-exposure to **10** did reveal significant cytotoxic-effects at 0.01 μM ($p < 0.05$) (Figure 2.10). Finally, compound **11** co-exposure significantly attenuated EtOH-induced PI-uptake at the 0.10 and 1.0 μM concentrations ($p < 0.05$); notably, both of these groups were still toxic when compared to the EtOH-naïve CTRL ($p < 0.05$). In CTRL cultures, co-exposure to **11** revealed significant cytotoxic-effects at 0.01 μM ($p < 0.05$) (Figure 2.11).

Table 2.2. Summary of ANOVA results of analog compound effects on propidium iodide uptake measured in ethanol (EtOH) and control-treated organotypic hippocampal slice cultures.

Compound	**Main Effects**		**Interaction** [$F_{3, 56}$ (p)]
	EtOH Treatment [$F_{1, 56}$ (p)]	Concentration [$F_{3, 56}$ (p)]	
2a	5.83 (**0.02**)	14.22 (**0.0001**)	13.46 (**0.0001**)
4a	2.05 (0.16)	1.35 (0.27)	6.39 (**0.001**)
5a	9.40 (**0.003**)	7.11 (**0.001**)	4.70 (**0.005**)
6	12.64 (**0.001**)	4.58 (**0.006**)	3.24 (**0.03**)
7	5.67 (**0.02**)	2.65 (0.06)	10.30 (**0.0001**)
9b	0.14 (0.71)	3.05 (**0.04**)	5.04 (**0.004**)
9d	0.08 (0.77)	4.41 (**0.008**)	8.08 (**0.0001**)
10	0.00 (0.95)	1.04 (0.38)	6.74 (**0.0006**)
11	13.84 (**0.0005**)	0.33 (0.80)	2.48 (0.07)

Considered significant if $p < 0.05$. Significant effects are in bold.

A



B

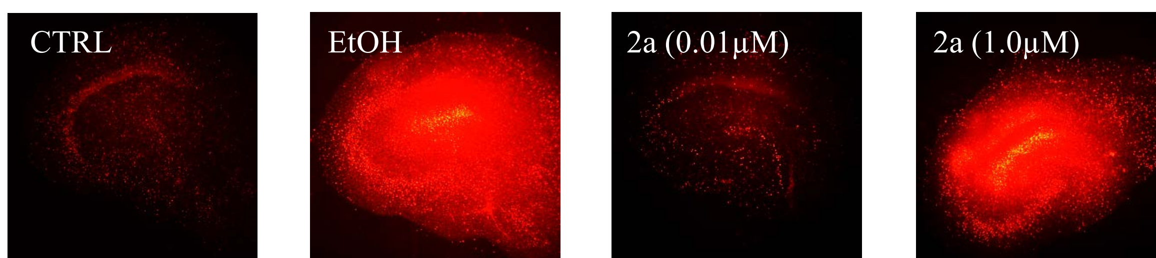
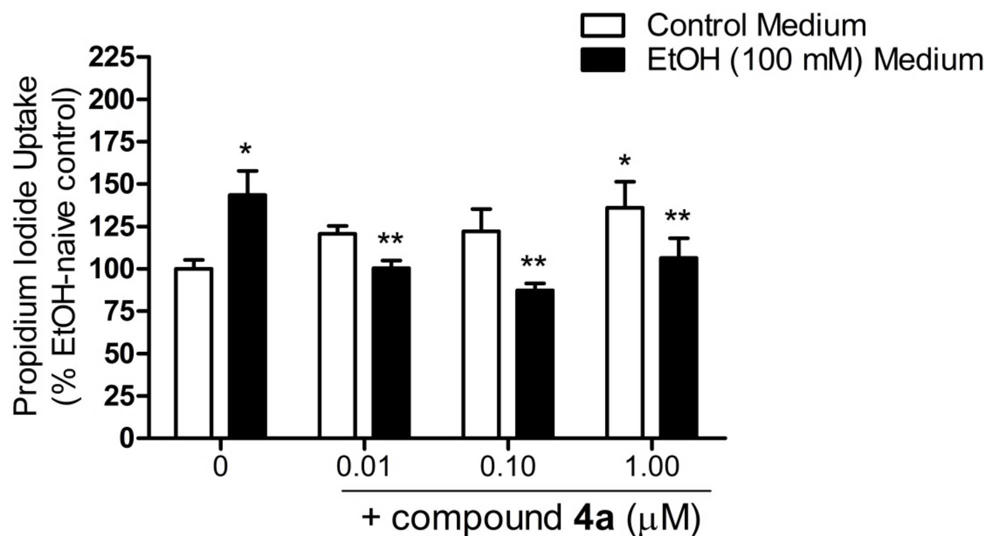


Figure 2.3. (A) Effects compound **2a** on propidium iodide (PI) uptake at 48 h in EtOH-naïve DMSO CTRL and EtOH (100) mM treated organotypic hippocampal slices cultures. Compound **2a** demonstrated no cytotoxic effects in CTRL cultures at 48 h. Exposure to 100 mM EtOH for 48 h resulted in significant increases of PI uptake compared to CTRL values within hippocampus. This increase was attenuated by the co-exposure to 0.01 and 0.10 μM of compound **2a.** * = $p < 0.05$ vs CTRL; ** = $p < 0.05$ vs EtOH, n = 8. (B) Representative images of the effects of 48 h exposure to EtOH (100 mM) and/or compound **2a** on PI uptake.

A



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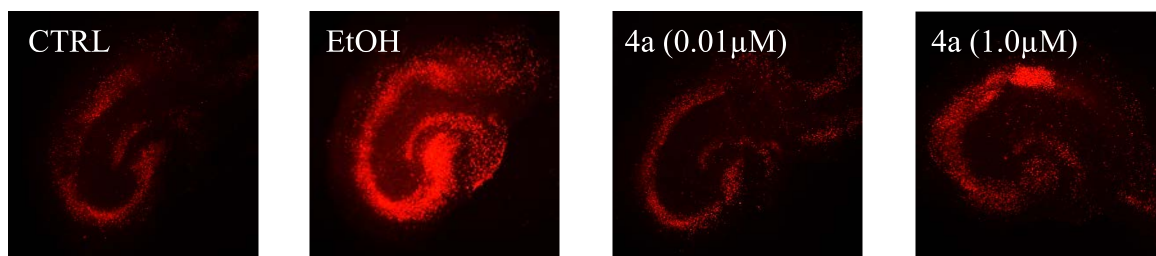
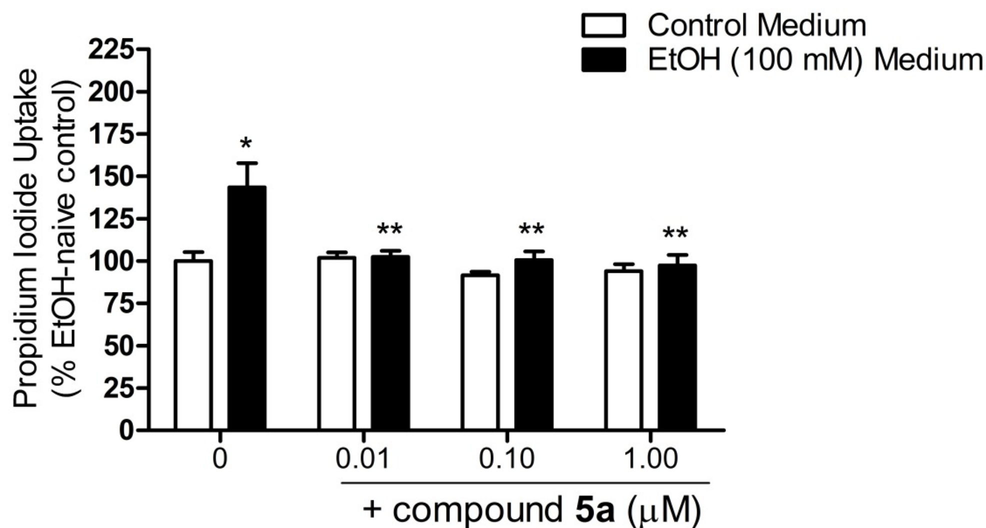


Figure 2.4. (A) Effects compound **4a** on propidium iodide (PI) uptake at 48 h in EtOH-naïve DMSO CTRL and EtOH (100) mM treated organotypic hippocampal slices cultures. Compound **4a** demonstrated cytotoxic effects in CTRL cultures at the 1 µM concentration at 48 h. Exposure to 100 mM EtOH for 48 h resulted in significant increases of PI uptake compared to CTRL values within hippocampus. This increase was attenuated by co-exposure to all concentrations (0.01-1.0 µM) of **4a**. * = $p < 0.05$ vs CTRL; ** = $p < 0.05$ vs EtOH, $n = 8$. (B) Representative images of the effects of 48 h exposure to EtOH (100 mM) and/or **4a** on PI uptake.

A



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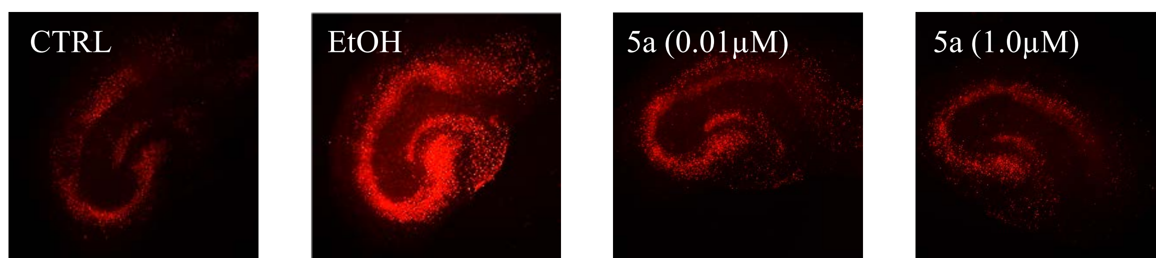
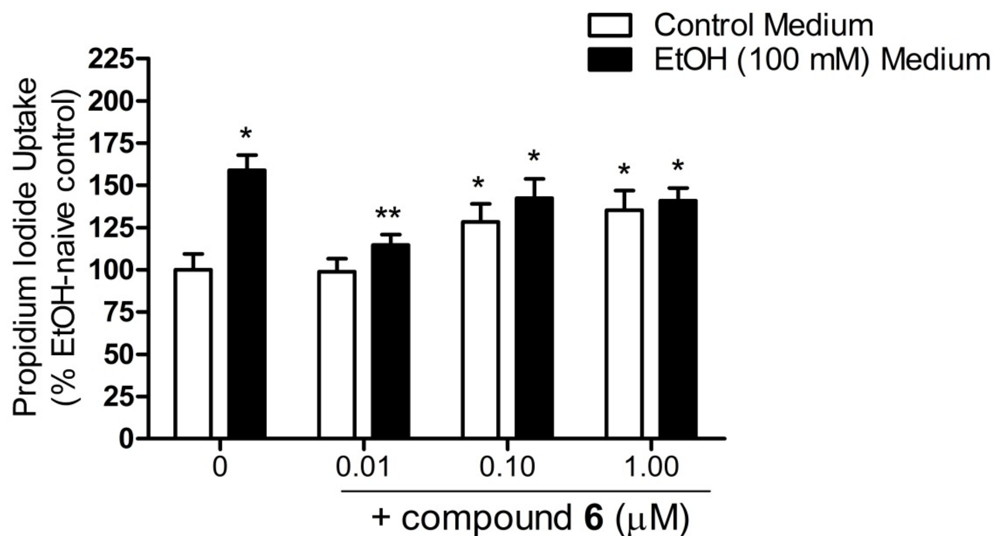


Figure 2.5. (A) Effects compound **5a** on propidium iodide (PI) uptake at 48 h in EtOH-naïve DSMO CTRL and EtOH (100) mM treated organotypic hippocampal slices cultures. Compound **5a** demonstrated no cytotoxic effects in CTRL cultures at 48 h. Exposure to 100 mM EtOH for 48 h resulted in significant increases of PI uptake compared to CTRL values within hippocampus. This increase was attenuated by co-exposure to all concentrations (0.01-1.0 μM) of **5a**. * = $p < 0.05$ vs CTRL; ** = $p < 0.05$ vs EtOH, $n = 8$. (B) Representative images of the effects of 48 h exposure to EtOH (100 mM) and/or **5a** on PI uptake.

A



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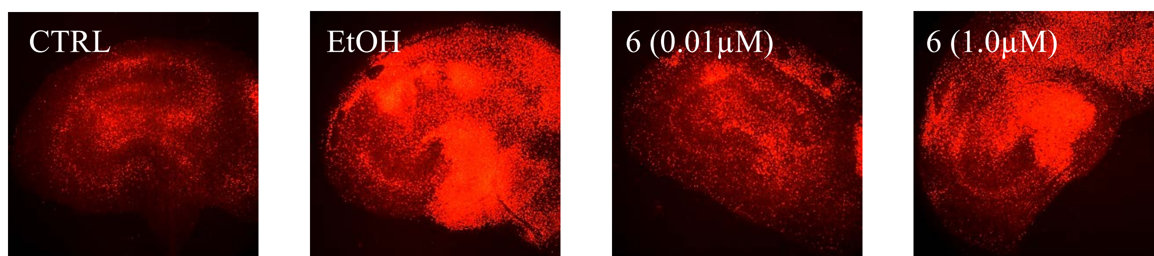
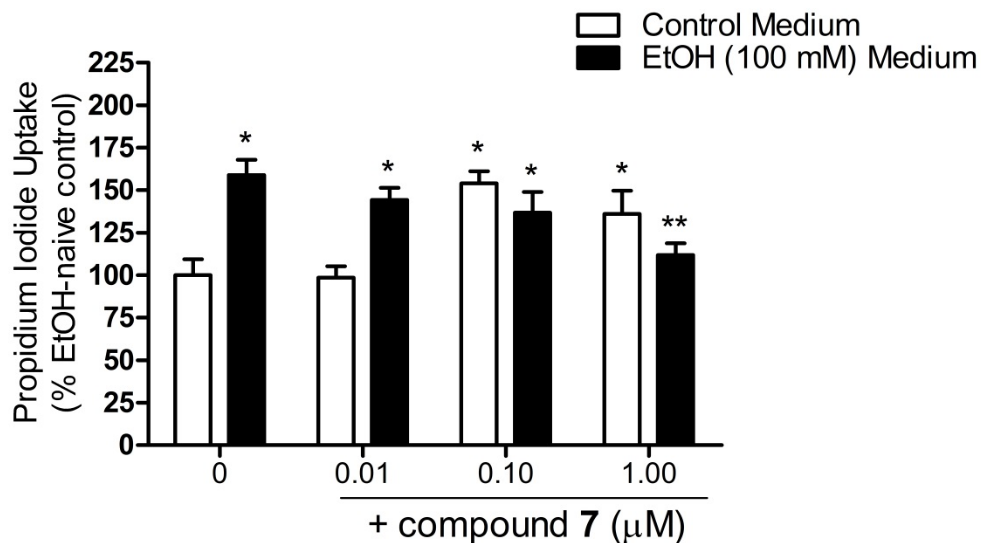


Figure 2.6. (A) Effects compound **6** on propidium iodide (PI) uptake at 48 h in EtOH-naïve DMSO CTRL and EtOH (100) mM treated organotypic hippocampal slices cultures. Compound **6** demonstrated cytotoxic effects in CTRL cultures at both the 0.10 and 1.0 μM concentrations at 48 h. Exposure to 100 mM EtOH for 48 h resulted in significant increases of PI uptake compared to CTRL values within hippocampus. This increase was attenuated by co-exposure to 0.01 μM of **6**. * = $p < 0.05$ vs CTRL; ** = $p < 0.05$ vs EtOH, $n = 8$. (B) Representative images of the effects of 48 h exposure to EtOH (100 mM) and/or **6** on PI uptake.

A



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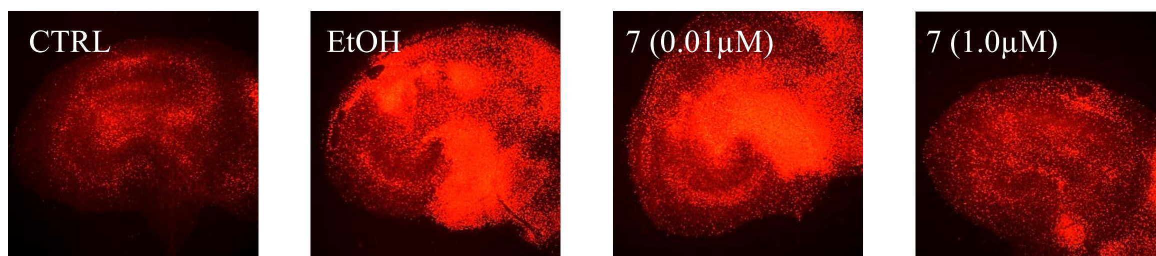
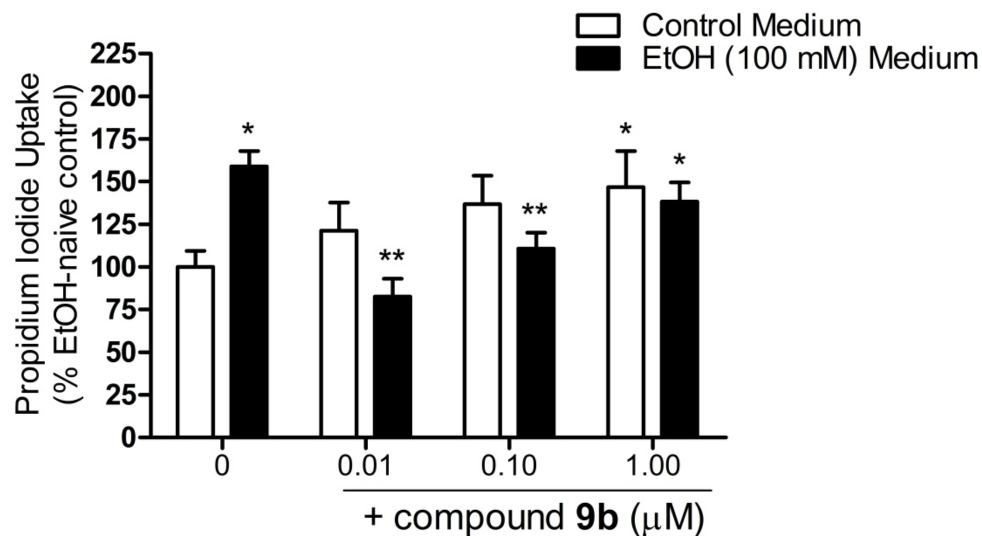


Figure 2.7. (A) Effects compound **7** on propidium iodide (PI) uptake at 48 h in EtOH-naïve DMSO CTRL and EtOH (100) mM treated organotypic hippocampal slices cultures. Compound **7** demonstrated cytotoxic effects in CTRL cultures at both the 0.10 and 1.0 μM concentrations at 48 h. Exposure to 100 mM EtOH for 48 h resulted in significant increases of PI uptake compared to CTRL values within hippocampus. This increase was attenuated by the co-exposure to highest (1.0 μM) concentration of **7**. * = $p < 0.05$ vs CTRL; ** = $p < 0.05$ vs EtOH, $n = 8$. (B) Representative images of the effects of 48 h exposure to EtOH (100 mM) and/or **7** on PI uptake.

A



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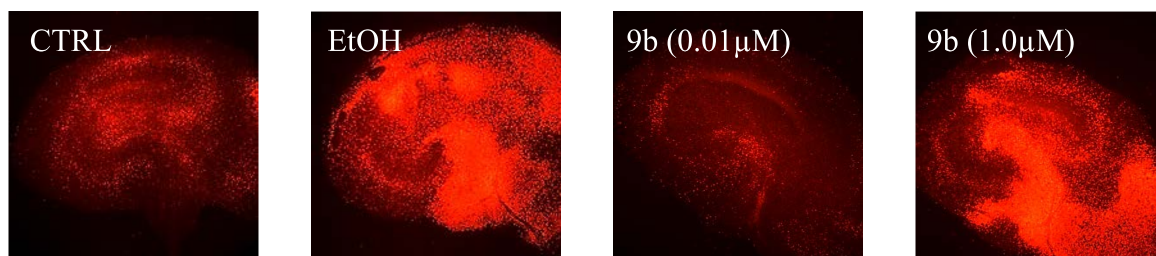
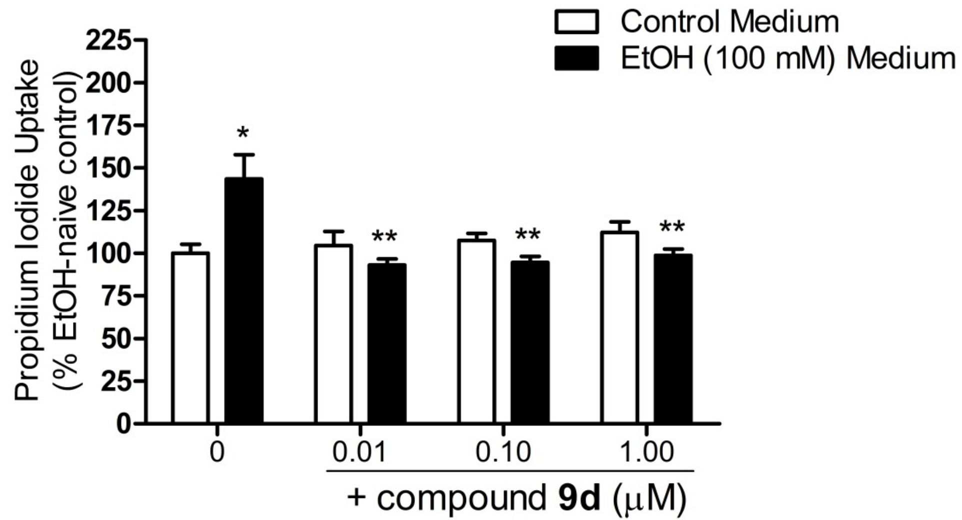


Figure 2.8. (A) Effects compound **9b** on propidium iodide (PI) uptake at 48 h in EtOH-naïve DMSO CTRL and EtOH (100) mM treated organotypic hippocampal slices cultures. Compound **9b** demonstrated cytotoxic effects in CTRL cultures at the 1.0 μM concentration at 48 h. Exposure to 100 mM EtOH for 48 h resulted in significant increases of PI uptake compared to CTRL values within hippocampus. This increase was attenuated by co-exposure to 0.01 and 0.10 μM of **9b**. * = $p < 0.05$ vs CTRL; ** = $p < 0.05$ vs EtOH, $n = 8$. (B) Representative images of the effects of 48 h exposure to EtOH (100 mM) and/or **9b** on PI uptake.

