A Thesis

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The Lethal and Toxic Effects of 3,4-Methylenedioxymethamphetamine (MDMA) and Methylone in Combination with Alcohol or Nicotine

by

Abdulrahman Omar Mohammed Ba Gunaid

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Master of Science in Pharmaceutical Sciences Degree in Pharmacology and Toxicology

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The University of Toledo
December 2022

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An Abstract of

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3,4-Methylenedioxy-methamphetamine (MDMA) and 3,4-methylenedioxy-N-methylcathinone (Methylone) have been abused, particularly by teens and young adults. These drugs are known for their distinct psychoactive effects that result from increased synaptic levels of the monoamine neurotransmitters dopamine (DA), serotonin (5-hydroxytryptamine; 5-HT), and norepinephrine (NE). MDMA and methylone are often abused and taken in conjunction with other licit and illicit drugs in nightclubs and concert environments. In particular, alcohol and tobacco are used. Tobacco can be a source of nicotine. MDMA and methylone overdose results in acute toxicity and lethality, but since use usually occurs in combination with alcohol or nicotine these drugs may contribute to those effects. The lethal and toxic effects of MDMA and methylone were examined in combination with alcohol and nicotine as a model of drug overdose in 5 days post fertilization (dpf) larval zebrafish (*Danio rerio*). Lethal concentrations (LC_{50}) for all drugs were determined. Combinations of MDMA and alcohol produced greater lethality than either alone, e.g., addition of the maximal non-lethal concentration (MNLC) of alcohol reduced the LC_{50} for MDMA, and addition of the MNLC of MDMA reduced the LC_{50} for

alcohol. The addition of the MNLC of MDMA to nicotine also produced a small reduction in the LC_{50} value. However, when the MNLC of nicotine was added to MDMA, the LC_{50} value was ***increased*** (i.e., it was protective). The same pattern of effects was observed for methylone, including exacerbation of lethality by ethanol and reductions in lethality by nicotine. Based on these results using this zebrafish model, when MDMA and methylone are combined with alcohol, greater lethality and toxicity occur than either drug alone. On the other hand, the combination of MDMA and methylone with MNLC of nicotine showed a promising reversal in lethality and toxicity. This may provide a rationale for developing a treatment for MDMA/methylone overdose, for which there are no current treatments aside from symptomatic relief.

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List of Abbreviations

5-HT …….5-Hydroxytryptamine

Ace……. Acetaldehyde
Ach……. Acetylcholine
COMT.....Catechol-O-Methyltransferase
CYP2D6.....Cytochrome P2D6

DA.....Dopamine
DAT .....Dopamine Transporter
DI .....Distilled Water
dpf .....Days Post Fertilization

e.g.....for example
EtOH .....Ethanol

hpf .....Hours Post Fertilization

LC_{50}.....The concentrations of the chemical that kills 50% of the test animals during the observation period

MDMA ……3,4-Methylenedioxymethamphetamine
MDMC.....3,4-Methylenedioxy-*N*-methylcathinone
MNLC.....Maximum Non-Lethal Concentration

NA.....Norepinephrine
nAChRs.....Nicotinic acetylcholine receptors
NET.....Norepinephrine transporter
NPS .....New Psychoactive Substances

pH.....An expression of hydrogen ion concentration in water

SERT…….Serotonin transporter

List of Symbols

< .....Less than
% .....Percent

µS/cm .....MicroSiemens

A.M. .....Ante meridiem, before noon

°C .....The degree *Celsius*

g.....Gram

hr. .....Hour

L.....Liter
LC50.....Concentration required kill 50% of the population.

µg..... Microgram
µL..... Microliter
µM..... Micromolar
mg..... Milligram
mL..... Milliliter
mM..... Millimolar

P.M.....Post meridiem, after midday
p.....A statistical measurement used to validate a hypothesis against observed data.

Chapter 1

Introduction

1.1 Khat

In the Arabian Peninsula and East African countries, there is a plant that is scientifically known as *Catha edulis*. Common names for *Catha edulis* include gat, graba, Somali tea, and most commonly, khat (Thomas and Williams 2013). The khat plant has buds and leaves that can be used either fresh or dried. They have a harsh faintly sweet taste and a mild scent (Alemu, Zeleke, and Takele 2018). In Yemen, Djibouti, Kenya, Ethiopia, Eritrea, Somalia, and Uganda, khat is chewed for its euphoric and stimulating effects produced by its psychoactive ingredients. Leaves and branches of khat contain cathinone (the β-ketone analogue of amphetamine) and cathine (norpseudoephedrine) (Abafita and Bulga 2020; Thomas and Williams 2013). These are two active chemicals in khat that alter the mood of its users (Godifey Wubneh, Mulaw Desta, and Amsalu Kahsay 2021). Traditional healers use Khat medicinally, as it is thought to help with headaches, colds, body aches, fever, arthritis, and even depression in some of these communities (Thomas and Williams 2013; Huffman 2021). Khat has been introduced to Europe and the United States through migration of people from Northeastern Africa and the Arabian Peninsula. Now, not only Northeastern Africa and the Arabian Peninsula have a growing

public health problem associated with khat use, but also Europe and the United States (Bedada et al. 2018).