A



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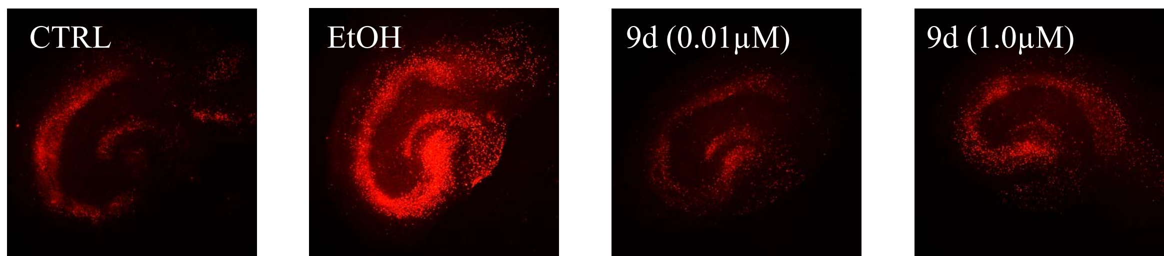
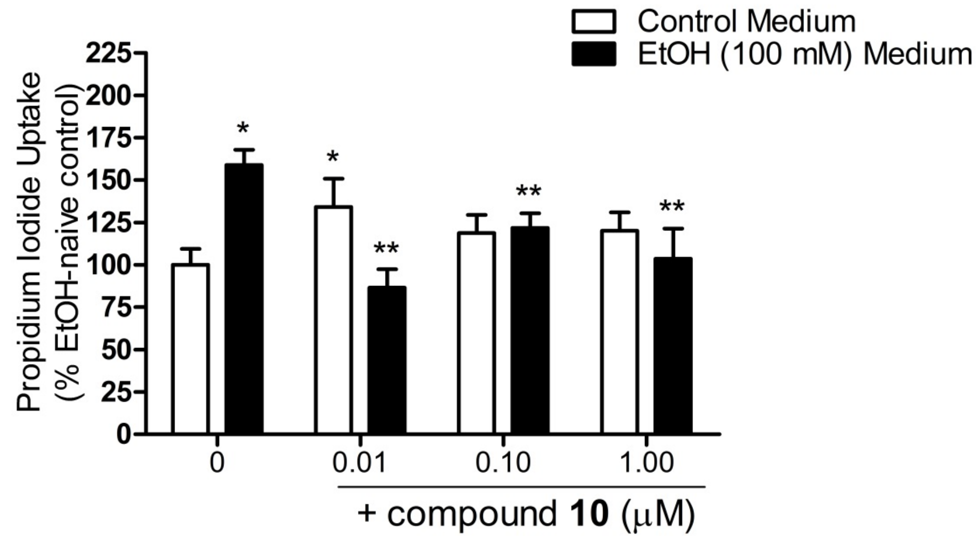


Figure 2.9. (A) Effects compound **9d** on propidium iodide (PI) uptake at 48 h in EtOH-naïve DMSO CTRL and EtOH (100) mM treated organotypic hippocampal slices cultures. Compound **9d** demonstrated no cytotoxic effects in CTRL cultures at 48 h. Exposure to 100 mM EtOH for 48 h resulted in significant increases of PI uptake compared to CTRL values within hippocampus. This increase was attenuated by co-exposure to all concentrations (0.01-1.0 μM) of **9d**. * = $p < 0.05$ vs CTRL; ** = $p < 0.05$ vs EtOH, $n = 8$. (B) Representative images of the effects of 48 h exposure to EtOH (100 mM) and/or **9d** on PI uptake.

A



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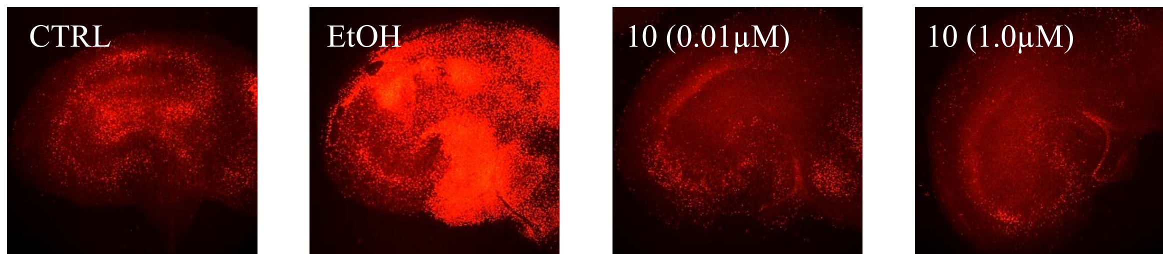
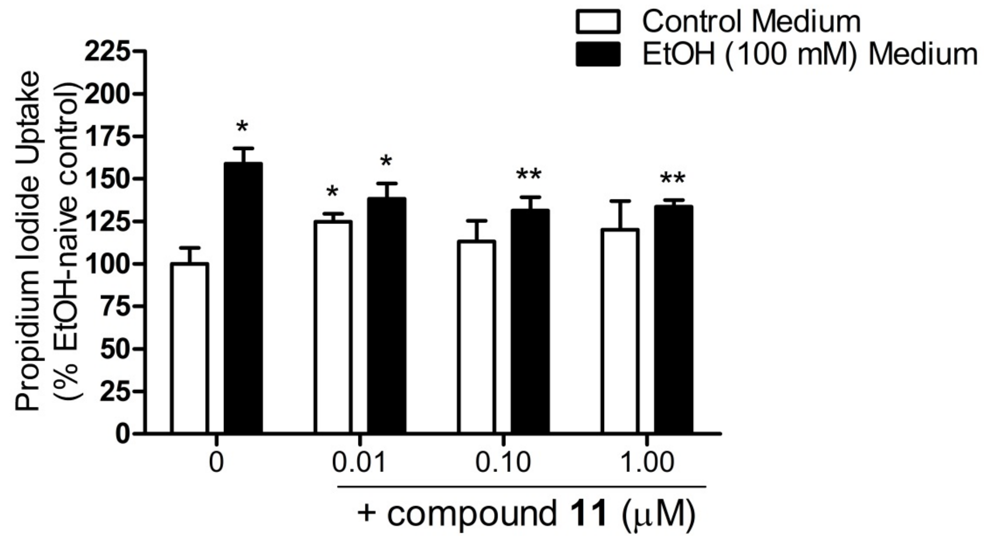


Figure 2.10. (A) Effects compound **10** on propidium iodide (PI) uptake at 48 h in EtOH-naïve DMSO CTRL and EtOH (100) mM treated organotypic hippocampal slices cultures. Compound **10** demonstrated cytotoxic effects in CTRL cultures at the lowest (0.01 μM) concentration at 48 h. Exposure to 100 mM EtOH for 48 h resulted in significant increases of PI uptake compared to CTRL values within hippocampus. This increase was attenuated by co-exposure to **10** at all concentrations (0.01-1.0 μM). * = $p < 0.05$ vs CTRL; ** = $p < 0.05$ vs EtOH, $n = 8$. (B) Representative images of the effects of 48 h exposure to EtOH (100 mM) and/or **10** on PI uptake.

A



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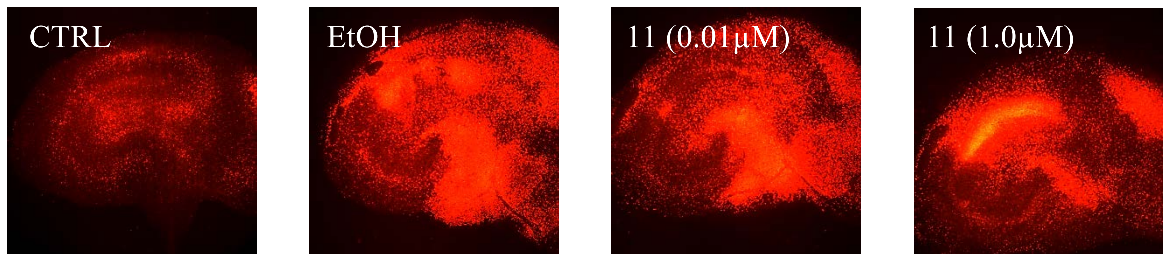


Figure 2.11. (A) Effects compound **11** on propidium iodide (PI) uptake at 48 h in EtOH-naïve DMSO CTRL and EtOH (100) mM treated organotypic hippocampal slices cultures. Compound **11** demonstrated cytotoxic effects in CTRL cultures at the 0.01 μM concentration at 48 h. Exposure to 100 mM EtOH for 48 h resulted in significant increases of PI uptake compared to CTRL values within hippocampus. This increase was attenuated by co-exposure to 0.10 and 1.0 μM of **11**. * = $p < 0.05$ vs CTRL; ** = $p < 0.05$ vs EtOH, $n = 8$. (B) Representative images of the effects of 48 h exposure to EtOH (100 mM) and/or **11** on PI uptake.

2.5. Discussion

Both clinical and pre-clinical studies have demonstrated that excessive EtOH exposure can predict worse neurological outcomes, and that the development of neurobiological deficits due to EtOH may serve to drive the development of AUD or worsen its existing condition (Koob & Le Moal, 1997). Thus, addressing the underlying neurobiological deficits induced by AUD may be an essential feature for an effective pharmacotherapy (Van Skike et al., 2016). Natural product compounds represent an untapped resource in drug R & D for AUD, as the complex chiral structures observed with natural product compounds may provide an enhanced fit to receptors and other target proteins (Mentel et al., 2009). Additionally, approximately 60% of drugs on the market are directly or indirectly derived from natural product sources (Newman, 2008), as natural product screens are associated with a higher hit rate than screens of synthetic product libraries. Specifically, HTS of natural product libraries presents with an average 0.3% hit rate vs 0.001% observed with synthetic compound libraries (Tegos & Mylonakis, 2012). In collaboration with scientists at the University of Kentucky Center for Pharmaceutical Research and Innovation (CPRI), our lab previously screened several compounds from the CPRI natural products repository for their ability to attenuate 48 h EtOH (100 mM)-induced increases in propidium iodide (PI) uptake in a rat OHSC model (Wang et al., 2015; Shaaban et al., 2017; Zhang et al., 2017). As the creation of natural product compound analogs can further increase potency and/or efficacy (Kerns & Di, 2003), the purpose of the present study was to screen novel analogs of previously identified natural product compounds for their ability to attenuate binge-like EtOH-induced hippocampal cytotoxicity *in vitro*.

The hypothesis for the current studies was that analogs of natural products previously shown to attenuate EtOH-induced neurodegeneration or neurobehavioral deficits would attenuate EtOH-induced cytotoxicity with enhanced potency and/or efficacy over their parental compound structures. Our results partially supported our hypothesis, as 9 of the 20 analogs tested were cytoprotective our screens. Interestingly, all hit compound analogs which can be discussed structurally (due to IP limitations, see *Method*) came from 3 parent structure families: oxazole carboxamide (spoxazomicin C and *N*-salicyloyl-2-aminopropan-1-ol), oxachelin, or isoflavonoid. Both oxazole carboxamide and oxachelin structures were isolated from *Streptomyces* sp. RM-14-6, an extremophile bacteria isolate from the underground Ruth Mullins coal mine (Shaaban et al., 2017). Specifically, compound **2a** is an analog of spoxazomicin C, which previously did not show cytoprotection in our model (Shaaban et al., 2017; Zhang et al., 2017). Compound **4a** is not a true analog, but rather the R-enantiomer of *N*-salicyloyl-2-aminopropan-1-ol, which is chiral in structure (Zhang et al., 2017). As mentioned previously, chiral structures are asymmetric. Due to this, the compound can exist in different configurations based on planes of symmetry, which chemists denote as the "right hand" (R) and left hand (S)-enantiomer (Cahn, Ingold, & Prelog, 1966). Compound **4b** was the S-enantiomer, which demonstrated no cytoprotective effects in our model. This finding is interesting as the effects of drugs such as baclofen (Lorrai, Maccioni, Gessa, & Colombo, 2016), bPiDI (Maggio et al., 2018), and modafinil (Maggio et al., 2018) on EtOH self-administration in rats has been shown to be enantiomer dependent, specifically showing greater efficacy with the R-enantiomer. Notably, both of these compounds are structurally related to spoxazomicin D, which

previously demonstrated potent neuroprotective effects in our model (Shaaban et al., 2017), but was advanced to *in vivo* screening for EtOH self-administration in alcohol preferring (P) rats and had no effect on drinking behavior (unpublished data). Unfortunately, spoxamicin D is a novel isolate (Shaaban et al., 2017), and very little is known about the therapeutic targets of related spoxazomicins A-C, beyond their anti-parasitic effects (Inahashi et al., 2011).

Also isolated from *Streptomyces* sp. RM-14-6, oxachelin previously demonstrated no cytoprotective effects in our model. However, the oxachelin analog, compound **5a,** was cytoprotective against EtOH-induced cytotoxicity at all concentrations tested (Shaaban et al., 2017; Zhang et al., 2017). A related compound, oxachelin C, did demonstrate potent cytoprotective effects against EtOH-induced neurotoxicity in our model (Shaaban et al., 2017); however, it did not pass *in silico* BBB screens, suggesting oxachelin C would not be successful at prevention EtOH-induced neurobiological or neurobehavioral effects *in vivo*. Oxachelin is known to chelate (i.e., bind and remove) iron (Sontag et al., 2006), and pathological accumulation of iron in the brain is a hallmark of nearly all neurodegenerative diseases (Dusek, Schneider, & Aaseth, 2016). Indeed, a positive-feedback relationship between oxidative stress, neuroimmune signaling, iron accumulation, and neurotoxicity has been proposed (Urrutia, Mena, & Núñez, 2014). Thus, future studies should evaluate whether compound **5a** may exert its neuroprotective effects through its action on iron levels.

While we have not previously screened isoflavonoid compounds in our lab, prior research has found that administration of isoflavonoids isolated from the kudzu plant (e.g., puerarin, daidzin, daidzein [DZ]) reduce EtOH intake in both rodents (Lin & Li,

1998; Benlhabib, Baker, Keyler & Singh, 2004) and humans (Lukas et al., 2005; 2013; Penetar et al., 2012; Penetar, Toto, Lee, & Lukas, 2015). The ability of isoflavonoids to reduce the *in vitro* excitotoxic effects (Lutz et al., 2015a) and *in vivo* anxiogenic effects of EtOH withdrawal has also been observed (Overstreet et al., 2003; Benlhabib et al., 2004). However, the effect of these compounds on EtOH-exposure induced neurotoxicity has not been explored. Isoflavonoid analog compounds **9b** and **9d** both demonstrated potent cytoprotective effects in our model. Notably, isoflavonoids are reported to exert a myriad of effects within the CNS (e.g., estrogen receptor binding, anti-inflammatory, antioxidant, cytoprotective) (Miadoková, 2009; Wozniak, Janda, Kapusta, Oleszek, & Matkowski 2010; Sakran, Selim, & Zidan, 2014; Aras et al., 2015). Interestingly, 4 structurally-related compounds (**9a**, **9c**, **9e**, **9f**) did not exert cytoprotection in our model. As all compounds retained a similar composition of hydroxyl (OH) groups in Ring B at both R3′ and R5′, and these structural substitutions from the basic common isoflavonoid structure modulate different isoflavonoid antioxidant effects (Promden, Monthakantirat, Umehara, Noguchi, & De-Eknamkul, 2014), compound **9b** and **9d** likely exert their cytoprotective effects in our model via another mechanism. Interestingly, compound **9b** and **9d** only diverges structurally from the other analogs via their R8 (ring A) and R4’ (ring B) substitution pattern (Figure 2.1). Compound **9b** and **9d** both share the addition of fluorine at R4′, and fluorinated natural products have been shown to increase metabolic stability, binding affinity, lowering of surface tension, hydrophobicity, and lipophobicity (for a review, see Monsen & Luzzio, 2017). However, **9b** and **9d** differ in that compound **9b** contains the addition of a piperidine at R8 (**9d** has a hydrogen at this position). While **9e** also has this piperidine, but shows no cytoprotection (but it lacks a fluorine), it is still

interesting as piperidine is conserved in lobeline, and sigma-1 receptor (σ1R) antagonists; and lobeline and σ1R antagonists have been shown to reduce EtOH consumption in rodents *in vivo* (Bell, Eiler II, Cook, & Rahman, 2009; Farook, Lewis, Gaddis, Littleton, & Barron, 2009; Sabino et al., 2009). Lobeline primarily acts as an agonist at the nicotinic α4β2 subunit, and activity at this receptor has been shown to decrease NF-κB and production of the pro-inflammatory cytokines IL-1β and IL-6 in SHEP1-hα4β2 cells (Hosur & Loring, 2010). A study by Yao and colleagues (2010) has also demonstrated that administration of σ1R antagonists inhibits activation of NF-κB pathways in BV2 microglia cultures. Furthermore, our lab has demonstrated that σ1R antagonists are protective against CIE-induced neurotoxicity in OHSC (Reynolds et al., 2016). These data suggests that isoflavonoids such as **9b** and **9d** have potential for the use in reducing EtOH-induced neurotoxicity, as well as an important structure-activity relationship between fluorine and piperidine in our model.

Interestingly, while both oxachelin and spoxazomicin C did not pass *in silico* BBB screens prior to cytotoxicity testing in our prior studies (Shaaban et al., 2017), both analog compounds **2a** and **5a** did pass these screens (Zhang et al., 2017). Furthermore, both compound **2a** and **5a** were structurally modified only via glycosylation (the addition of a sugar). Glycosylation of natural product compounds has been previously employed to improve drug potency, mechanism, pharmacodynamic, pharmacokinetic, and ADME properties (Gantt, Peltier-Pain, & Thorson, 2011; Goff & Thorson, 2012; Zhang et al., 2017). However, the current structure-activity relationships also suggest an impact of glycosylation on improving BBB permeability (Egleton et al., 2001; Fichna et al., 2013), which may support future lead optimization studies for compounds intended for the

treatment of neurological deficits due to AUD (Wang et al., 2015; Shaaban et al., 2017; Zhang et al., 2017).

However, a limitation of this study is that CPRI-withholding of parental and analog structures for several hit compounds (**1**, **6**, **7**, **10**, and **11**) to preserve I.P. limits the interpretation of several findings, as well as any decision making towards lead compound advancement. Additionally, each compound was only screened a single time. This is normal practice in initial stage HTS, as testing and replication of very large chemical libraries is burdensome; however, there is the possibility of a false positive or a false negative result (Kelly, 2009). Furthermore, while significant attenuation of EtOH-induced cytotoxicity by the potent antioxidant Trolox has been observed in the current model, and may suggest EtOH exerts its neurodegenerative effects via oxidative stress, no additional markers (e.g., neuroimmune mediators, proteases, NMDAR activation) have been assessed to firmly characterize the mechanism of cytotoxicity within our model (Saunders, 2016). Furthermore, while PI has been correlated with other markers of cell death (Wilkins et al., 2006), analysis of this marker is time dependent, presenting the opportunity for user error. Even though they are more time consuming, employment of specific and reliable markers, such as the neuron-specific marker NeuN (Kim, Adelstein, & Kawamoto, 2009), may help elucidate specific effects of compounds on certain cell types (neurons vs glia), as well as serve to better verify findings of cell death, before advancing a compound into further screening phases.

While the findings from the current study should be interpreted as preliminary, they suggest important structure-activity relationships that may support future target identification (e.g., via incorporation of sugars, fluorine, or piperidine), and given the

impact of glycosylation on improving BBB permeability (Egleton et al., 2001; Fichna et al., 2013), may also aid in informing optimization studies in the context of advancing lead compounds for the treatment of the neurological deficits resulting from AUD (Wang et al., 2015; Shaaban et al., 2017; Zhang et al., 2017). Future research should be conducted to characterize the mechanism by which the 9 lead compounds identified here (**2a**, **4a**, **5a**, **6**, **7**, **9b**, **9d**, **10**, **11**) exert their cytoprotective effects to further explore the potential of these novel compound analogs as potential treatment options in reducing the neurotoxicity caused by EtOH.

CHAPTER THREE: Study Two: Effect of a Novel Isoflavonoid Analog on Binge Ethanol-Induced Neuroimmune Signaling *In Vitro*

3.1. Introduction

There is significant difficulty in developing a successful treatment for AUD, as EtOH does not act at 1 specific target, but rather causes neurotoxicity through several mechanisms, including activation of pro-inflammatory neuroimmune cascades (Blanco et al., 2005; Fernandez-Lizarbe et al., 2009; Alfonso-Loeches et al., 2010; Pascual et al., 2011) and excitotoxicity (Lovinger, 1993; Davis & Wu, 2001; Nagy & Laszlo, 2002; Prendergast et al., 2004). While EtOH-induced excitotoxicity primarily involves interactions with the glutamatergic NMDAR (Lovinger, 1993; Tsai, 1998; Wirkner et al., 1999; Davis & Wu, 2001; Nagy & Laszlo, 2002; Ron, 2004), several reports suggests that these interactions may be secondary to the induction of persistent neuroimmune activation (for a review, see Crews et al., 2015); therefore, therapeutic strategies which address this mechanism could be effective at ameliorating the toxicity of both pathologies. Increased markers for the pro-inflammatory transcription factor, nuclear factor kappa-light-chain-enhancers of activated B cells (NF-κB) (Liu et al., 2006; Okvist et al., 2007) and upregulated neuroimmune gene expression have also been observed (He and Crews, 2008). In pre-clinical studies, activation of a second pro-inflammatory transcription factor, activator protein 1 (AP-1), has also been observed in response to EtOH (Fried et al., 2001; Blanco, et al., 2005). Both NF-κB and AP-1 are known to increase the expression of cytokines (TNF-a, IL-1β, IL-6, MCP-1), and these effects are associated with inflammation and cell death (for a review, see Blanco and Guerri, 2006). Alfonso-Loeches and colleagues (2010) found that activation of NF-κB in response to

chronic EtOH exposure was associated with increased levels of IL-1β, TNF-α, IL-6, and several oxidases (e.g., COX-2 and iNOS) in the cerebral cortex of female wide-type mice. This increase in COX-2 and iNOS by NF-κB could lead to oxidative stress through the generation of ROS (Sun et al., 2001). As ROS can directly activate NF-κB, oxidative stress may also contribute to the persistent innate immune response observed following chronic EtOH consumption (for a review, see Crews et al., 2013).

While several mechanisms may be involved in the upstream signaling which results in NF-κB transcription, evidence suggests EtOH-induced activation of NF-κB is dependent on signaling through toll-like receptors (TLRs). In the previously mentioned study by Alfonso-Loeches and colleagues (2010), knockdown of TLR4 abolished the activation of NF-κB pathways and induction of inflammatory mediators in primary astrocyte cultures. The TLR pathway is highly conserved in rodents and humans and represents the canonical mechanism of NF-κB activation (Hoesel & Schmid, 2013). Protein expression for several TLRs (i.e. TLR2, TLR3, TLR4) and high-mobility group box 1 (HMGB1), an endogenous danger signaling cytokine and TLR4 agonist, is upregulated following chronic EtOH exposure in mice, rat brain slice cultures, and the post-mortem brains of alcoholics (Crews et al., 2013). HMGB1 is known to stimulate TLR4 and lead to the production of ROS and downstream activation of NF-κB (for a review, see Tang et al., 2011). HMGB1 has also been shown to induce ROS and NF-κB activation via action at the receptor for advanced glycation end-products (RAGE), although the role of this receptor in EtOH-induced neuroinflammation is less clear (Tang, et al., 2011; Crews et al., 2015). Notably, brain slice cultures do show that EtOH-induces signaling through HMGB1-TLR4, resulting in IL-1β release; further, neutralizing

antibodies to HMGB1 or siRNAs against HMGB1-TLR4 attenuate this EtOH-dependent induction of IL-1β (Crews et al., 2013). Ultimately, this upregulation in pro-inflammatory mediators has been associated with an increased expression of "pro death signals" (e.g., caspase 3 and calpain) and apoptotic cell death (Vallés et al., 2004; Zhao et al., 2015). However, apoptosis is not observed in EtOH-exposed microglia and astroglia deficient in TLR4 or in TLR4 knockout mice (Blanco et al., 2005; Fernandez-Lizarbe et al., 2009; Alfonso-Loeches et al., 2010; Pascual et al., 2011). This suggests a direct role of TLR4-dependent neuroimmune signaling in the promotion EtOH-induced neurotoxicity. Furthermore, TLR4 knockout mice exhibit less EtOH-induced cognitive and motor impairments than wild type mice (Pascual et al., 2011) and reduced self-administration of EtOH is observed in alcohol preferring (P) rats following amygdala-specific TLR4 inhibition (Liu et al., 2011).

3.2. Experimental Rationale

These studies suggest a role for HMGB1-TLR4 in EtOH-induced excitotoxicity, neurodegeneration, and behavioral deficits. Isoflavonoids are polyphenolic compounds which can be found in several legumes (soy, peanuts, chick peas) (Barnes, 2010). Isoflavonoids readily distribute to all tissues, including the brain (Chandrasekharan & Aglin, 2013), and are known influence several processes within CNS, including estrogen receptor binding, neuroimmune signaling, intracellular redox status, and to have antioxidant and cytoprotective properties (Miadoková, 2009; Wozniak et al., 2010; Sakran et al., 2014; Aras et al., 2015; Subedi et al., 2017). Additionally, prior research has found that the administration of isoflavonoids (e.g., puerarin, daidzin, daidzein [DZ])

reduces EtOH intake in both rodents (Lin & Li, 1998; Benlhabib et al., 2004) and humans (Lukas et al., 2005; 2013; Penetar et al., 2012; 2015). The ability of isoflavonoids to reduce the *in vitro* cytotoxic (Lutz et al., 2015a) and *in vivo* anxiogenic effects of EtOH withdrawal has also been observed (Overstreet et al., 2003; Benlhabib et al., 2004). Although the precise mechanism by which isoflavonoids attenuate consumption is not yet known, the anti-inflammatory capacity of these compounds may underlie their protective properties. Isoflavonoids have been shown to directly scavenge reactive oxygen, nitrogen, and chlorine species (Boersma et al., 1999; Patel et al., 2001). Moreover, oral administration of the isoflavonoids genistein and DZ decreased serum RNS levels in LPS-challenged rats (Yen & Lai, 2003), suggesting that isoflavonoids scavenge increased free radicals produced during inflammation and may prevent persistent neuroimmune activation and resultant cell damage. However, independent of their antioxidant effects, isoflavonoids have been shown to modulate neuroimmune signaling through upstream regulation of the DNA-binding capacity of both NF-κB and AP-1 (Park et al., 2007; Chinta, Ganesan, Reis-Rodrigues, Lithgow, & Andersen, 2013) and inhibition of the production of cytokines and chemokines such as IL-1β, IL-6, IL-12, and TNF-α in diverse models of inflammation *in vitro* (Morimoto, Watanabe, Yamori, Takebe, & Wakatsuki, 2009; Ji et al., 2012; Gao et al., 2014; Lutz et al., 2015a), *in vivo* (Paradkar Blum, Berhow, Baumann, & Kuo, 2004; Ganai, Khan, Malik, & Farooqi, 2015), and in humans (Lesinski et al., 2015). Furthermore, the inhibitory effect of DZ on IL-6 and IL-8 in monocytes may be mediated through its action at the TLR2 and TLR4 receptors (Morimoto et al., 2009). In a recent study, Subedi and colleagues (2017) found that equol, a DZ gut metabolite, inhibited LPS-induced TLR4 activation, MAPK activation,

NF-kB-mediated transcription of inflammatory mediators, NO production, and secretion of TNF-α and IL-6 from murine microglia cells. A downregulation of neuronal apoptosis was also observed in LPS-treated N2a cells co-exposed to equol (Subedi et al., 2017).

In Study 1 we identified a novel isoflavonoid analog which attenuated 48 h binge EtOH-induced cytotoxicity at 0.01 and 0.10 μM (the lowest concentrations tested). Further, 4 structurally-related compounds (**9a**, **9c**, **9e**, **9f**) did not demonstrate this efficacy in our model. As all compounds retained a similar composition of hydroxyl (OH) groups in Ring B at both R3′ and R5′, and these structural substitutions from the basic common isoflavonoid structure modulate different isoflavonoid antioxidant effects (Promden et al., 2014), compound **9b** likely exerts its cytoprotective effects in our model via another mechanism. Compound **9b** only diverges structurally via its R8 (ring A) and R4' (ring B) substitution pattern (Study 1, Figure 2.1). Notably, the addition of fluorine at R4′ is interesting, as fluorinated natural products display beneficial biological properties, such as increased metabolic stability, binding affinity, lowering of surface tension, hydrophobicity, and lipophobicity (for a review, see Monsen & Luzzio, 2017). The addition of a piperidine at R8, while not unique to compound **9b**, is also interesting as it conserved in lobeline, and σ-1 receptor (σ1R) antagonists; and lobeline and σ1R antagonists have been shown to reduce EtOH consumption in rodents *in vivo* (Bell et al., 2009; Farook et al., 2009; Sabino et al., 2009). Lobeline primarily acts as an agonist at the nicotinic α4β2 subunit, and activity at this receptor has been shown to decrease NF-κB and production of the pro-inflammatory cytokines IL-1β and IL-6 in SHEP1-hα4β2 cells (Hosur & Loring, 2010). A study by Yao and colleagues (2010) has also

demonstrated that administration of σ1R antagonists inhibits activation of NF-κB pathways in BV2 microglia cultures. Furthermore, our lab has demonstrated that σ1R antagonists are protective against CIE-induced neurotoxicity in OHSC (Reynolds et al., 2016).

Previous studies have used the OHSC model for the examination of EtOH-induced neurodegeneration (Collins, Zou, & Neafsey, 1998; Bulter et al., 2013; Reynolds et al., 2015a) and neuroimmune signaling (Moon et al., 2014; Lutz et al., 2015a,b). OHSCs provide a good model for the examination of EtOH-induced changes on neuroimmune signaling as neuron-glia interactions remain intact in this model (Benediktsson et al., 2005; Haber et al., 2009; Dailey & Waite, 1999). Therefore, in the present study, we evaluated a lower concentration range of compound **9b** (0.0001-0.01 μM) for inhibitory effects on EtOH-induced hippocampal cytotoxicity and altered neuroimmune signaling *in vitro* using the exposure method described in Study 1, to establish dose-response relationships. As **9b** was cytoprotective at 0.01 μM in Study 1, and isoflavonoids have been shown to have dose dependent anti-apoptotic actions down to 0.05 μM in a previous study (Adams et al., 2012), we expected 0.01 μM to remain the most cytoprotective concentration. As DZ is a prototypical isoflavonoid, and has been previously shown to reduce EtOH intake (Lin & Li, 1998; Benlhabib et al., 2004; Lukas et al., 2005; 2013; Penetar et al., 2012; 2015) and modulate neuroimmune signaling (Morimoto et al., 2009; Subedi et al., 2017), it was used as a comparison compound. Based on these previous studies, we hypothesized that co-exposure to DZ would inhibit EtOH-induced pro-inflammatory mediator release into medium during a 48 h EtOH exposure. Additionally, as **9b** exhibited structural similarities to lobeline and σ-1R

antagonist, compounds which have been shown to attenuate neuroimmune signaling (Hosur & Loring, 2010; Wu et al., 2015), and were unique from DZ, we hypothesized that **9b** might have more anti-inflammatory potential than DZ.

3.3. Method

The following section closely follows methods detailed in Chapter 2. For the reader's convenience, changes and additions have been underlined.

3.3.1. 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 thickness 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, 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 method produces 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.

3.3.2. EtOH and Compound Treatment

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 1 mL of culture medium with 0.01% DMSO (DMSO vehicle control [CTRL]; Fisher) or 1 mL of culture medium with the addition of 0.01% DMSO and 100 mM EtOH. Additional cultures were exposed to CTRL or EtOH (100 mM) medium with the addition of compound **9b** or DZ (diluted to 0.01% DMSO as previously describe). As the previous study revealed cytoprotective effects at the lowest concentration tested (0.01 μM), a lower concentration range (0.0001-0.01 μM) was used in the current study to better characterize the effects of this compound. All culture media also received the addition of propidium iodide (PI; 7.48 μM) during the 48 h exposure (discussed below in

Measurement of Cytotoxicity via Propidium Iodide Staining). 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. CTRL groups received similar treatment, without the addition of (receiving EtOH-naïve 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.

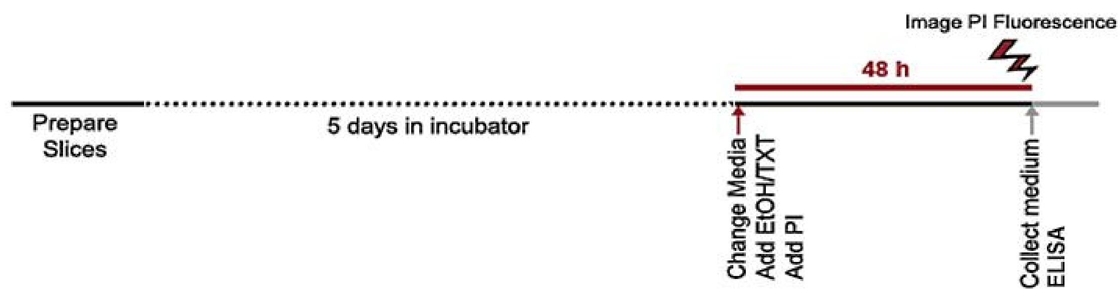


Figure 3.1. Diagram showing the timeline of experimental procedures and treatments.

3.3.3. Measurement of Cytotoxicity via Propidium Iodide Staining

Similar to Study 1, all cultures received the addition of propidium iodide (PI; 7.48 μM) during the 48 h exposure. Propidium iodide (PI) allows for the quantification of cell death 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). Once 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 (e.g. NeuN, GFAP, BrdU) *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). Densitometry using Image J software (National Institutes of Health, Bethesda, MD) was used to measure the intensity of the PI fluorescence within the entire hippocampal slice. A background measurement of fluorescence was taken from the visual field surrounding each slice and was subsequently subtracted from the measurement of each slice before analysis. To control for the variability between each screen, 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 slice (after Mulholland et al., 2005).

3.3.4. ELISA Measurements of HMGB1, TNF-α, IL-6, and IL-10 Release

Following the assessment of PI uptake in hippocampal cultures, slices were discarded and pooled culture media was collected for use in Enzyme-Linked Immunosorbent Assay (ELISA). Sample media were stored at -80°C until use. Previous studies have found that binge EtOH exposure increases TNF-α mRNA and induces release of HMGB1 into hippocampal-entorhinal (HEC) slice culture medium (Crews et al., 2013; Coleman, Zou, & Crews, 2017). *In vivo* studies also suggest a role for IL-6 in EtOH-induced neurological and neurobehavioral deficits (Heberlein et al., 2014; Zago et al., 2016 Casachahua, 2016). Thus, the presence of released HMGB1 (NeoBioLab, #RH0016), TNF-α (Invitrogen, #KRC3011C), and IL-6 (R & D Systems kit, #R6000B) was determined via ELISA according to the manufacturer's instructions. HMGB1 can lead to the initiation of pro- and anti-inflammatory cascades dependent on its redox state (Venereau et al., 2012), thus, levels of the prototypical anti-inflammatory cytokine IL-10 (Invitrogen, #KRC0101) were also assessed. While no direct model translation has been characterized in hippocampal slice cultures, Lutz et al. (2015a,b) found that exposure of hippocampal slice cultures to LPS (10 ug/mL) produced increases in TNF-α release into culture medium at 24 h that persisted through hour 72. As LPS has been demonstrated to induce all of our neuroimmune mediators of interest, LPS (10 ug/mL; Lutz et al., 2015a,b) was used as a positive control for all assays except IL-6, for which R & D systems provided a specific positive control. All standards and samples were run in duplicate. Absorbance was measured at 450 nm using a Beckman Coulter DTX 880 Mulitmodal Detector (Lagerhausstrasse, Austria) with Beckman Coulter Multimode Detection Software (v.20.0.12). Mean concentrations of inflammatory mediators for each

sample was determined based upon a standard curve (defined by known samples provided by each kit) that was analyzed along with the experimental samples. To control for the variability between each screen, cytokine concentrations are reported as percent of control.

3.3.5. Statistical Analysis

Statistical analyses were conducted to assess the inhibitory effect of compound **9b** and DZ on EtOH-induced cytotoxicity and neuroimmune signaling. Study 2 was conducted two times, using two different rat litters. Data from each cytotoxicity screen were converted into percent control values, yielding a total 16 slices per treatment group (EtOH treatment × compound concentration). Data from each ELISA assay were converted into percent control values, yielding a total of three pooled media samples per treatment group (EtOH treatment × compound concentration). Data generated from the above method was analyzed using a 2-factor ANOVA (EtOH treatment × compound concentration), conducted in Prism 5.0 (Graph Pad Software Inc., San Diego, CA, USA). Similar to Study 1, the effect of sex was not assessed. When appropriate, post-hoc tests were conducted using Tukey. The level of significance was set at $p < 0.05$.

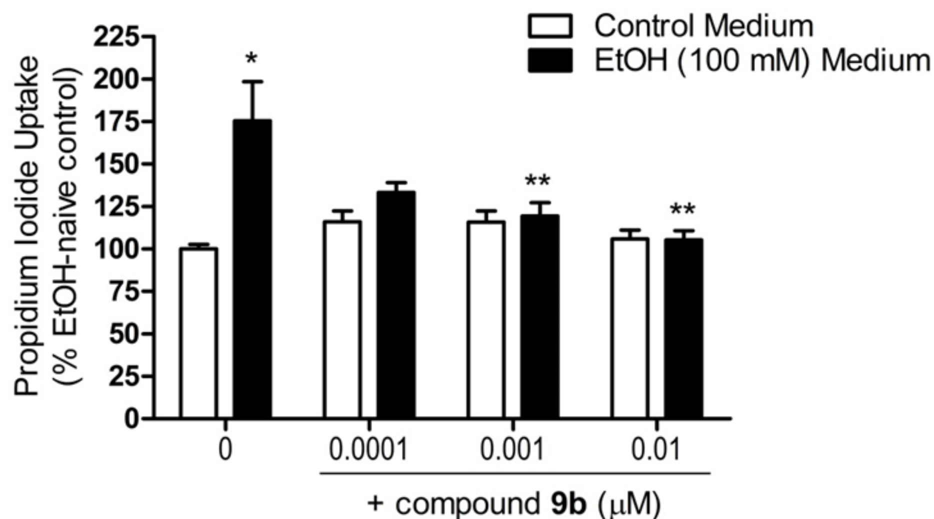
3.4. Results

3.4.1. Co-Exposure to Compound 9b or Daidzein Attenuates PI-Uptake Following 48 h EtOH-Exposure

Studies were conducted to examine the effects of compound **9b** (0.0001-0.01 μM) for its ability to attenuate EtOH (100mM)-induced cytotoxicity in OHSC, as determined

by uptake of PI at 48 h. DZ (0.0001-0.01 μM), a prototypical isoflavonoid which is known to reduce EtOH intake and withdrawal *in vivo* and *in vitro* (refer to chapter introduction), was used as a comparison compound. The effects of **9b** and DZ on EtOH-naïve treated hippocampal explants were also examined. For statistical analyses, a 2-factor ANOVA was conducted (EtOH treatment x compound concentration) to establish any concentration-response relationships in **9b** or DZ treated tissue. Consistent with our previous natural product screens (Wang et al., 2015; Shaaban et al., 2017) and Study 1, EtOH (100 mM) exposure for 48 h resulted in significant uptake of PI compared to CTRL treated cultures for both experiments ($p < 0.001$). For compound **9b**, within the hippocampus, a significant main effect of EtOH treatment [$F(1,116)=11.32$, $p < 0.001$] and compound concentration [$F(3,116)=3.56$, $p < 0.05$] was observed. Additionally, a significant interaction between EtOH treatment x compound concentration was detected in cultures co-exposed to **9b,** $F(3,116)=6.21$, $p < 0.001$. Post hoc analyses revealed co-exposure to **9b** significantly attenuated EtOH-induced PI-uptake at 0.001 and 0.10 μM ($p < 0.05$), and had no cytotoxic effects in EtOH-naïve CTRL cultures (Figure 3.2). Similarly, for DZ, a significant main effect of EtOH treatment [$F(1,120)=4.55$, $p < 0.05$] and compound concentration [$F(3,120)=4.04$, $p < 0.01$] was observed, as well as a significant EtOH treatment x compound concentration interaction, $F(3,120)=8.65$, $p < 0.0001$. Post hoc analyses revealed co-exposure to DZ significantly attenuated EtOH-induced PI-uptake at all concentrations tested (0.0001-0.10 μM ($p < 0.05$). Notably, DZ was found to have significant cytotoxic-effects in EtOH-naïve CTRL cultures at the 0.001 μM concentration ($p < 0.05$) (Figure 3.3).

A



B

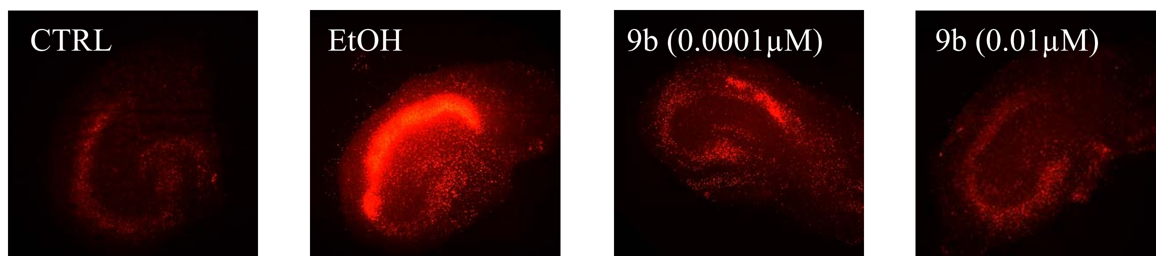
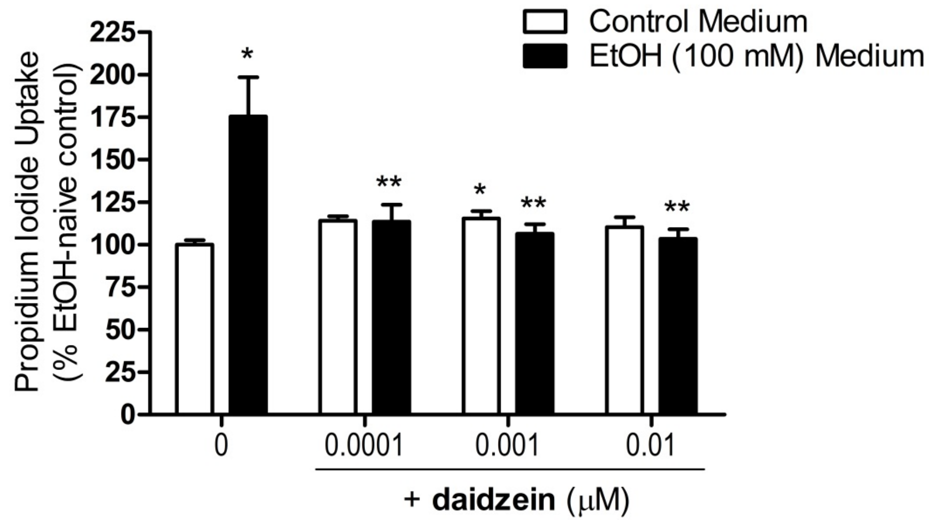


Figure 3.2. (A) Effect of compound **9b** on propidium iodide (PI) uptake at 48 h in EtOH-naïve DMSO CTRL and EtOH (100 mM)-treated organotypic hippocampal slices cultures. Compound **9b** demonstrated no cytotoxic effects in CTRL cultures at 48 h. Exposure to 100 mM EtOH for 48 h resulted in significant increases of PI uptake compared to CTRL values within hippocampus. This increase was attenuated by co-exposure to 0.001 and 0.01 μM of compound **9b.** * = $p < 0.05$ vs CTRL; ** = $p < 0.05$ vs EtOH, n = 15-16. (B) Representative images of the effects of 48 h exposure to EtOH (100 mM) and/or compound **9b** on PI uptake.

A



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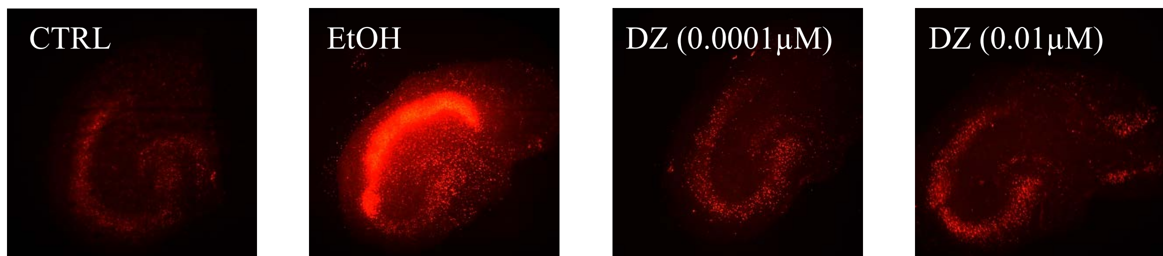

Figure 3.3. (A) Effect of daidzein (DZ) on propidium iodide (PI) uptake at 48 h in EtOH-naïve DMSO CTRL and EtOH (100 mM)-treated organotypic hippocampal slices cultures. DZ demonstrated cytotoxic effects in CTRL cultures at the 0.001 μM concentration at 48 h. Exposure to 100 mM EtOH for 48 h resulted in significant increases of PI uptake compared to CTRL values within hippocampus. This increase was attenuated by the co-exposure to all concentrations (0.0001-0.01 μM) of DZ. * = $p < 0.05$ vs CTRL; ** = $p < 0.05$ vs EtOH, n = 15-16. (B) Representative images of the effects of 48 h exposure to EtOH (100 mM) and/or DZ on PI uptake.

3.4.2. Co-Exposure to Compound 9b or Daidzein Attenuates HMGB1 and IL-6 Release Following 48 h EtOH-Exposure

To investigate whether compound **9b** or DZ may exert their effect via modulation of neuroimmune mediators, ELISAs were performed on the 48 h culture media for HMGB1, TNF-α, IL-6, and IL-10. For statistical analyses, a 2-factor ANOVA was conducted (EtOH treatment x compound concentration) to establish any concentration-response relationships. Initial data cleaning revealed no detectable levels (when compared to the ELISA standard curve) of TNF-α or IL-10 in any group except for the LPS (10 ug/mL) positive control group. Thus, no statistical analyses were conducted on these data. Consistent with previous data indicating and dose-dependent 85% increase in HMGB1 release following a 48 h exposure to EtOH (25-100mM) in rat HEC slice cultures (Coleman et al., 2017), and IL-6 is increased following EtOH consumption both *in vivo* (Alfonso-Loeches et al., 2010) and in human (Heberlein et al., 2014; Zago et al., 2016), EtOH (100mM) exposure for 48 h resulted in significantly higher HMGB1 and IL-6 release into EtOH medium compared to EtOH-naïve CTRL medium ($p < 0.05$). For compound **9b**, analyses on media for HMGB1 release revealed a significant main effect of compound concentration, $F(3,16)=7.16$, $p < 0.01$. Additionally, a significant interaction between EtOH treatment x compound concentration was detected, $F(3,16)=13.97$, $p < 0.0001$. Post hoc analyses revealed that co-exposure to **9b** significantly attenuated EtOH-induced HMGB1 release at all concentrations tested (0.0001-0.10 μM) ($p < 0.05$), and had no effect on HMGB1 release into EtOH-naïve CTRL medium (Figure 3.4a). Analyses on media assessing for **9b**'s effects on IL-6 release revealed a significant main effect of EtOH treatment [$F(1,16)=5.79$, $p < 0.05$] and

compound concentration [$F(3,16)=86.93$, $p < 0.0001$]. Additionally, a significant interaction between EtOH treatment x compound concentration was detected, $F(3,16)=29.77$, $p < 0.0001$. Post hoc analyses revealed co-exposure to **9b** significantly attenuated EtOH-induced IL-6 release at all concentrations tested (0.0001- 0.10 µM) ($p < 0.05$). Further, this reduction of IL-6 by **9b** was to a level below that of both EtOH-naïve CTRL and **9b** treated EtOH-naïve CTRL media ($p < 0.05$). Compound **9b** co-exposure had no significant effect on IL-6 release into CTRL medium when compared to the naïve-CTRL (Figure 3.5a). For DZ, analyses on media for HMGB1 release revealed a significant both a significant main effect of EtOH treatment [$F(1,16)=22.67$, $p < 0.001$] and compound concentration [$F(3,16)=6.30$, $p < 0.01$]. Additionally, a significant interaction between EtOH treatment x compound concentration was detected, $F(3,16)=3.79$, $p < 0.05$. Post hoc analyses revealed that co-exposure to DZ significantly attenuated EtOH-induced HMGB1 release at all concentrations tested (0.0001- 0.10 µM) ($p < 0.05$), and had no effect on HMGB1 release into EtOH-naïve CTRL medium (Figure 3.4b). Analyses on media assessing for DZ's effects on IL-6 release revealed a significant main effect of EtOH treatment [$F(1,16)=383.41$, $p < 0.0001$] and compound concentration [(F(3,16)=415.73, $p < 0.0001$]. Additionally, a significant interaction between EtOH treatment x compound concentration was detected, $F(3,16)=115.86$, $p < 0.0001$. Post hoc analyses revealed co-exposure to DZ significantly attenuated EtOH-induced IL-6 release at all concentrations tested (0.0001- 0.10 µM) ($p < 0.001$). Further, this reduction of IL-6 by DZ was to a level below that of both EtOH-naïve CTRL and DZ treated EtOH-naïve CTRL media ($p < 0.001$). DZ co-exposure had no effect on IL-6 release into CTRL medium when compared to the naïve-CTRL (Figure 3.5b).

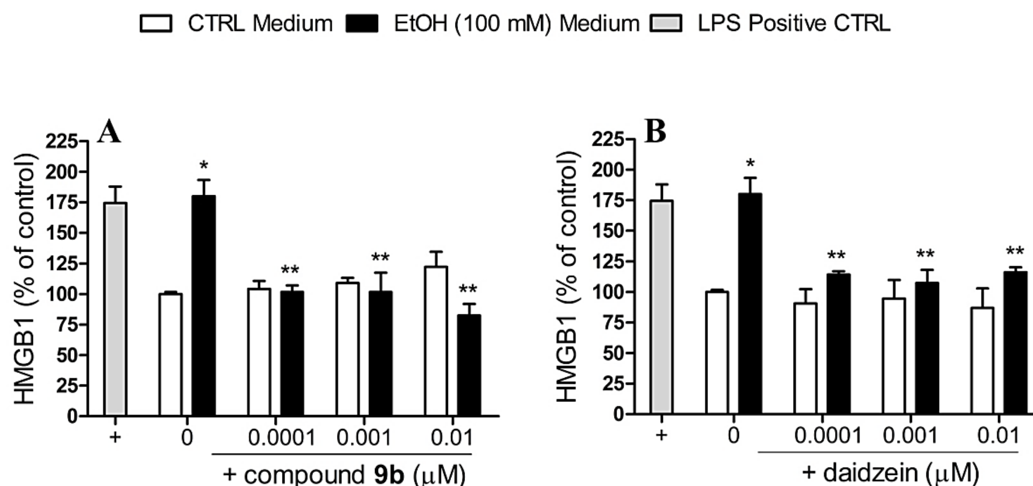

Figure 3.4. Effects of compound (A) **9b** and (B) daidzein on HMGB1 release into the media of EtOH-naïve DMSO CTRL and EtOH (100 mM)-treated organotypic hippocampal slices cultures. Exposure to EtOH (100mM) for 48 h resulted in significantly higher HMGB1 release into medium compared to CTRL medium. (A) **9b** significantly attenuated EtOH-induced HMGB1 release into culture medium at all concentrations tested (0.0001- 0.10 μM), and had no effects on HMGB1 release into CTRL medium. (B) Daidzein significantly attenuated EtOH-induced HMGB1 release into culture medium at all concentrations tested (0.0001- 0.10 μM), and had no effects on HMGB1 release into CTRL medium. LPS (10 ug/mL) was used as a positive control. * = $p < 0.05$ vs CTRL; ** = $p < 0.05$ vs EtOH, $n = 3$.

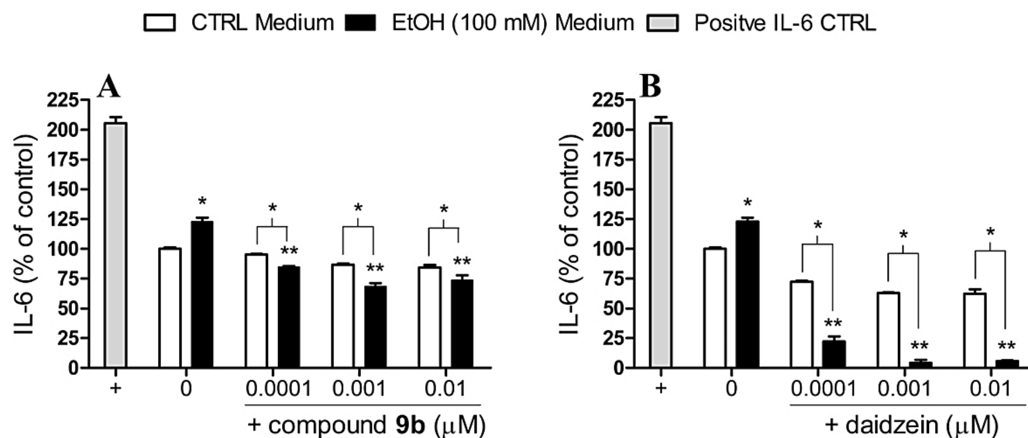

Figure 3.5. Effects of compound (A) **9b** and (B) daidzein on IL-6 release into the media of EtOH-naïve DMSO CTRL and EtOH (100 mM)-treated organotypic hippocampal slices cultures. Exposure to EtOH (100mM) for 48 h resulted in significantly higher IL-6 release into medium compared to CTRL medium. (A) **9b** significantly attenuated EtOH-induced IL-6 release into culture medium at all concentrations tested (0.0001- 0.10 μM), and had no effects on IL-6 release into CTRL medium. (B) Daidzein significantly attenuated EtOH-induced IL-6 release into culture medium at all concentrations tested (0.0001- 0.10 μM), and had no effects on IL-6 release into EtOH-naïve CTRL medium. Both compounds reduced EtOH-induced increases in IL-6 release levels below that observed in CTRL and compound-treated CTRL media. A kit provided (R & D Systems) IL-6 positive control was used. * = $p < 0.05$ vs CTRL or corresponding compound CTRL; ** = $p < 0.05$ vs EtOH, $n = 3$.

3.5. Discussion

Screens for natural product analogs from our lab identified a novel isoflavonoid analog, compound **9b**, which completely attenuated 48 h EtOH-induced cytotoxicity in hippocampal slice cultures (Study 1). Several isoflavonoids (e.g., puerarin, daidzin, DZ) have been shown to reduce EtOH intake *in vivo* (Lin & Li, 1998; Benlhabib et al., 2004) and in humans (Lukas et al., 2005; 2013; Penetar et al., 2012; 2015). Although the precise mechanism by which isoflavonoids attenuate consumption is not yet known, recent studies suggests that DZ may inhibit pro-inflammatory neuroimmune signaling through action at TLR4 (Morimoto et al., 2009; Subedi et al., 2017). HMGB1-TLR4 signaling and downstream activation of neuroimmune mediators has been shown to contribute to EtOH-induced excitotoxicity, neurodegeneration, and behavioral deficits (Blanco et al., 2005; Fernandez-Lizarbe et al., 2009; Alfonso-Loeches et al., 2010; Pascual et al., 2011; Liu et al., 2011). Thus, the present studies sought to evaluate the cytoprotective, and potentially anti-inflammatory, properties of compound **9b** in the same *in vitro* model of EtOH-induced cytotoxicity, with the addition of a neuroimmune component. However, as **9b** was found to have its greatest cytoprotective effect against EtOH-induced cytotoxicity at 0.01 μM (the lowest concentration tested in Study 1; Figure 2.8), lower concentrations were evaluated to establish a dose-dependent response. Specifically, OHSC were co-exposed to EtOH and **9b** for 48 h, at which point cultures were assessed for PI uptake, a measure of cell death, and the release of inflammatory mediators (HMGB1, TNF-α, IL-6, IL-10) into the culture medium. In prior studies, including Study 1, we found that a 48 h EtOH exposure results in significant cytotoxicity (Wang et al., 2015; Shaaban et al, 2017). While prior studies have demonstrated dose-dependent increases in HMGB1

release and TNF-α mRNA following a 48 h exposure to EtOH (25-100mM) in rat HEC slice cultures (Coleman et al., 2017), the interactions between EtOH and these mediators in OHSC, as well as the impact of isoflavonoids such as **9b** on these responses, is not yet known. Thus, as the effects of DZ have been evaluated in other models of AUD (Lin & Li, 1998; Benlhabib et al., 2004; Lukas et al., 2005; 2013; Penetar et al., 2012; 2015) and has known effects on neuroimmune signaling (Morimoto et al., 2009; Subedi et al., 2017), it was used as a comparison compound to assess any potential value of observed effects of **9b** in our model.

We have previously shown that compound **9b** (0.01 μM) completely attenuates EtOH-induced cytotoxicity at 48 h (Study 1). Therefore, in order to assess dose-dependent responses, Study 2 aimed to characterize the cytoprotective effects of **9b** at a lower concentration range (0.0001-0.01 μM). As the prototypical isoflavonoids daidzein and genistein have been shown to have dose dependent anti-apoptotic actions down to 0.05 μM in a previous study (Adams et al., 2012), we expected 0.01 μM to remain the most cytoprotective concentration. We found that **9b** dose-dependently inhibited PI-uptake; and consistent with our hypothesis, the 0.01 μM co-exposure treatment was the most effective at attenuating EtOH-induced cytotoxicity (Figure 3.2). When compared with DZ (0.0001-0.01 μM), **9b** was slightly less potent. Specifically, DZ was cytoprotective at the 0.0001 μM concentration, compared to **9b,** which was only cytoprotective down to the 0.001 μM concentration (Figure 3.3). These data suggest that isoflavonoids such as **9b** and DZ have potential for the use in reducing EtOH-induced neurotoxicity.

In addition to further characterizing the cytoprotective effects of **9b**, the current study aimed to assess its potential in reducing EtOH-induced release of pro-inflammatory mediators. Previous studies have shown that EtOH exposure upregulates the TL4R agonist HMGB1, which then leads to the activation of downstream signaling cascades and cytokine (e.g., TNF-α, IL-6) release (Crews et al., 2015). Additionally, inhibition of these cascades has been shown to afford neuroprotection (Blanco et al., 2005; Fernandez-Lizarbe et al., 2009; Alfonso-Loeches et al., 2010; Pascual et al., 2011; Wu et al., 2015; Subedi et al., 2017). DZ and its metabolite, eqoul, have been shown to attenuate LPS-induced cytokine release through action at the TLR4 (Morimoto et al., 2009; Subedi et al., 2017). Interestingly, **9b** exhibited structural similarities to lobeline and σ-1R antagonists, compounds which have been shown to attenuate EtOH consumption (Bell et al., 2009; Farook et al., 2009; Sabino et al., 2009) and have inhibitory effects on pro-inflammatory neuroimmune signaling (Hosur & Loring, 2010; Yao et al., 2010), and were unique from DZ. Thus, we hypothesized that co-exposure to **9b** or DZ would inhibit EtOH-induced pro-inflammatory mediator release into medium during a 48 h EtOH exposure. Additionally, due to its unique structural properties, we hypothesized that **9b** might have more anti-inflammatory potential than DZ. In the current study, DZ and **9b** reduced EtOH-induced HMGB1 and IL-6 during EtOH exposure (Figure 3.4; 3.5), but **9b** did not exhibit more anti-neuroinflammatory potential than DZ. Specifically, while **9b** and DZ inhibited the upstream release of HMGB1 at similar potencies (Figure 3.4), DZ co-exposure had a larger effect (Cohen's d= 27.0, 0.01 μM) than **9b** (Cohen's d= 7.5, 0.01 μM) on IL-6 release into media during a 48 h EtOH exposure (Figure 3.5). Notably, isoflavonoids are phytoestrogens, and DZ is known to have a high affinity for estrogen

receptors (Kostelac, Rechkemmer, & Briviba, 2003). Estradiol has been demonstrated to directly reduce NF-κB activity and cytokine production in macrophages *in vitro* (Deshpande, Khalili, Pergolizzi, Michael, & Chang, 1997), and estrogen treatment in male mice has been shown to decrease IL-6 secretion in a model of EtOH-administration and burn injury (Messingham, Heinrich, & Kovacs, 2001). Interestingly, this suggests effects on NF-κB which are independent of HMGB1-TLR4 (Deshpande et al., 1997). Thus, this potentiated effect of DZ on IL-6 release may reflect a greater affinity for estrogen receptor binding compared to compound **9b**; in drug R & D, a lower affinity for estrogen receptors is desirable for pharmacotherapies expected to be administered to female populations (Dinsdale & Ward, 2010). However, future studies should evaluate the estrogen binding affinity of **9b**, as everything discussed here is just speculation. Furthermore, while direct correlations cannot be conducted due to the pooling of media samples, observable disparities in the cytoprotective effects of **9b** and DZ, compared to their inhibition of IL-6 release, suggests that **9b** may be exerting its cytoprotective effects via additional mechanisms not tested in the current study. These findings support the potential use of isoflavonoids such as **9b** and DZ to reduce pro-inflammatory neuroimmune signaling during EtOH exposure.

However, the conclusions of the current study are limited, as only a few neuroimmune markers were measured. The original hypothesis stated that EtOH exposure would enhance pro-inflammatory cytokine release, which in turn would be inhibited by compounds that afforded cytoprotection. As such, we chose to focus on HMGB1 and TNF-α, as these markers have been well established in EtOH models *in vitro* (Crews et al., 2013; Coleman et al., 2017). However, while our hypothesis was

supported by our HMGB1 findings, TNF-α levels were below the limits of detection in our culture media. Thus, we measured IL-6 as an alternative pro-inflammatory cytokine, due to its presence in serum in clinical AUD populations (Heberlein et al., 2014; Zago et al., 2016) and recent findings suggesting binge EtOH intake is dependent on IL-6 *in vivo* (Casachahua, 2016). As HMGB1 can also have anti-inflammatory action based on its redox state (Venereau et al., 2012), we also attempted to measure IL-10 (an anti-inflammatory cytokine). However, similar to TNF-α, IL-10 levels were below the limits of detection in our culture medium. While TNF-α and IL-10 ELISAs have been performed by Lutz and colleagues (2015) using medium from OHSC, suggesting these mediators can be detected in culture medium, initial assays for these ELISAs lacked a positive control (R & D systems provided an IL-6 positive control with kit). Thus, an additional post-hoc study was conducted to assess the effect of LPS (10 ug/mL) on release of HMGB1, TNF-α, and IL-10 into control medium. Unfortunately, while LPS (10 ug/mL) did produce detectable levels of release in all assays, both the TNF-α and IL-10 ELISAs presented with low standard signal, complicating the interpretation of their results. The low standard signal may have been due to the use of previously opened and re-sealed ELISA kits. Future studies should evaluate these, and additional, neuroimmune markers in order to further assess how neuroimmune signaling is affected by EtOH exposure and isoflavonoid treatment.

In summary, compound **9b** reduced EtOH-induced cytotoxicity and neuroimmune signaling during a 48 h exposure period. However, contrary to our hypothesis, **9b** was not as potent as the comparison compound DZ. Future studies should continue to investigate the possible therapeutic role of neuroimmune signaling modulation during EtOH

exposure, as well as the potential use of isoflavonoid compounds such as **9b** and DZ for the treatment of EtOH-induced neurotoxicity.

CHAPTER FOUR: General Discussion

Approximately 14% of Americans currently meet criteria for AUD, and this statistic is only expected to rise (Grant et al., 2017). While there are 4 medications approved by the FDA to treat AUD, disulfiram (Antabuse©), naltrexone (Revia©, Vivitrol©), and acamprosate (Campral©), they have high failure rates (Johnson, 2008; Moos and Moos, 2006; Heinz et al., 2009); however, among persons 12 and older needing treatment for AUD, there is a self-indicated desire for effective pharmacotherapy-based treatment options (SAMHSA, 2014). The lack of efficacy of these pharmacotherapies may be because they work at preventing future drug consumption by lowering its value to the user, and not by targeting the underlying neurological dysfunction induced by alcohol (Koob & Le Moal, 1997; Van Skike et al., 2016). However, drug research and development is an expensive and high risk venture, costing an average of $2.6 billion per new drug brought to market in the United States (DiMasi et al., 2016). Natural product compounds could provide an advantage in drug R & D for neurodegenerative diseases, as they display complex chiral structures that may confer an enhanced fit to receptors and other target proteins (Mentel, Blankenfeldt, & Breinbauer, 2009). Initial screens of several natural product compounds identified 3 compounds which attenuate 48 h EtOH (100mM)-induced cytotoxicity in a rodent OHSC (Wang et al., 2015; Shaaban et al., 2017; Zhang et al., 2017). However, during compound screening and optimization, analogs can be developed which have enhanced characteristics over their parental structures (Kerns & Di, 2003). Thus, the current studies aimed to assess the effects of natural product compound analogs on EtOH-induced cytotoxicity within our model. Additionally, as EtOH may exert its neurotoxicity via

induction of pro-inflammatory neuroimmune signaling (Crews et al., 2015), we aimed to assess whether compound hits in our model inhibited pro-inflammatory neuroimmune mediators. In Study 1, 9 compound analogs were identified which protected against 48 h EtOH (100 mM)-induced cytotoxicity. In Study 2, we found that an isoflavonoid analog, **9b**, attenuated EtOH-induced cytotoxicity and release of pro-inflammatory mediators HMGB1 and IL-6 into culture media at 48 h. Additionally, as **9b** is a novel analog, Study 2 investigated the effects of the prototypical isoflavonoid, daidzein (DZ), for comparison. DZ was also found to significantly attenuate EtOH-induced cytotoxicity and release neuroimmune mediators at 48 h. However, DZ was more potent than compound **9b** at attenuating EtOH-induced cytotoxicity and IL-6 release, exhibiting these effects at all concentrations tested. Isoflavonoids are known phytoestrogens, and estradiol can directly inhibit NF-κB activity and cytokine production (Deshpande et al., 1997; Messingham et al., 2001). These findings, while tentative, may suggest **9b** has a lower affinity for the estrogen receptor than DZ, a property which is desirable when developing drugs to be administered to female populations (Dinsdale & Ward, 2010).

While Study 1 identified 9 novel compound analogs which afford cytoprotection in our model (compounds **2a**, **4a**, **5a**, **6**, **7**, **9b**, **9d**, **10**, and **11)**, Study 2 only further evaluated a single compound (compound **9b**) for inhibitory effects on EtOH-induced pro-inflammatory mediators. Compounds **2a**, **4a**, and **5a** are all analogs of compound structures isolated from *Streptomyces* sp. RM-14-6, an extremophile bacteria isolate from the underground Ruth Mullins coal mine (Shaaban et al., 2017). Compounds **2a** and **4a** were revealed to be structurally related to spoxamicin D, which previously demonstrated potent neuroprotective effects in our model (Shaaban et al., 2017), but was advanced to *in*

vivo screening for EtOH self-administration in alcohol preferring (P) rats and had no effect on drinking behavior (unpublished data). While this does not mean that these compound analogs would not be effective *in vivo*, it did reduce their interest when compared to the isoflavonoid analogs (**9b** and **9d**), as isoflavonoids have proven effective at reducing EtOH intake in both rodents (Lin & Li, 1998; Benlhabib et al., 2004) and humans (Lukas et al., 2005; 2013; Penetar et al., 2012; 2015). Isoflavonoids have also been shown to reduce the severity of EWD, suggesting they may be useful administered at various phases of dependence (Overstreet et al., 2003; Benlhabib et al., 2004; Lutz et al., 2015a). Compound **5a** was revealed to be an analog of oxachelin, which previously demonstrated no cytoprotective effects in our model (Shaaban et al., 2017; Zhang et al., 2017). A related compound, oxachelin C, was cytoprotective; however, it did not pass *in silico* BBB screens (Shaaban et al., 2017). While compound **5a** did pass *in silico* screens, it is still a very large compound (Figure reference). *In silico* predictions are not perfect (Kelly, 2009), and doubts about **5a**'s BBB permeability have been raised (personal communication, Dr. Yinan Zhang). Furthermore, the lack of knowledge surrounding several hit compound structures (**6**, **7**, **10**, and **11**), restricted our ability to determine the value of their use.

Notably, Study 1 was useful in providing information on whether compounds were cytoprotective against the effects of EtOH, but it was not useful in providing information on the mechanism(s) by which those compounds exerted their cytoprotection. HMGB1-TLR4 signaling and downstream activation of neuroimmune mediators has been shown to contribute to EtOH-induced excitotoxicity, neurodegeneration, and behavioral deficits (Blanco et al., 2005; Fernandez-Lizarbe et al.,

2009; Alfonso-Loeches et al., 2010; Pascual et al., 2011; Liu et al., 2011). Therefore, Study 2 evaluated the effects of compound **9b**, and a comparison compound DZ, on EtOH-induced pro-inflammatory neuroimmune mediators. Specifically, we chose to examine the effects of **9b** on EtOH-induced changes in HMGB1, TNF-α, and IL-6 as these mediators have been well established in binge-EtOH models *in vitro* and in rodents (Crews et al., 2013; Casachahua, 2016; Coleman et al., 2017). We also examined the effects of EtOH on the release of the anti-inflammatory cytokine IL-10, as HMGB1 can have anti-inflammatory effects dependent on its redox state (Venereau et al., 2012). While the literature primarily discusses an increase in IL-10 release in the context of acute EtOH exposure, Marshall, McKnight, Blose, Lysle, and Thiele (2017) have shown that IL-10 is decreased in the amygdala of male C57BL/6J mice following a 4-day binge EtOH exposure. Similarly, Peng and colleagues (2017) found both M1 (pro-inflammatory) and M2 (anti-inflammatory)-like microglia expression in the rat hippocampus and entorhinal cortex following a similar 4 day binge, suggesting overlapping patterns of neuroimmune signaling may occur. As mentioned previously, we demonstrated inhibitory effects of both **9b** and DZ against EtOH-induced HMGB1 and IL-6 release into culture media at 48 h. However, we could not assess these compounds' effects on TNF-α or IL-10 as we did not find detectable levels of these cytokines released into media following a 48 h EtOH exposure. Unlike our measure of cytotoxicity (Saunders, 2016), prior to the current studies, a timeline characterizing the release of these mediators in response to EtOH in our model was not established. One important hindrance this lack of timeline presents is that we are prevented from making any causal interpretations between EtOH-induced neuroimmune signaling and cytotoxicity in

OHSC. A review by Wang, Yang, and Tracey (2004) found that secretion of TNF-α in response to systematic inflammation occurs on a separate timeline from HMGB1 release. Specifically, HMGB1 release correlates with the onset of cell death, but is delayed in response to early TNF-α release. Notably, TNF-α release was observed to be bi-phasic, with an additional peak in release observed 4-10 h following HMGB1 release (Wang et al., 2004). Thus, it is possible that we are simply missing the time point for TNF-α and IL-10 release with a single 48 h measurement within our model.

Alternatively, while TNF-α release has been shown in several *in vitro* and rodent models, Zahr, Luong, Sullivan, and Pfefferbaum (2010) did not find this cytokine to be upregulated in the liver or brain (anterior cerebellar vermis, cingulate cortex, frontal cortex, hippocampus, hypothalamus, striatum) of male Wister rats following a 4-day binge EtOH exposure. Furthermore, human studies have found that measures of alcohol consumption, craving, and EWD severity are more strongly associated with serum levels of IL-6 than TNF-α (Heberlein et al., 2014). More recent studies have failed to find any relationship with TNF-α, associating only increased IL-6 and decreased IL-10 serum levels with AUD measures (Zago et al., 2016). These studies call into question the importance of TNF-α, compared to IL-6, as a target for the screening of novel pharmacotherapies for AUD.

As previously discussed, the OHSC culture model was chosen as it has been validated in the study of EtOH-induced changes in the brain (Bulter et al., 2013; Reynolds et al., 2015a; Reynolds et al., 2016) and preserves neuron-glia interactions which are important in the development of this neuropathology (for a review, see Crews et al., 2015). Furthermore, the hippocampus also lends clinical relevancy, as this region

has been implicated in the development of many of the neurobehavioral deficits observed in alcoholics (Sullivan, Rosenbloom, & Pfefferbaum, 2000; Chanraud, et al., 2007; Pitel et al., 2007; Makris et al., 2008; Müller, 2013; Ozoy et al., 2013; Kutlu & Gould, 2016). Notably, OHSC used in these studies were taken from rat pups at post-natal (PND) day 8, and although the effects of EtOH were not analyzed until 7 DIV (or PND day 15), this time period still involves overlap with the third trimester "brain growth spurt" equivalent period in rats (PND 1-10) (West, 1987). Additionally, similar OHSC preparations have been used to study the effects of EtOH exposures and neuroimmune signaling on fetal brain development (Barron, Mulholland, Littleton, & Prendergast, 2008; Moon et al., 2014). However, OHSCs taken from postnatal rat pups do maintain structural and functional integrity over time (Gutierrez & Heinemann, 1999), and observations of OHSC taken from PND 6 pups and aged for 24 days revealed a similar distribution of hippocampal glutamate receptors to PND 30 rat brain *in situ* (Martens & Wree, 2001). This suggests that effects observed in cultures taken from postnatal pups may hold implications for the adolescent or adult rat brain. Moreover, our lab has reliably translated several findings from OHSC to adult rat *in vivo* studies (Reynolds et al., 2015c; Reynolds et al., 2016). In kind with the current study, Tajuddin, Kim, and Collins (2018) revealed that a 4-day 100 mM EtOH exposures results in neurodegeneration through induction of HMGB1-TLR4 signaling in adult-age HEC cultures. Thus, while the current studies provide support that changes neuroimmune signaling may contribute to EtOH-induced toxicity in the brain, the implication that the therapeutic targets identified in these studies may be relevant to EtOH-induced neurotoxicity during development should also be considered.

The current studies are the first to our knowledge to demonstrate inhibitory effects of isoflavonoids and isoflavonoid analogs against binge-like EtOH-induced cytotoxicity and pro-inflammatory neuroimmune signaling. The main findings from these studies are that structural modifications to natural product compounds (e.g., via glycosylation, fluorination, addition of piperidine) can have a positive effect on BBB permeability and/or cytoprotection. Furthermore, isoflavonoids may exert their cytoprotective effects via modulation of pro-inflammatory neuroimmune signaling. Future research should continue to explore the precise mechanism by which the novel analogs identified in these studies exert their cytoprotective effects.

References

Adachi, Mizoi, Fukunaga, Ogawa, Ueno, Imamichi, & Adachi, J. (1991). Degrees of alcohol intoxication in 117 hospitalized cases. *Journal of Studies on Alcohol,52*(5), 448-453.

Adams, S. M., Aksenova, M. V., Aksenov, M. Y., Mactutus, C. F., & Booze, R. M. (2012). Soy isoflavones genistein and daidzein exert anti-apoptotic actions via a selective ER-mediated mechanism in neurons following HIV-1 Tat1–86 exposure. *PLoS One*, *7*(5), e37540.

Adinoff, B. (2004). Neurobiologic Processes in Drug Reward and Addiction. *Harvard Review of Psychiatry, 12*(6), 305-320.

Agartz, I., Momenan, R., Rawlings, R., Kerich, M., & Hommer, D. (1999). Hippocampal Volume in Patients With Alcohol Dependence. *Archives of General Psychiatry, 56*(4), 356-363.

Alfonso-Loeches, S., Pascual-Lucas, M., Blanco, A., Sanchez-Vera, I., & Guerri, C. (2010). Pivotal Role of TLR4 Receptors in Alcohol-Induced Neuroinflammation and Brain Damage. *Journal of Neuroscience, 30*(24), 8285-8295.

Alling, C., & Boström, K. (1980). Demyelination of the mamillary bodies in alcoholism. A combined morphological and biochemical study. *Acta Neuropathologica, 50*(1), 77-80.

American Psychiatric Association. (2013). Diagnostic and statistical manual of mental disorders (5th ed.). Washington, DC.

Arain, M., Haque, M., Johal, L., Mathur, P., Nel, W., Rais, A., ... & Sharma, S. (2013). Maturation of the adolescent brain. *Neuropsychiatric disease and treatment*, *9*, 449.

Aras, A.B., Guven, M., Akman, T., Ozkan, A., Sen, H.M., Duz, U., ... & Cosar, M. (2015). Neuroprotective effects of daidzein on focal cerebral ischemia injury in rats. *Neural regeneration research*, *10*(1), 146.

Balosso, S., Liu, J., Bianchi, M.E., & Vezzani, A. (2014). Disulfide-containing high mobility group box-1 promotes N-methyl-D-aspartate receptor function and excitotoxicity by activating Toll-like receptor 4-dependent signaling in hippocampal neurons. *Antioxidants & redox signaling*, *21*(12), 1726-1740.

Barnes, S. (2010). The biochemistry, chemistry and physiology of the isoflavones in soybeans and their food products. *Lymphatic research and biology*, *8*(1), 89-98.

Barnham, K. J., Masters, C. L., & Bush, A. I. (2004). Neurodegenerative diseases and oxidative stress. *Nature reviews Drug discovery*, *3*(3), 205.

Barron, S., Mulholland, P.J., Littleton, J.M., & Prendergast, M.A. (2008). Age and gender differences in response to neonatal ethanol withdrawal and polyamine challenge in organotypic hippocampal cultures. *Alcoholism: Clinical and Experimental Research*, *32*(6), 929-936.

Basso, M., & Bornstein, R. (2000). Neurobehavioural consequences of substance abuse and HIV infection. *Journal of Psychopharmacology, 14*(3), 228-237.

Battino, M., Bullon, P., Wilson, M., & Newman, H. (1999). Oxidative injury and inflammatory periodontal diseases: the challenge of anti-oxidants to free radicals

and reactive oxygen species. *Critical Reviews in Oral Biology & Medicine*, *10*(4), 458-476.

Bava, S., & Tapert, S. F. (2010). Adolescent brain development and the risk for alcohol and other drug problems. *Neuropsychology review*, *20*(4), 398-413.

Bell, R.L., Eiler II, B.J., Cook, J.B., & Rahman, S. (2009). Nicotinic receptor ligands reduce ethanol intake by high alcohol–drinking HAD-2 rats. *Alcohol*, *43*(8), 581-592.

Benediktsson, A. M., Schachtele, S. J., Green, S. H., & Dailey, M. E. (2005). Ballistic labeling and dynamic imaging of astrocytes in organotypic hippocampal slice cultures. *Journal of neuroscience methods*, *141*(1), 41-53.

Benlhabib, E., Baker, J.I., Keyler, D.E., & Singh, A.K. (2004). Kudzu root extract suppresses voluntary alcohol intake and alcohol withdrawal symptoms in P rats receiving free access to water and alcohol. *Journal of medicinal food*, *7*(2), 168-179.

Beresford, T. P., Arciniegas, D. B., Alfers, J., Clapp, L., Martin, B., Du, Y., ... & Davatzikos, C. (2006). Hippocampus volume loss due to chronic heavy drinking. *Alcoholism: Clinical and Experimental Research*, *30*(11), 1866-1870.

Bienkowski, P., Krzascik, P., Koros, E., Kostowski, W., Scinska, A., & Danysz, W. (2001). Effects of a novel uncompetitive NMDA receptor antagonist, MRZ 2/579 on ethanol self-administration and ethanol withdrawal seizures in the rat. *European journal of pharmacology*, *413*(1), 81-89.

Blanco, A., & Guerri, C. (2006). Alcohol and neuroinflammation: Involvement of astroglial cells and TLR4/IL-1RI receptors. *Inmunologia, 25*(3), 188-200.

Blanco, A., Valles, S., Pascual, M., & Guerri, C. (2005). Involvement of TLR4/type IIL-1 receptor signaling in the induction of inflammatory mediators and cell death induced by ethanol in cultured astrocytes. *Journal Of Immunology,175*(10), 6893-6899.

Bleich, Bandelow, Javaheripour, Müller, Degner, Wilhelm, . . . Kornhuber. (2003). Hyperhomocysteinemia as a new risk factor for brain shrinkage in patients with alcoholism. *Neuroscience Letters, 335*(3), 179-182.

Boersma, B.J., Patel, R.P., Kirk, M., Jackson, P.L., Muccio, D., Darley-Usmar, V.M., & Barnes, S. (1999). Chlorination and nitration of soy isoflavones. *Archives of biochemistry and biophysics*, *368*(2), 265-275.

Borchardt, J. (2002). The Beginnings of Drug Therapy: Ancient Mesopotamian Medicine. *Drug News & Perspectives, 15*(3), 187-192.

Boyadjieva, N., & Sarkar, D. (2013). Microglia Play a Role in Ethanol-Induced Oxidative Stress and Apoptosis in Developing Hypothalamic Neurons. *Alcoholism: Clinical and Experimental Research, 37*(2), 252-262.

Brebner, K., Hayley, S., Zacharko, R., Merali, Z., & Anisman, H. (2000). Synergistic effects of interleukin-1β, interleukin-6, and tumor necrosis factor-α: central monoamine, corticosterone, and behavioral variations. *Neuropsychopharmacology*, *22*(6), 566.

Butler, T.R., Berry, J.N., Sharrett-Field, L.J., Pauly, J.R., & Prendergast, M.A. (2013). Long-Term Ethanol and Corticosterone Co-Exposure Sensitize the Hippocampal CA 1 Region Pyramidal Cells to Insult During Ethanol Withdrawal in an NMDA

GluN2B Subunit-Dependent Manner. *Alcoholism: Clinical and Experimental Research*, *37*(12), 2066-2073.

Cahn, R.S., Ingold, C., & Prelog, V. (1966). Specification of molecular chirality. *Angewandte Chemie International Edition in English*, *5*(4), 385-415.

Casachahua, J.D. (2016). The role of cytokines in binge-like ethanol consumption and ethanol-induced sedation (Dissertation). Retrieved from https://cdr.lib.unc.edu/indexablecontent/uuid:29248153-76af-437f-911b b6a1a822bf0a?dl=true

Center for Behavioral Health Statistics and Quality. (2015). *Behavioral health trends in the United States: Results from the 2014 National Survey on Drug Use and Health* (HHS Publication No. SMA 15–4927, NSDUH Series H-50). Retrieved from https://www.samhsa.gov/data/

Chandrasekharan, S., & Aglin, A. (2013). Pharmacokinetics of dietary isoflavones. *J Steroids Horm Sci S*, *12*, 004.

Chanraud, S., Martelli, C., Delain, F., Kostogianni, N., Douaud, G., Aubin, H. J., Reynaud, M. and Martinot, J.L. (2007). Brain morphometry and cognitive performance in detoxified alcohol-dependents with preserved psychosocial functioning. *Neuropsychopharmacology, 32*, 429-438.

Chastain, G. (2006). Alcohol, neurotransmitter systems, and behavior. *Journal Of General Psychology, 133*(4), 329-335.

Cheng, F., Li, W., Zhou, Y., Shen, J., Wu, Z., Liu, G., ... & Tang, Y. (2012). admetSAR: a comprehensive source and free tool for assessment of chemical ADMET properties [Computer Program]. *ACS Publications*, 3099-3105.

Cherry, J., Olschowka, J., & O'Banion, M. (2014). Neuroinflammation and M2 microglia: The good, the bad, and the inflamed. *Journal Of Neuroinflammation,11*(1), 98.

Chinta, S.J., Ganesan, A., Reis-Rodrigues, P., Lithgow, G.J., & Andersen, J.K. (2013). Anti-inflammatory role of the isoflavone diadzein in lipopolysaccharide-stimulated microglia: implications for Parkinson's disease. *Neurotoxicity research*, *23*(2), 145-153.

Cohen, J. (1988). Statistical power analysis for the behavioral sciences (2nd ed.). Hillsdale, NJ: Lawrence Earlbaum Associates.

Coleman, L.G., Zou, J., & Crews, F.T. (2017). Microglial-derived miRNA let-7 and HMGB1 contribute to ethanol-induced neurotoxicity via TLR7. *Journal of neuroinflammation*, *14*(1), 22.

Collins, M., & Neafsey, E. (2012). Ethanol and adult CNS neurodamage: Oxidative stress, but possibly not excitotoxicity. *Frontiers in Bioscience (Elite Edition), 4*, 1358-67.

Collins, M.A., Zou, J.Y. & Neafsey, E.J. (1998). Brain damage due to episodic alcohol exposure in vivo and in vitro: furosemide neuroprotection implicates edema-based mechanism. *FASEB J, 12*, 221–230.

Colton, C. (2009). Heterogeneity of Microglial Activation in the Innate Immune Response in the Brain. *Journal of Neuroimmune Pharmacology, 4*(4), 399-418.

Crews, F., Nixon, K., Kim, D., Joseph, J., Shukitt-Hale, B., Qin, L., & Zou, J. (2006). BHT blocks NF-κB activation and ethanol-induced brain damage. *Alcoholism: Clinical and Experimental Research*, *30*(11), 1938-1949.

Crews, F., Sarkar, D., Qin, L., Zou, J., Boyadjieva, N., & Vetreno, R. (2015). Neuroimmune Function and the Consequences of Alcohol Exposure. *Alcohol Research: Current Reviews, 37*(2), 331-41, 344-51.

Crews, F.T., Qin, L., Sheedy, D., Vetreno, R.P., & Zou, J. (2013). High mobility group box 1/Toll-like receptor danger signaling increases brain neuroimmune activation in alcohol dependence. *Biological psychiatry*, *73*(7), 602-612.

Crews, F. T., Vetreno, R. P., Broadwater, M. A., & Robinson, D. L. (2016). Adolescent alcohol exposure persistently impacts adult neurobiology and behavior. *Pharmacological reviews*, *68*(4), 1074-1109.

Dailey, M.E., & Waite, M. (1999). Confocal imaging of microglial cell dynamics in hippocampal slice cultures. *Methods*, *18*(2), 222-230.

Davis, & Syapin. (2004). Ethanol increases nuclear factor-κB activity in human astroglial cells. *Neuroscience Letters, 371*(2), 128-132.

Davis, K., & Wu, M. (2001). Role of glutamatergic and GABAergic systems in alcoholism. *Journal of Biomedical Science, 8*(1), 7-19.

Davidson, M., Shanley, B., & Wilce, P. (1995). Increased NMDA-induced excitability during ethanol withdrawal: a behavioural and histological study. *Brain Research*, *674*(1), 91-96.

Dawson, D.A., & Archer, L.D. (1993). Relative frequency of heavy drinking and the risk of alcohol dependence. *Addiction*, *88*, 1509-1518.

De Bellis, M.D., Clark, D.B., Beers, S.R., Soloff, P.H., Boring, A.M., Hall, J., ... & Keshavan, M.S. (2000). Hippocampal volume in adolescent-onset alcohol use disorders. *American Journal of Psychiatry*, *157*(5), 737-744.

de la Monte, S.M., & Kril, J.J. (2014). Human alcohol-related neuropathology. *Acta Neuropathologica*, *127*(1), 71-90.

Deshpande, R., Khalili, H., Pergolizzi, R.G., Michael, S. D., & Chang, M. D. Y. (1997). Estradiol Down-Regulates LPS-Induced Cytokine Production and NFkB Activation in Murine Macrophages. *American journal of reproductive immunology*, *38*(1), 46-54.

DiMasi, J.A., Grabowski, H.G., & Hansen, R.W. (2016). Innovation in the pharmaceutical industry: new estimates of R&D costs. *Journal of health economics*, *47*, 20-33.

Dinsdale, E.C., & Ward, W.E. (2010). Early exposure to soy isoflavones and effects on reproductive health: a review of human and animal studies. *Nutrients*, *2*(11), 1156-1187.

Doremus-Fitzwater, T.L., Gano, A., Paniccia, J.E., & Deak, T. (2015). Male adolescent rats display blunted cytokine responses in the CNS after acute ethanol or lipopolysaccharide exposure. *Physiology & behavior*, *148*, 131-144.

Dusek, P., Schneider, S.A., & Aaseth, J. (2016). Iron chelation in the treatment of neurodegenerative diseases. *Journal of Trace Elements in Medicine and Biology*, *38*, 81-92.

Dwivedi, A.K., Chatterjee, K., & Singh, R. (2017). Lifetime alcohol consumption and severity in alcohol dependence syndrome. *Industrial Psychiatry Journal*, *26*(1), 34.

Egleton, R.D., Mitchell, S.A., Huber, J.D., Palian, M.M., Polt, R., & Davis, T. P. (2001). Improved blood-brain barrier penetration and enhanced analgesia of an opioid

peptide by glycosylation. *Journal of Pharmacology and Experimental Therapeutics*, *299*(3), 967-972.

Ewing, S.W.F., Sakhardande, A., & Blakemore, S.J. (2014). The effect of alcohol consumption on the adolescent brain: a systematic review of MRI and fMRI studies of alcohol-using youth. *NeuroImage: Clinical*, *5*, 420-437.

Fadda, & Rossetti. (1998). Chronic ethanol consumption:from neuroadaptation to neurodegeneration. *Progress in Neurobiology, 56*(4), 385-431.

Farook, J.M., Lewis, B., Gaddis, J.G., Littleton, J.M., & Barron, S. (2009). Lobeline, a nicotinic partial agonist attenuates alcohol consumption and preference in male C57BL/6J mice. *Physiology & behavior*, *97*(3-4), 503-506.

Faul, F. & Erdfelder, E. (1992). GPOWER: A priori-, post hoc-, and compromise power analyses for MS-DOS [Computer Program]. Bonn, Germany: Bonn University.

Fein, G., Sclafani, V., Cardenas, V., Goldmann, H., Tolou-Shams, M., & Meyerhoff, D. (2002). Cortical Gray Matter Loss in Treatment-Naïve Alcohol Dependent Individuals. *Alcoholism: Clinical and Experimental Research, 26*(4), 558-564.

Fernandez-Lizarbe, S., Pascual, M., & Guerri, C. (2009). Critical role of TLR4 response in the activation of microglia induced by ethanol. *The Journal of Immunology*, *183*(7), 4733-4744.

Fichna, J., Mazur, M., Grzywacz, D., Kamysz, W., Perlikowska, R., Piekielna, J., ... & Chen, C. (2013). Novel glycosylated endomorphin-2 analog produces potent centrally-mediated antinociception in mice after peripheral administration. *Bioorganic & medicinal chemistry letters*, *23*(24), 6673-6676.

Frank-Cannon, T., Alto, L., Mcalpine, F., & Tansey, M. (2009). Does neuroinflammation fan the flame in neurodegenerative diseases? *Molecular Neurodegeneration, 4*(1), 47.

Fried, Kotarsky, & Alling. (2001). Chronic ethanol exposure enhances activating protein-1 transcriptional activity in human neuroblastoma cells. *Alcohol, 24*(3), 189-195.

Gahwiler, B.H., Capogna, M., Debanne., D., McKinney, R.A., & Thompson, S.M. (1997). Organotypic slice cultures: a technique that has come of age. *Trends in Neurosciences*, *20*(10), 471-477.

Galm, & Shen. (2007). Natural Product Drug Discovery: The Times Have Never Been Better. *Chemistry & Biology, 14*(10), 1098-1104.

Ganai, A.A., Khan, A.A., Malik, Z.A., & Farooqi, H. (2015). Genistein modulates the expression of NF-κB and MAPK (p-38 and ERK1/2), thereby attenuating d-Galactosamine induced fulminant hepatic failure in Wistar rats. *Toxicology and applied pharmacology*, *283*(2), 139-146.

Gantt, R.W., Peltier-Pain, P., & Thorson, J.S. (2011). Enzymatic methods for glyco (diversification/randomization) of drugs and small molecules. *Natural product reports*, *28*(11), 1811-1853.

Gao, Y., Fang, L., Liu, F., Zong, C., Cai, R., Chen, X., & Qi, Y. (2014). Suppressive effects of irisflorentin on LPS-induced inflammatory responses in RAW 264.7 macrophages. *Experimental Biology and Medicine*, *239*(8), 1018-1024.

Giacomotto, J., & Ségalat, L. (2010). High-throughput screening and small animal models, where are we? *British journal of pharmacology*, *160*(2), 204-216.

Giovannini, M.G., Scali, C., Prosperi, C., Bellucci, A., Pepeu, G., & Casamenti, F. (2003). Experimental brain inflammation and neurodegeneration as model of Alzheimer's disease: protective effects of selective COX-2 inhibitors. *International journal of immunopathology and pharmacology*, *16*(2; SUPP), 31-40.

Goff, R.D., & Thorson, J.S. (2012). Enhancement of cyclopamine via conjugation with nonmetabolic sugars. *Organic letters*, *14*(10), 2454-2457.

Goral, J., Choudhry, M.A., & Kovacs, E.J. (2004). Acute ethanol exposure inhibits macrophage IL-6 production: role of p38 and ERK1/2 MAPK. *Journal of leukocyte biology*, *75*(3), 553-559.

Grant, B., Chou, S., Saha, T., Pickering, R., Kerridge, B., Ruan, W., . . . Hasin, D. (2017). Prevalence of 12-Month Alcohol Use, High-Risk Drinking, and DSM-IV Alcohol Use Disorder in the United States, 2001-2002 to 2012-2013: Results From the National Epidemiologic Survey on Alcohol and Related Conditions. *JAMA Psychiatry, 74*(9), 911-923.

Grant, B. F., & Dawson, D. A. (1997). Age at onset of alcohol use and its association with DSM-IV alcohol abuse and dependence: results from the National Longitudinal Alcohol Epidemiologic Survey. *Journal of substance abuse*, *9*, 103-110.

Grant, B.F., Goldstein, R.B., Saha, T.D., Chou, S.P., Jung, J., Zhang, H., Pickering, R.P., Ruan, W.J., Smith, S.M., Huang, B., & Hasin, D.S. (2015). Epidemiology of DSM-5 alcohol use disorder: results from the National Epidemiologic Survey on Alcohol and Related Conditions III. *JAMA Psychiatry*, *72*, 757-766.

Grant, K.A., Valverius, P., Hudspith, M., & Tabakoff, B. (1990). Ethanol withdrawal seizures and the NMDA receptor complex. *European journal of pharmacology*, *176*(3), 289-296.

Griffin, W. C., Lopez, M. F., Yanke, A. B., Middaugh, L. D., & Becker, H. C. (2009). Repeated cycles of chronic intermittent ethanol exposure in mice increases voluntary ethanol drinking and ethanol concentrations in the nucleus accumbens. *Psychopharmacology*, *201*(4), 569-580.

Guerri, C., & Grisolia, S. (1980). Changes in glutathione in acute and chronic alcohol intoxication. *Pharmacology Biochemistry and Behavior*, *89*, 197-203.

Haber, M., Vautrin, S., Fry, E.J., & Murai, K.K. (2009). Subtype-specific oligodendrocyte dynamics in organotypic culture. *Glia*, *57*(9), 1000-1013.

Hamelink, C., Hampson, A., Wink, D. A., Eiden, L. E., & Eskay, R. L. (2005). Comparison of cannabidiol, antioxidants, and diuretics in reversing binge ethanol-induced neurotoxicity. *The Journal of Pharmacology and Experimental Therapeutics*, *314*(2), 780-788.

Haorah J., Schall K., Ramirez S. H., Persidsky Y. (2008). Activation of protein tyrosine kinases and matrix metalloproteinases causes blood-brain barrier injury: novel mechanism for neurodegeneration associated with alcohol abuse. *Glia*, *56*, 78–88.

Harms, A.S., Lee, J.K., Nguyen, T.A., Chang, J., Ruhn, K.M., Treviño, I., & Tansey, M.G. (2012). Regulation of microglia effector functions by tumor necrosis factor signaling. *Glia*, *60*(2), 189-202.

Harper, C., & Kril, J. (1985). Brain atrophy in chronic alcoholic patients: A quantitative pathological study. *Journal of Neurology, Neurosurgery & Psychiatry, 48*(3), 211-7.

Harper, C., Kril, J., & Holloway, R. (1985). Brain shrinkage in chronic alcoholics: A pathological study. *British Medical Journal (Clinical Research Ed.), 290*(6467), 501.

Harris, R.A., Bajo, M., Bell, R.L., Blednov, Y.A., Varodayan, F.P., Truitt, J., ... & Roberts, A.J. (2016). Genetic and pharmacologic manipulation of TLR4 has minimal impact on ethanol consumption in rodents. *Journal of Neuroscience*, 2002-16.

Harris, B. R., Gibson, D. A., Prendergast, M. A., Blanchard, J. A., Holley, R. C., Hart, S. R., ... & Littleton, J. M. (2003). The neurotoxicity induced by ethanol withdrawal in mature organotypic hippocampal slices might involve cross-talk between metabotropic glutamate type 5 receptors and N-methyl-D-aspartate receptors. *Alcoholism: Clinical and Experimental Research*, *27*(11), 1724-1735.

Harvey, A. (2008). Natural products in drug discovery. *Drug Discovery Today,13*(19), 894-901.

He, & Crews. (2008). Increased MCP-1 and microglia in various regions of the human alcoholic brain. *Experimental Neurology, 210*(2), 349-358.

Heberlein, A., Käser, M., Lichtinghagen, R., Rhein, M., Lenz, B., Kornhuber, J., ... & Hillemacher, T. (2014). TNF-α and IL-6 serum levels: neurobiological markers of alcohol consumption in alcohol-dependent patients? *Alcohol*, *48*(7), 671-676.

Heinz, A., Beck, A., Grüsser, S. M., Grace, A. A., & Wrase, J. (2009). Identifying the neural circuitry of alcohol craving and relapse vulnerability. *Addiction Biology, 14*, 108-118.

Henkel, T., Brunne, R.M., Müller, H., & Reichel, F. (1999). Statistical investigation into the structural complementarity of natural products and synthetic compounds. *Angewandte Chemie International Edition*, *38*(5), 643-647.

Hoesel, B., & Schmid, J. (2013). The complexity of NF-kappa B signaling in inflammation and cancer. *Molecular Cancer, 12*(1), 86.

Hoffman, P. L., & Tabakoff, B. (1994). The role of the NMDA receptor in ethanol withdrawal. In Toward a molecular basis of alcohol use and abuse (pp. 61-70). Birkhäuser Basel.

Hosur, V., & Loring, R. H. (2010). α4β2 Nicotinic Receptors Partially Mediate Anti-Inflammatory Effects through JAK2-STAT3 but not Calcium or cAMP signaling. *Molecular pharmacology*, mol-110.

Hunot, S., & Hirsch, E. C. (2003). Neuroinflammatory processes in Parkinson's disease. *Annals of neurology*, *53*(S3).

Hu, Leak, Shi, Suenaga, Gao, Zheng, & Chen. (2015). Microglial and macrophage polarization—new prospects for brain repair. *Nature Reviews. Neurology, 11*(1), 56-64.

Inahashi, Y., Iwatsuki, M., Ishiyama, A., Namatame, M., Nishihara-Tsukashima, A., Matsumoto, A., ... & Takahashi, Y. (2011). Spoxazomicins A–C, novel antitrypanosomal alkaloids produced by an endophytic actinomycete,

Streptosporangium oxazolinicum K07-0460T. *The Journal of antibiotics*, *64*(4), 303-307.

Jernigan, T., Schafer, K., Butters, N., & Cermak, L. (1991). Magnetic resonance imaging of alcoholic Korsakoff patients. *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology, 4*(3), 175-86.

Jha, Lee, & Suk. (2016). Functional polarization of neuroglia: Implications in neuroinflammation and neurological disorders. *Biochemical Pharmacology, 103*, 1-16.

Ji, H., Li, X., & Zhang, H. (2009). Natural products and drug discovery. Can thousands of years of ancient medical knowledge lead us to new and powerful drug combinations in the fight against cancer and dementia? *EMBO Reports, 10*(3), 194-200.

Ji, G., Zhang, Y., Yang, Q., Cheng, S., Hao, J., Zhao, X., & Jiang, Z. (2012). Genistein suppresses LPS-induced inflammatory response through inhibiting NF-κB following AMP kinase activation in RAW 264.7 macrophages. *PLoS One*, *7*(12), e53101.

Johnson, B.A. (2008). Update on neuropharmacological treatments for alcoholism: scientific basis and clinical findings. *Biochemical Pharmacology*, *75*(1), 34–56.

Kelly, J. (2009). *Principles of CNS Drug Development*. Chichester, UK: John Wiley & Sons.

Kendrick, N. (2014). A gene’s mRNA level does not usually predict its protein level. Retrieved from https://kendricklabs.com/wpcontent/uploads/2016/08/ WP1_mRNAvsProtein_KendrickLabs.pdf

Kerns, & Di. (2003). Pharmaceutical profiling in drug discovery. *Drug Discovery Today, 8*(7), 316-323.

Kim, K. K., Adelstein, R. S., & Kawamoto, S. (2009). Identification of neuronal nuclei (NeuN) as Fox-3, a new member of the Fox-1 gene family of splicing factors. *Journal of Biological Chemistry*, jbc-M109.

Knapp, D. J., Whitman, B. A., Wills, T. A., Angel, R. A., Overstreet, D. H., Criswell, H. E., ... & Breese, G. R. (2011). Cytokine involvement in stress may depend on corticotrophin releasing factor to sensitize ethanol withdrawal anxiety. *Brain, behavior, and immunity*, *25*, S146-S154.

Koob, G., & Le Moal, M. (1997). Drug abuse: Hedonic homeostatic dysregulation. *Science (Washington), 278*(5335), 52-58.

Kostelac, D., Rechkemmer, G., & Briviba, K. (2003). Phytoestrogens modulate binding response of estrogen receptors α and β to the estrogen response element. *Journal of agricultural and food chemistry*, *51*(26), 7632-7635.

Krishnaiah, Y.S. (2010). Pharmaceutical technologies for enhancing oral bioavailability of poorly soluble drugs. *J Bioequiv Availab*, *2*(2), 28-36.

Kutlu, M.G., & Gould, T.J. (2016). Effects of drugs of abuse on hippocampal plasticity and hippocampus-dependent learning and memory: contributions to development and maintenance of addiction. *Learning & Memory*, *23*(10), 515-533.

Lau, A., & Tymianski, M. (2010). Glutamate receptors, neurotoxicity and neurodegeneration. *Pflügers Archiv-European Journal of Physiology*, *460*(2), 525-542.

Lenroot, R. K., & Giedd, J. N. (2006). Brain development in children and adolescents: insights from anatomical magnetic resonance imaging. *Neuroscience & biobehavioral reviews*, *30*(6), 718-729.

Lesinski, G.B., Reville, P.K., Mace, T.A., Young, G.S., Ahn-Jarvis, J., Thomas-Ahner, J., ... & Schwartz, S. (2015). Consumption of soy isoflavone enriched bread in men with prostate cancer is associated with reduced proinflammatory cytokines and immunosuppressive cells. *Cancer Prevention Research*, *8*(11), 1036-1044.

Levy, M.D. (2000). The drug discovery and development process in the new millennium. *Canadian Journal of Gastroenterology and Hepatology*, *14*(7), 641-643.

Lin, M.T., & Beal, M.F. (2006). Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature*, *443*(7113), 787.

Lin, R.C., & Li, T.K. (1998). Effects of isoflavones on alcohol pharmacokinetics and alcohol-drinking behavior in rats. *The American journal of clinical nutrition*, *68*(6), 1512S-1515S.

Lipinski, C.A., Lombardo, F., Dominy, B.W., & Feeney, P.J. (2001). Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced drug delivery reviews*, *46*(1-3), 3-26.

Lisdahl, K.M., Thayer, R., Squeglia, L.M., McQueeny, T.M., & Tapert, S.F. (2013). Recent binge drinking predicts smaller cerebellar volumes in adolescents. *Psychiatry Research: Neuroimaging*, *211*(1), 17-23.

Liu, S., & Malik, A. (2006). NF- Kappa B activation as a pathological mechanism of septic shock and inflammation. *American Journal of Physiology: Lung Cellular and Molecular Physiology, 290*(4), L622-L645.

Liu, Zhan, Li, Xu, Ma, Zhang, . . . Xuetao Cao. (2011). Intracellular MHC class II molecules promote TLR-triggered innate immune responses by maintaining activation of the kinase Btk. *Nature Immunology, 12*(5), 416-24.

Lorrai, I., Maccioni, P., Gessa, G. L., & Colombo, G. (2016). r (+)-Baclofen, but not s (−)-Baclofen, alters alcohol self-administration in alcohol-Preferring rats. *Frontiers in psychiatry*, *7*, 68.

Lovinger, D.M. (1993). Excitotoxicity and alcohol-related brain damage. *Alcoholism: Clinical and Experimental Research*, *17*(1), 19-27.

Lucas, S.M., Rothwell, N.J., & Gibson, R.M. (2006). The role of inflammation in CNS injury and disease. *British journal of pharmacology*, *147*(S1), S232- 240.

Lukas, S.E., Penetar, D., Berko, J., Vicens, L., Palmer, C., Mallya, G., ... & Lee, D.Y.W. (2005). An extract of the Chinese herbal root kudzu reduces alcohol drinking by heavy drinkers in a naturalistic setting. *Alcoholism: Clinical and Experimental Research*, *29*(5), 756-762.

Lukas, S.E., Penetar, D., Su, Z., Geaghan, T., Maywalt, M., Tracy, M., ... & Lee, D.Y.W. (2013). A standardized kudzu extract (NPI-031) reduces alcohol consumption in nontreatment-seeking male heavy drinkers. *Psychopharmacology*, *226*(1), 65-73.

Lutz, J.A., Carter, M., Fields, L., Barron, S., & Littleton, J. M. (2015b). Altered Relation Between Lipopolysaccharide-Induced Inflammatory Response and Excitotoxicity

in Rat Organotypic Hippocampal Slice Cultures During Ethanol Withdrawal. Alcoholism: *Clinical and Experimental Research*, *39*(5), 827-835.

Lutz, J.A., Carter, M., Fields, L., Barron, S., & Littleton, J.M. (2015a). The dietary flavonoid rhamnetin inhibits both inflammation and excitotoxicity during ethanol withdrawal in rat organotypic hippocampal slice cultures. *Alcoholism: Clinical and Experimental Research*, *39*(12), 2345-2353.

Maggio, S. E., Saunders, M. A., Baxter, T. A., Nixon, K., Prendergast, M. A., Zheng, G., ... & Bell, R. L. (2018). Effects of the nicotinic agonist varenicline, nicotinic antagonist r-bPiDI, and DAT inhibitor (R)-modafinil on co-use of ethanol and nicotine in female P rats. *Psychopharmacology*, 1-15.

Makris, N., Oscar-Berman, M., Jaffin, S.K., Hodge, S.M., Kennedy, D.N., Caviness, V. S., ... & Harris, G. J. (2008). Decreased volume of the brain reward system in alcoholism. *Biological psychiatry*, *64*(3), 192-202.

Mann, K., Agartz, I., Harper, C., Shoaf, S., Rawlings, R., Momenan, D., . . . Heinz. (2001). Neuroimaging in alcoholism: Ethanol and brain damage. *Alcoholism: Clinical and Experimental Research, 25*(5), 104S.

Maroso, M., Balosso, S., Ravizza, T., Liu, J., Bianchi, M.E., & Vezzani, A. (2011). Interleukin-1 type 1 receptor/Toll-like receptor signalling in epilepsy: the importance of IL-1beta and high-mobility group box 1. *Journal of internal medicine*, *270*(4), 319-326.

Marshall, S. A., McKnight, K. H., Blose, A. K., Lysle, D. T., & Thiele, T. E. (2017). Modulation of binge-like ethanol consumption by IL-10 signaling in the basolateral amygdala. *Journal of Neuroimmune Pharmacology*, *12*(2), 249-259.

Marshall, S.A., Geil, C.R., and Nixon, K. (2016). Prior Binge Ethanol Exposure Potentiates the Microglial Response in a Model of Alcohol-Induced Neurodegeneration. *Brain Sciences, 6*(2), 16.

Martens, U., & Wree, A. (2001). Distribution of [3 H] MK-801,[3 H] AMPA and [3 H] Kainate binding sites in rat hippocampal long-term slice cultures isolated from external afferents. *Anatomy and embryology*, *203*(6), 491-500.

Mayer, S., Harris, B.R., Gibson, D.A., Blanchard, J.A., Prendergast, M.A., Holley, R.C., & Littleton, J. (2002). Acamprosate, MK-801, and ifenprodil inhibit neurotoxicity and calcium entry induced by ethanol withdrawal in organotypic slice cultures from neonatal rat hippocampus. *Alcoholism: Clinical and Experimental Research*, *26*(10), 1468-1478.

Mechtcheriakov, S., Brenneis, C., Egger, K., Koppelstaetter, F., Schocke, M., & Marksteiner, J. (2006). A wide-spread distinct pattern of cerebral atrophy in patients with alcohol addiction revealed by voxel-based morphometry. *Journal of Neurology, Neurosurgery & Psychi*atry, *78*, 610-614.

Mentel, M., Blankenfeldt, W., & Breinbauer, R. (2009). The active site of an enzyme can host both enantiomers of a racemic ligand simultaneously. *Angewandte Chemie International Edition*, *48*(48), 9084-9087.

Messingham, K.A., Heinrich, S.A., & Kovacs, E.J. (2001). Estrogen restores cellular immunity in injured male mice via suppression of interleukin-6 production. *Journal of Leukocyte Biology*, *70*(6), 887-895.

Miadoková, E. (2009). Isoflavonoids—an overview of their biological activities and potential health benefits. *Interdisciplinary toxicology*, *2*(4), 211-218.

Michelucci, Heurtaux, Grandbarbe, Morga, & Heuschling (2009). Characterization of the microglial phenotype under specific pro-inflammatory and anti-inflammatory conditions: Effects of oligomeric and fibrillar amyloid-β. *Journal of Neuroimmunology, 210*(1), 3-12.

Mirshahi, T., & Woodward, J. J. (1995). Ethanol sensitivity of heteromeric NMDA receptors: effects of subunit assembly, glycine and NMDAR1 Mg2+-insensitive mutants. *Neuropharmacology*, *34*(3), 347-355.

Monsen, P.J., & Luzzio, F.A. (2017). Selective fluorination of natural products. *ARKIVOC*, 117-147.

Moon, K.H., Tajuddin, N., Brown, J., Neafsey, E.J., Kim, H.Y., & Collins, M.A. (2014). Phospholipase A2, Oxidative Stress, and Neurodegeneration in Binge Ethanol-Treated Organotypic Slice Cultures of Developing Rat Brain. *Alcoholism: Clinical and Experimental Research*, *38*(1), 161-169.

Moos, Rudolf H., & Moos, Bernice S. (2006). Rates and predictors of relapse after natural and treated remission from alcohol use disorders. *Addiction, 101*(2), 212-222.

Morimoto, M., Watanabe, T., Yamori, M., Takebe, M., & Wakatsuki, Y. (2009). Isoflavones regulate innate immunity and inhibit experimental colitis. *Journal of gastroenterology and hepatology*, *24*(6), 1123-1129.

Morrisett, R.A., Rezvani, A.H., Overstreet, D., Janowsky, D.S., Wilson, W.A., & Swartzwelder, H.S. (1990). MK-801 potently inhibits alcohol withdrawal seizures in rats. *European journal of pharmacology*, *176*(1), 103-105.

Mulholland, P.J., Harris, B.R., Self, R.L., Little, H.J., Littleton, J.M., Prendergast, M.A. (2005). Corticosterone increases damage and cytosolic calcium accumulation associated with EWD in rat hippocampal slice cultures. *Alcohol Clin Exp Res*, *29*, 871-881.

Müller, C.P. (2013). Episodic memories and their relevance for psychoactive drug use and addiction. *Frontiers in behavioral neuroscience*, *7*, 34.

Nagel, B.J., Schweinsburg, A.D., Phan, V., & Tapert, S.F. (2005). Reduced hippocampal volume among adolescents with alcohol use disorders without psychiatric comorbidity. *Psychiatry Research: Neuroimaging*, *139*(3), 181-190.

Nagy, J., & László, L. (2002). Increased sensitivity to NMDA is involved in alcohol-withdrawal induced cytotoxicity observed in primary cultures of cortical neurones chronically pre-treated with ethanol. *Neurochemistry international*, *40*(7), 585-591.

National Institute on Alcohol Abuse and Alcoholism (NIAAA). NIAAA Council Approves Definition of Binge Drinking. *NIAAA Newsletter*, No. 3, Winter 2004.

Nevo, I., & Hamon, M. (2004). Neurotransmitter and neuromodulatory mechanisms involved in alcohol abuse and alcoholism. *Alcoholism-Clinical and Experimental Research, 28*(8), 40A.

Newman, D. (2008). Natural products as leads to potential drugs: An old process or the new hope for drug discovery? *Journal of Medicinal Chemistry, 51*(9), 2589-99.

Newman, D., & Cragg, G. (2007). Natural Products as Sources of New Drugs over the Last 25 Years. *Journal of Natural Products, 70*(3), 461-477.

Nicolas, J.M., Catafau, A.M., Estruch, R., Lomena, F.J., Salamero, M., Herranz, R., … Urbano-Marquez, A. (1993). Regional cerebral blood flow-SPECT in chronic alcoholism: relation to neuropsychological testing. *Journal of Nuclear Medicine*, *34*, 1452-1459.

Nixon, K., & McClain, J.A. (2010). Adolescence as a critical window for developing an alcohol use disorder: current findings in neuroscience. *Current opinion in psychiatry*, *23*(3), 227.

Noël, X., Paternot, J., Van der Linden, M., Sferrazza, R., Verhas, M., Hanak, C., … & Verbanck, P. (2001). Correlation between inhibition, working memory and delimited frontal area blood flow measure by 99mTc-Bicisate SPECT in alcohol-dependent patients. *Alcohol and Alcoholism*, *36*(6), 556-563.

Noraberg, J., Kristensen, B.W., & Zimmer, J. (1999). Markers for neuronal degeneration in organotypic slice cultures. *Brain Research Protocols*, *3*(3), 278-290.

Noraberg, J., Poulsen, F.R., Blaabjerg, M., Kristensen, B.W., Bonde, C., Montero, M., ... & Zimmer, J. (2005). Organotypic hippocampal slice cultures for studies of brain damage, neuroprotection and neurorepair. *Current Drug Targets-CNS & Neurological Disorders*, *4*(4), 435-452.

Norberg, E., Gogvadze, V., Ott, M., Horn, M., Uhlén, P., Orrenius, S., & Zhivotovsky, B. (2008). An increase in intracellular Ca2+ is required for the activation of mitochondrial calpain to release AIF during cell death. *Cell Death and Differentiation*, *15*(12), 1857-1864.

Nordmann, Ribière, & Rouach. (1992). Implication of free radical mechanisms in ethanol-induced cellular injury. *Free Radical Biology and Medicine, 12*(3), 219-240.

Obernier, J., Bouldin, T., & Crews, F. (2002). Binge Ethanol Exposure in Adult Rats Causes Necrotic Cell Death. *Alcoholism: Clinical and Experimental Research, 26*(4), 547-557.

Ökvist, J., Kuzmin, B., Merino-Martinez, P., . . . Baune, B. (2007). Neuroadaptations in Human Chronic Alcoholics: Dysregulation of the NF-κB System (NF-κB system in alcoholics). *PLoS ONE, 2*(9), E930.

Orihuela, R., McPherson, C., & Harry, G. (2016). Microglial M1/M2 polarization and metabolic states. *British Journal of Pharmacology, 173*(4), 649-665.

Overstreet, D.H., Kralic, J.E., Morrow, A.L., Ma, Z.Z., Zhang, Y.W., & Lee, D.Y. (2003). NPI-031G (puerarin) reduces anxiogenic effects of alcohol withdrawal or benzodiazepine inverse or 5-HT2C agonists. *Pharmacology Biochemistry and Behavior*, *75*(3), 619-625.

Ozsoy, S., Durak, A.C., & Esel, E. (2013). Hippocampal volumes and cognitive functions in adult alcoholic patients with adolescent-onset. *Alcohol*, *47*(1), 9-14.

Paoletti, P., & Neyton, J. (2007). NMDA receptor subunits: function and pharmacology. *Current opinion in pharmacology*, *7*(1), 39-47.

Paradkar, P.N., Blum, P.S., Berhow, M.A., Baumann, H., & Kuo, S.M. (2004). Dietary isoflavones suppress endotoxin-induced inflammatory reaction in liver and intestine. *Cancer letters*, *215*(1), 21-28.

Park, J.S., Woo, M.S., Kim, D.H., Hyun, J.W., Kim, W.K., Lee, J.C., & Kim, H.S. (2007). Anti-inflammatory mechanisms of isoflavone metabolites in lipopolysaccharide-stimulated microglial cells. *Journal of Pharmacology and Experimental Therapeutics*, *320*(3), 1237-1245.

Parks, M.H., Morgan, V.L., Pickens, D.R., Price, R.R., Dietrich, M.S., Nickel, M.K., & Martin, P.R. (2003). Brain fMRI activation associated with self-paced finger tapping in chronic alcohol-dependent patients. Alcoholism, *Clinical and Experimental Research*, *27*(4), 704-711.

Parsons, O., & Leber, W. (1981). The Relationship between Cognitive Dysfunction and Brain Damage in Alcoholics: Causal, Interactive, or Epiphenomenal? *Alcoholism: Clinical and Experimental Research, 5*(2), 326-343.

Parsons, O.A., & Nixon, S.J. (1993). Neurobehavioral sequelae of alcoholism. *Neurologic Clinics*, *11*(1), 205-18.

Pascual, Baliño, Alfonso-Loeches, Aragón, & Guerri. (2011). Impact of TLR4 on behavioral and cognitive dysfunctions associated with alcohol-induced neuroinflammatory damage. *Brain Behavior and Immunity, 25*, S80-S91.

Patel, R.P., Boersma, B.J., Crawford, J.H., Hogg, N., Kirk, M., Kalyanaraman, B., ... & Darley-Usmar, V. (2001). Antioxidant mechanisms of isoflavones in lipid systems: paradoxical effects of peroxyl radical scavenging. *Free Radical Biology and Medicine*, *31*(12), 1570-1581.

Penetar, D.M., Toto, L.H., Farmer, S.L., Lee, D.Y.W., Ma, Z., Liu, Y., & Lukas, S.E. (2012). The isoflavone puerarin reduces alcohol intake in heavy drinkers: a pilot study. *Drug & Alcohol Dependence*, *126*(1), 251-256.

Penetar, D.M., Toto, L.H., Lee, D.Y.W., & Lukas, S.E. (2015). A single dose of kudzu extract reduces alcohol consumption in a binge drinking paradigm. *Drug & Alcohol Dependence*, *153*, 194-200.

Peng, H., Nickell, C. R. G., Chen, K. Y., McClain, J. A., & Nixon, K. (2017). Increased expression of M1 and M2 phenotypic markers in isolated microglia after four-day binge alcohol exposure in male rats. *Alcohol*, *62*, 29-40.

Pfefferbaum, A., Lim, K., Zipursky, R., Mathalon, D., Rosenbloom, M., Lane, B., . . . Sullivan, E. (1992). Brain Gray and White Matter Volume Loss Accelerates with Aging in Chronic Alcoholics: A Quantitative MRI Study. *Alcoholism: Clinical and Experimental Research, 16*(6), 1078-1089.

Pfefferbaum, A., Sullivan, E., Mathalon, D., & Lim, K. (1997). Frontal Lobe Volume Loss Observed with Magnetic Resonance Imaging in Older Chronic Alcoholics. *Alcoholism: Clinical and Experimental Research, 21*(3), 521-529.

Pitel, A. L., Beaunieux, H., Witkowski, T., Vabret, F., Guillery-Girard, B., Quinette, P., ... & Eustache, F. (2007). Genuine episodic memory deficits and executive dysfunctions in alcoholic subjects early in abstinence. *Alcoholism: Clinical and Experimental Research*, *31*(7), 1169-1178.

Prendergast, M.A., Harris, B.R., Mullholland, P.J., Blanchard, J.A., 2nd, Gibson, D.A., Holley, R.C. and Littleton, J.M. (2004) Hippocampal CA1 region neurodegeneration produced by ethanol withdrawal requires activation of intrinsic polysynaptic hippocampal pathways and function of N-methyl-D-aspartate receptors. *Neurosci,* 124, 869-77.

Promden, W., Monthakantirat, O., Umehara, K., Noguchi, H., & De-Eknamkul, W. (2014). Structure and antioxidant activity relationships of isoflavonoids from Dalbergia parviflora. *Molecules*, *19*(2), 2226-2237.

Pruett, S.B., & Fan, R. (2009). Ethanol inhibits LPS-induced signaling and modulates cytokine production in peritoneal macrophages in vivo in a model for binge drinking. *BMC immunology*, *10*(1), 49.

Pruett, S., Schwab, C., Zheng, Q., & Fan, R. (2004). Suppression of Innate Immunity by Acute Ethanol Administration: A Global Perspective and a New Mechanism Beginning with Inhibition of Signaling through TLR3. *Journal of Immunology, 173*(4), 2715-2724.

Qin, L., & Crews F.T. (2012). Chronic ethanol increases systemic TLR3 agonist-induced neuroinflammation and neurodegeneration. *Journal of Neuroinflammation, 9*(1), 130.

Ramachandran, V., Watts, L., Maffi, S., Chen, J., Schenker, S., & Henderson, G. (2003). Ethanol-induced oxidative stress precedes mitochondrially mediated apoptotic death of cultured fetal cortical neurons. *Journal of Neuroscience Research, 74*(4), 577-588.

Rani, C. S., & Ticku, M. K. (2006). Comparison of chronic ethanol and chronic intermittent ethanol treatments on the expression of GABA A and NMDA receptor subunits. *Alcohol*, *38*(2), 89-97.

Ransohoff, R. (2016). A polarizing question: Do M1 and M2 microglia exist? *Nature Neuroscience, 19*(8), 987-91.

Reynolds, A. R., Saunders, M. A., & Prendergast, M. A. (2016). Ethanol Stimulates Endoplasmic Reticulum Inositol Triphosphate and Sigma Receptors to Promote Withdrawal-Associated Loss of Neuron-Specific Nuclear Protein/Fox-3. *Alcoholism: Clinical and Experimental Research*, *40*(7), 1454-1461.

Reynolds, A.R., Williams, L.A., Saunders, M.A., & Prendergast, M.A. (2015). Group 1 mGlu-family proteins promote neuroadaptation to ethanol and withdrawal-associated hippocampal damage. *Drug and alcohol dependence*, *156*, 213-220.

Reynolds, A.R., Saunders, M.A., Honoree', W.B., Winchester, S.R., Elgumati, I.S., & Prendergast, M.A. (2015). Acute oral administration of the novel, competitive and selective glucocorticoid receptor antagonist ORG 34517 reduces the severity of ethanol withdrawal and related hypothalamic–pituitary–adrenal axis activation. *Drug and alcohol dependence*, *154*, 100-104.

Reynolds, A.R., Berry, J.N., Sharrett-Field, L., & Prendergast, M.A. (2015). Ethanol withdrawal is required to produce persisting N-methyl-d-aspartate receptor-dependent hippocampal cytotoxicity during chronic intermittent ethanol exposure. Alcohol, 49(3), 219-227.

Rivest, S. (2009). Regulation of innate immune responses in the brain. *Nature Reviews Immunology, 9*(6), 429-39.

Ron, D. (2004) Signaling cascades regulating NMDA receptor sensitivity to ethanol. *The Neuroscientist*, *10*(4), 325-336.

Sabino, V., Cottone, P., Zhao, Y., Iyer, M. R., Steardo Jr, L., Steardo, L., ... & Zorrilla, E. P. (2009). The σ-receptor antagonist BD-1063 decreases ethanol intake and

reinforcement in animal models of excessive drinking. *Neuropsychopharmacology*, *34*(6), 1482.

Sacks, Gonzales, Bouchery, Tomedi, & Brewer. (2015). 2010 National and State Costs of Excessive Alcohol Consumption. *American Journal of Preventive Medicine, 49*(5), E73-E79.

Sakran, M., Selim, Y., & Zidan, N. (2014). A new isoflavonoid from seeds of Lepidium sativum L. and its protective effect on hepatotoxicity induced by paracetamol in male rats. *Molecules*, *19*(10), 15440-15451.

Saunders, M.A., Berry, J.N., Butler, T.R., Sharrett-Field, L.J., Reynolds, A.R., & Prendergast, M.A. Calpain activation following acute withdrawal from corticosterone and ethanol co-exposure promotes the loss of hippocampal synaptophysin immunoreactivity. Manuscript in preparation.

Saunders, M.A. (2016). Novel spoxazomicins derived from *Streptomyces* sp. RM-14−6 attenuate ethanol-induced cytotoxicity *in vitro* (Master's Thesis). Retrieved from https://uknowledge.uky.edu/psychology_etds/94

Schlorff, Husain, & Somani. (1999). Dose and Time Dependent Effects of Ethanol on Antioxidant System in Rat Testes. *Alcohol, 18*(2), 203-214.

Schmidt, O., Infanger, I., Heyde, M., Ertel, C., & Stahel, E. (2004). The Role of Neuroinflammation in Traumatic Brain Injury. *European Journal of Trauma,30*(3), 135-149.

Shaaban, K.A., Saunders, M.A., Zhang, Y., Tran, T., Elshahawi, S.I., Ponomareva, L.V., . . . Thorson, J.S. (2017). Spoxazomicin D and Oxachelin C, potent

neuroprotective carboxamides from the appalachian coal fire-associated isolate streptomyces sp. RM-14.6. *Journal of Natural Products, 80*(1), 2-11.

Sharrett-Field, L., Butler, T. R., Berry, J. N., Reynolds, A. R., & Prendergast, M. A. (2013). Mifepristone pretreatment reduces ethanol withdrawal severity in vivo. *Alcoholism: Clinical and Experimental Research*, *37*(8), 1417-1423.

Sontag, B., Gerlitz, M., Paululat, T., Rasser, H. F., Gruen-Wollny, I., & Hansske, F. G. (2006). Oxachelin, a novel iron chelator and antifungal agent from Streptomyces sp. GW9/1258. *The Journal of antibiotics*, *59*(10), 659.

Substance Abuse and Mental Health Services Administration, Results from the 2013 National Survey on Drug Use and Health: Summary of National Findings, NSDUH Series H-48, HHS Publication No. (SMA) 14-4863. Rockville, MD: Substance Abuse and Mental Health Services Administration, 2014.

Sullivan, E. V., Marsh, L., Mathalon, D. H., Lim, K. O., & Pfefferbaum, A. (1995). Anterior hippocampal volume deficits in nonamnesic, aging chronic alcoholics. *Alcoholism: Clinical and Experimental Research*, *19*(1), 110-122.

Sullivan, Mathalon, Lim, Marsh, & Pfefferbaum (1998). Patterns of regional cortical dysmorphology distinguishing schizophrenia and chronic alcoholism. *Biological Psychiatry, 43*(2), 118-131.

Sullivan, E., & Pfefferbaum, V. (2005). Neurocircuitry in alcoholism: A substrate of disruption and repair. *Psychopharmacology, 180*(4), 583-594.

Sullivan, E., Rosenbloom, M., & Pfefferbaum, A. (2000). Pattern of Motor and Cognitive Deficits in Detoxified Alcoholic Men. *Alcoholism: Clinical and Experimental Research, 24*(5), 611-621.

Sullivan, E., Rosenbloom, M., Serventi, K., Deshmukh, A., & Pfefferbaum, A. (2003). Effects of Alcohol Dependence Comorbidity and Antipsychotic Medication on Volumes of the Thalamus and Pons in Schizophrenia. *American Journal of Psychiatry, 160*(6), 1110-1116.

Sun, A.Y., Ingelman-Sundberg, M., Neve, E., Matsumoto, H., Nishitani, Y., Minowa, Y., . . . Sun, G.Y. (2001). Ethanol and oxidative stress. *Alcoholism: Clinical and Experimental Research, 25*(5), 237S.

Sunazuka, T., Hirose, T., & Omura, S. (2008). Efficient total synthesis of novel bioactive microbial metabolites. *Accounts Of Chemical Research, 41*(2), 302-314.

Sunkesula, S.R.B., Swain, U., & Babu, P.P. (2002). Binge ethanol exposure in adult rats causes necrotic cell death. *Alcoholism: Clinical and Experimental Research*, *26*, 547-557.

Suryanarayanan, A., Carter, J. M., Landin, J. D., Morrow, A. L., Werner, D. F., & Spigelman, I. (2016). Role of interleukin-10 (IL-10) in regulation of GABAergic transmission and acute response to ethanol. *Neuropharmacology*, *107*, 181-188.

Tajuddin, N., Kim, H. Y., & Collins, M. A. (2018). PARP Inhibition Prevents Ethanol-Induced Neuroinflammatory Signaling and Neurodegeneration in Rat Adult-Age Brain Slice Cultures. *Journal of Pharmacology and Experimental Therapeutics*, *365*(1), 117-126.

Tang, D., Kang, R., Zeh, H., & Lotze, M. (2011). High-Mobility Group Box 1, Oxidative Stress, and Disease. *Antioxidants & Redox Signaling, 14*(7), 1315-1335.

Tegos, G., & Mylonakis, E. (2012). Antimicrobial drug discovery: emerging strategies. Cabi ebook.

Thomas, M. P., Monaghan, D. T., & Morrisett, R. A. (1998). Evidence for a causative role of N-methyl-D-aspartate receptors in an in vitro model of alcohol withdrawal hyperexcitability. *Journal of Pharmacology and Experimental Therapeutics*, *287*(1), 87-97.

Trevisan, L. A., Boutros, N., Petrakis, I. L., & Krystal, J. H. (1998). Complications of alcohol withdrawal: pathophysiological insights. *Alcohol Research*, *22*(1), 61.

Tsai, G. (1998). Glutamatergic neurotransmission in alcoholism. *Journal of biomedical science*, *5*(5), 309-320.

Urrutia, P. J., Mena, N. P., & Nunez, M. T. (2014). The interplay between iron accumulation, mitochondrial dysfunction, and inflammation during the execution step of neurodegenerative disorders. *Frontiers in pharmacology*, *5*, 38.

Vallés, S., Blanco, A., Pascual, M., & Guerri, C. (2004). Chronic Ethanol Treatment Enhances Inflammatory Mediators and Cell Death in the Brain and in Astrocytes. *Brain Pathology, 14*(4), 365-371.

Van Skike, C.E., Maggio, S.E., Reynolds, A.R., Casey, E.M., Bardo, M.T., Dwoskin, L.P., Prendergast, M.A., Nixon, K. (2016). Critical needs in drug discovery for cessation of alcohol and nicotine polysubstance abuse. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, *65*, 269-287.

Veatch, L.M., Becker, H.C. (2002) Electrographic and behavioral indices of ethanol withdrawal sensitization. *Brain Res*, *946*, 272-282.

Venereau, E., Casalgrandi, M., Schiraldi, M., Antoine, D. J., Cattaneo, A., De Marchis, F., ... & Shams, S. S. (2012). Mutually exclusive redox forms of HMGB1 promote

cell recruitment or proinflammatory cytokine release. *Journal of Experimental Medicine*, *209*(9), 1519-1528.

Vetreno, R.P., & Crews, F.T. (2015). Binge ethanol exposure during adolescence leads to a persistent loss of neurogenesis in the dorsal and ventral hippocampus that is associated with impaired adult cognitive functioning. *Frontiers in neuroscience*, *9*, 35.

Vezzani, Maroso, Balosso, Sanchez, & Bartfai (2011). IL-1 receptor/toll-like receptor signaling in infection, inflammation, stress and neurodegeneration couples hyperexcitability and seizures. *Brain Behavior and Immunity*, *25*(7), 1281-289.

Viviani, Bartesaghi, Gardoni, Vezzani, Behrens, Bartfai, . . . Viviani, B. (2003). Interleukin-1 beta Enhances NMDA Receptor-Mediated Intracellular Calcium Increase through Activation of the Src Family of Kinases. *Journal of Neuroscience, 23*(25), 8692-8700.

Wang, X., Reynolds, A. R., Elshahawi, S. I., Shaaban, K. A., Ponomareva, L. V., Saunders, M. A., ... & Sunkara, M. (2015). Terfestatins B and C, new p-terphenyl glycosides produced by Streptomyces sp. RM-5–8. *Organic letters*, *17*(11), 2796-2799.

Wang, J., Lanfranco, M.F., Gibb, S.L., Yowell, Q.V., Carnicella, S., & Ron, D. (2010). Long-lasting adaptations of the NR2B-containing NMDA receptors in the dorsomedial striatum play a crucial role in alcohol consumption and relapse. *Journal of Neuroscience*, *30*(30), 10187-10198

Wang, H., Yang, H., & Tracey, K.J. (2004). Extracellular role of HMGB1 in inflammation and sepsis. *Journal of internal medicine*, *255*(3), 320-331.

Ward, R., Lallemand, F., & De Witte, P. (2009). Biochemical and Neurotransmitter Changes Implicated in Alcohol-Induced Brain Damage in Chronic or 'Binge Drinking' Alcohol Abuse. *Alcohol and Alcoholism: International Journal of the Medical Council on Alcoholism, 44*(2), 128-35.

Ward, R., Zhang, Y., Crichton, R., Piret, B., Piette, J., & De Witte, P. (1996). Identification of the nuclear transcription factor NFκB in rat brain after in vivo ethanol administration. *FEBS Letters, 389*(2), 119-122.

West, J.R. (1987). Fetal alcohol-induced brain damage and the problem of determining temporal vulnerability: a review. *Alcohol and drug research*, *7*(5-6), 423-441.

Whitman, B.A., Knapp, D.J., Werner, D.F., Crews, F.T., & Breese, G.R. (2013). The Cytokine m RNA Increase Induced by Withdrawal from Chronic Ethanol in the Sterile Environment of Brain is Mediated by CRF and HMGB 1 Release. *Alcoholism: Clinical and Experimental Research*, *37*(12), 2086-2097.

Wilkins, L., Prendergast, M., Blanchard, J., Holley, R., Chambers, E., & Littleton, J. (2006). Potential Value of Changes in Cell Markers in Organotypic Hippocampal Cultures Associated With Chronic EtOH Exposure and Withdrawal: Comparison with NMDA-Induced Changes. *Alcoholism: Clinical and Experimental Research, 30*(10), 1768-1780.

Wilson, S., Bair, J. L., Thomas, K. M., & Iacono, W. G. (2017). Problematic alcohol use and reduced hippocampal volume: a meta-analytic review. *Psychological medicine*, *47*(13), 2288-2301.

Wirkner, K., Poelchen, W., Köles, L., Mühlberg, K., Scheibler, P., Allgaier, C., and Illes, P. (1999). Ethanol-induced inhibition of NMDA receptor channels. *Neurochemistry International*, *35*, 153-162.

Wozniak, D., Janda, B., Kapusta, I., Oleszek, W., & Matkowski, A. (2010). Antimutagenic and anti-oxidant activities of isoflavonoids from Belamcanda chinensis (L.) DC. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *696*(2), 148-153.

Wu, Z., Li, L., Zheng, L. T., Xu, Z., Guo, L., & Zhen, X. (2015). Allosteric modulation of sigma-1 receptors by SKF83959 inhibits microglia-mediated inflammation. *Journal of neurochemistry*, *134*(5), 904-914.

Yao, H., Yang, Y., Kim, K. J., Bethel-Brown, C., Gong, N., Funa, K., ... & Buch, S. (2010). Molecular mechanisms involving sigma receptor-mediated induction of MCP-1: implication for increased monocyte transmigration. *Blood*.

Yen, G.C., & Lai, H.H. (2003). Inhibition of reactive nitrogen species effects in vitro and in vivo by isoflavones and soy-based food extracts. *Journal of agricultural and food chemistry*, *51*(27), 7892-7900.

Zago, A., Moreira, P., Jansen, K., Lhullier, A.C., da Silva, R.A., de Oliveira, J.F., ... & de Mattos Souza, L.D. (2016). Alcohol Use Disorder and Inflammatory Cytokines in a Population Sample of Young Adults. *Journal of Alcoholism & Drug Dependence*, 1-5.

Zahr, N.M., Luong, R., Sullivan, E.V., & Pfefferbaum, A. (2010). Measurement of Serum, Liver, and Brain Cytokine Induction, Thiamine Levels, and Hepatopathology in Rats Exposed to a 4-Day Alcohol Binge Protocol. *Alcoholism: Clinical and Experimental Research*, *34*(11), 1858-1870.

Zhang, J., Hughes, R.R., Saunders, M.A., Elshahawi, S.I., Ponomareva, L.V., Zhang, Y., ... & Thorson, J.S. (2017). Identification of Neuroprotective Spoxazomicin and Oxachelin Glycosides via Chemoenzymatic Glycosyl-Scanning. *Journal of natural products*, *80*(1), 12-18.

Zhao, Yang, Bi, Yu, Jia, Fang, and Zang (2015). Acetylcholine attenuated TNF-α-induced apoptosis in H9c2 cells: Role of calpain and the P38-MAPK pathway. *Cellular Physiology and Biochemistry*, *36*(5), 1877-889.

Zimatkin, S.M., Pronko, S.P., Vasiliou, V., Gonzalez, F.J., & Deitrich, R.A. (2006). Enzymatic mechanisms of ethanol oxidation in the brain. *Alcoholism: clinical and experimental research*, *30*(9), 1500-1505.

Zimmer, J., Kristensen, B.W., Jakobsen, B., Noraberg, J. (2000). Excitatory amino acid neurotoxicity and modulation of glutamate receptor expression in organotypic brain slice cultures. *Amino Acids*, *19*, 7-21.

Zou, J., & Crews, F. (2010). Induction of Innate Immune Gene Expression Cascades in Brain Slice Cultures by Ethanol: Key Role of NF-κB and Proinflammatory Cytokines. *Alcoholism: Clinical and Experimental Research, 34*(5), 777-789.

Zou, J. Y., & Crews, F. T. (2005). TNFα potentiates glutamate neurotoxicity by inhibiting glutamate uptake in organotypic brain slice cultures: neuroprotection by NFκB inhibition. *Brain research*, *1034*(1), 11-24.

Curriculum Vitae

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Degrees Awarded

University of Kentucky, M.S. 2016
University of Kentucky, B.S. 2013

Professional Positions

Pre-doctoral Trainee 2016 – 2018
National Institute on Drug Abuse
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University of Kentucky

Teaching Assistant 2013 – 2016
University of Kentucky, Department of Psychology

Research Assistant 2015 – 2015
University of Kentucky, Department of Psychology

Laboratory Technician 2013
Neurobiological Effects of Alcohol
University of Kentucky, Department of Psychology

Undergraduate Research Intern 2014 – 2013
Neurobiological Effects of Alcohol
University of Kentucky, Department of Psychology

Scholastic Honors

Lipman Summer Research Award in Alcohol Dependence 2016
Annual BGSFN Spring Neuroscience Day, Outstanding poster award 2016
Symposium on Drug Discovery & Development, Outstanding poster award 2015
Psi Chi International Honor Society in Psychology 2009
University of Kentucky Presidential Scholarship 2009 – 2013

Publications

Maggio, S. E., **Saunders, M.A.**, Baxter, T. A., Nixon, K., Prendergast, M. A., Zheng, G., Crooks, P., Slack, R. D., Newman, A. H., Thorson, J. S., Dwoskin, L. P., & Bardo, M. T. (2018). Effects of the nicotinic agonist varenicline, the novel nicotinic antagonist r-bPiDI, and the dopamine transporter inhibitor r-modafinil

on co-use of alcohol and nicotine in female P rats. *Psychopharmacology*, *235*(5), 1439–1453.

Reynolds A.R., **Saunders, M.A.**, Sharrett-Field, L.J., Berry, J.N., Winchester, S., & Prendergast, M.A. (2017). Broad- spectrum protein kinase inhibition by the staurosporine analog KT-5720 reverses ethanol withdrawal-associated loss of NeuN/Fox-3. *Alcohol*, *64*, 37–43.

Shaaban, K.A., **Saunders, M.A.**, Zhang, Y., Tran, T., Elshahawi, S.I., Ponomareva, L.V., Wang, X., Zhang, J., Copley, G.C., Sunkara, M., Kharel, M.K., Morris, A.J., Hower, J.C., Tremblay, M.S., Prendergast, M.A., & Thorson, J.S. (2017). Spoxazomicin D and oxachelin C, potent neuroprotective carboxamides from the Appalachian coal fire-associated isolate *Streptomyces* sp. RM-14-6. *Journal of Natural Products*, *80* (1), 2–11.

Zhang, J., Hughes, R.R., **Saunders, M.A.**, Elshahawi, S.I., Ponomareva, L.V., Zhang, Y., Winchester, S.R., Scott, S.A., Sunkara, M., Morris, A.J., Prendergast, M.A., Shaaban, K.A., & Thorson, J.S. (2017). Identification of neuroprotective spoxazomicin and oxachelin glycosides via chemoenzymatic glycosyl-scanning. *Journal of Natural Products*, *80* (1), 12–18.

Reynolds, A.R., **Saunders, M.A.**, & Prendergast, M.A. (2016). Ethanol stimulates endoplasmic reticulum inositol triphosphate and sigma receptors to promote withdrawal- associated loss of neuron specific nuclear protein/Fox-3. *Alcoholism: Clinical and Experimental Research*, *40*(7), 1454-61.

Berry, J.N., **Saunders, M.A**., Sharrett-Field, L.J, Reynolds, A.R., Bardo, M.T., Pauly, J.R., & Prendergast, M.A. (2016). Corticosterone enhances N-methyl-D-aspartate receptor signaling to promote ventral tegmental area neurotoxicity in the reconstituted mesolimbic dopamine pathway. *Brain Research Bulletin*, *120*, 159–165.

Reynolds, A.R., Williams, L.A., **Saunders, M.A.**, & Prendergast, M.A. (2015). Group 1 mGlu- family proteins promote neuroadaptation to ethanol and withdrawal-associated hippocampal damage. *Drug and Alcohol Dependence*, *156*, 213–220.

Reynolds, A.R., **Saunders, M.A.**, Brewton, H.W., Winchester, S.R., Elgumati, I.E., & Prendergast, M.A. (2015). Acute oral administration of the novel, competitive and selective glucocorticoid receptor antagonist ORG 34517 reduces the severity of ethanol withdrawal and related hypothalamic- pituitary-adrenal axis activation. *Drug and Alcohol Dependence*, *154*, 100–104.

Wang, X., Reynolds, A.R., Elshahawi, S.I., Shaaban, K.A., Ponomareva, L.V., **Saunders, M.A.**, Elgumati, I.E., Zhang, Y., Copley, G.C., Hower, J.C., Sunkara, M., Morris, A.J., Kharel, M.K., Van Lanen, S.G., Prendergast, M.A., Thorson, J.S. (2015). Terfestatins B and C, new p-terphenyl glycosides produced by streptomyces sp. RM-5–8. *Organics Letters*, *17*(11), 2796–9.

Submitted Manuscripts

Maggio, S. E., **Saunders, M.A.**, Nixon, K., Prendergast, M. A., Zheng, G., Crooks, P., Dwoskin, L. P., Bell, R.L., & Bardo, M. T. Effects of the opioid antagonist naltrexone, the nicotinic partial agonist varenicline and the nicotinic α6β2* antagonist r-bPiDI on co-use of ethanol and nicotine in female P rats. (*submitted*

to Drug and Alcohol Dependence)

In Preparation

Saunders, M.A., Jagielo-Miller, J.E., & Prendergast, M.A. Daidzein induces cytoprotection against binge-like ethanol exposure *in vitro*: Role of High Mobility Group Box 1 protein? (*in preparation for Drug and Alcohol Dependence*)

Saunders, M.A.*, Berry, J.N.*, Butler, T.R., Sharrett-Field, L.J., Reynolds, A.R., & Prendergast, M.A. Calpain activation following acute withdrawal from corticosterone and ethanol co-exposure promotes the loss of hippocampal synaptophysin immunoreactivity. (*in preparation for Drug and Alcohol Dependence*)

Saunders, M.A.*, Sharrett-Field*, L.J., Jagielo-Miller, J.E., Prendergast, M.A. Plasma corticosterone levels are correlated with subsequent voluntary intake of ethanol using a chronic, intermittent access paradigm. (*in preparation for Drug and Alcohol Dependence*)

Rice, B.A., **Saunders, M.A.**, Prendergast, M.A. and Akins, C.K. Repeated blockade of the glucocorticoid receptor with PT 150 has dose-dependent effects on sign tracking, in male Japanese quail.

Rice, B.A., **Saunders, M.A.**, Eaton, S.E., Arthur, D. Technology in the classroom facilitates application of APA style, but not recall.

Prendergast, M.A., Butler, T.R., Smith, K.J., Reynolds, A.R**., Saunders, M.A.**, Little, H.J. The novel, competitive glucocorticoid receptor antagonist PT 150 reduces the severity of withdrawal from repeated binge-like ethanol administration.

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Signature