In its countries of origin, khat has been used to combat fatigue and elevate mood, because of its euphoric and psychostimulant effects (Alemu, Zeleke, and Takele 2018). Traditionally in these places *Catha edulis* is consumed as a chewable paste, tea, or sprinkled on food where the leaves and buds are chewed fresh or preserved by wrapping them in banana leaves. Users most often chew about 100 to 300 g of fresh leaves to form a bolus or quid (similar to oral tobacco consumption). This is usually held on one side of the mouth to allow one to swallow the juice produced. A normal khat chewing session lasts for about 3 to 4 hours (Mihretu et al. 2020; Ongeri et al. 2019). The chemical components and concentrations in Khat leaves vary from one plant to another due to different natural factors (Thomas and Williams 2013), as well as factors associated with its production and use. Indeed, this is one of the reasons for preservation of Khat using banana leaves, which prevents the breakdown of cathinone by oxidation.

Around 10 million people consume this plant regularly, most of these in traditional Khat-consuming nations, but with growing numbers of users world-wide. Long-term consumption of Khat causes adverse effects on organ systems of the body including the nervous, cardiovascular, respiratory, and reproductive systems (Alhazmi et al. 2022). Paranoia, delusions, and hallucinations also appear in long-term khat consumers (Nencini and Ahmed 1989), similar to what occurs with chronic use of other psychostimulant drugs. Furthermore, chronic khat users can show elevated blood pressure, tachycardia, hyperthermia, aggression, and confusion while consuming, while depression, irritability, anxiety, and cognitive impairment arise as withdrawal symptoms develop alongside khat

tolerance and dependence. These symptoms of chronic use and withdrawal are also similar to those occurring after using amphetamine-like substances (Katz et al. 2014; Thomas and Williams 2013). During the 1980s, khat was classified as a drug of abuse by the World Health Organization. The U.S. Drug Enforcement Administration has classified cathinone as a Schedule I drug, while cathine is a Schedule IV controlled substance (Sterling 2020).

1.2 MDMA

3,4-Methylenedioxy-methamphetamine (MDMA) is commonly known as “ecstasy” or “Molly”. It is a synthetic drug that is known to alter perception and mood. It is described as a stimulating drug that makes a user feel high, although its subjective effects are quite distinct from other amphetamines, like methamphetamine, with less overall stimulant and euphoric effects. MDMA and similar drugs are classified as “entactogens” producing more of a feeling of well-being than euphoria. This psychoactive drug is popular among teens and young adults for recreational purposes in social situations such as concerts, "rave" parties, and clubbing, due to its ability to trigger sensations, empathy, and cause increased energy and pleasure (Budzynska et al. 2018). It is common for MDMA manufacturers and users to mix other substances with MDMA, such as amphetamine, methamphetamine, and ephedrine, to produce with the purpose of producing a greater energizing effect. MDMA was initially manufactured in 1912 by a German company for use as an appetite suppressant (Monson et al. 2021). In the 1970s it was used to aid psychotherapy, something which is finally being recognized once again (Mitchell et al. 2021). MDMA became popular in the 1980s as a street drug due to its mood-altering

effects. After being initially placed on Schedule I by the DEA in 1985, its illicit use expanded in the 1990s among young adults and college students, used primarily as a “club” or “party” drug (Liang et al. 2017), and its use remains highly prevalent today. It is estimated that 0.9% (or approximately 2.6 million people) of people aged 12 or older in 2019 used MDMA (NIDA. 2022)

MDMA comes in various forms such as tablets, capsules, dissolved in a liquid, or as a powder form. The drug's slang name "Molly" is derived from the word “molecular” due to its striking appearance in its pure crystalline powder form. Although the crystalline powder often appears to be pure, adulterations with other chemicals used to dilute it and make it more effective is common (Elliott et al. 2019; Poyatos et al. 2021). More recently, “Ecstasy” has come to stand for a variety of drugs that have similar effects, in particular several of the MDMA-like cathinones, in the same manner that “heroin” on the street now often contains fentanyl or other opioids. The substances in “Ecstasy” sold illicitly may include the cathinones ethylone, methylone, mephedrone, or 3,4-methylenedioxypyrovalerone (MDPV), in addition to other drugs or adulterants. MDMA is most often taken orally in a pill form, snorted through the nose in its crystalline form, or dabbed onto the gums. Its effects can begin within 30 to 45 minutes, and its influence can last up to 6 hours, although the typical pattern of use involves binges of multiple doses on the same night.

As the drug is taken orally most often, the absorption of MDMA occurs in the intestinal track. It has about an 8-hour half-life in the body indicating a need of 5 half-lives for 95% to be eliminated. The primary subjective effects of MDMA involve its ability to elevate monoamine levels extracellularly (Vizeli and Liechti 2018). It triggers the release

of serotonin (5-hydroxytryptamine; 5-HT) and norepinephrine (NA) more selectively than dopamine (DA) from their respective axon terminals. Its primary effects are thought to be mediated by its serotonin releasing effects, although it has some effects on the other monoamine transmitters (Battaglia et al. 1987; Finnegan et al. 1988; Kalant 2001; O'hearn et al. 1988). There are two main ways that MDMA is metabolized. The primary route involves N-dealkylation, deamination, and oxidation followed by conjugation with glycine. A second route involves both O-demethylation and catechol-O-methyltransferase (COMT) catalyzing methylation. Cytochrome P2D6 enzyme (CYP2D6) is involved in this metabolic pathway, which may increase the risk of toxicity for those with a slower metabolism in individuals with reduced or less active CYP2D6 (Campbell and Rosner 2008).

MDMA works as an indirect monoaminergic agonist, prompting synapses to release significant amounts of serotonin, norepinephrine and, to a lesser extent, dopamine (Lizarraga et al. 2015). Serotonin has a role in many behavioral and psychological functions, including cognition, mood, sleep, pain, and appetite, among others (Vizeli and Liechti 2018). Serotonin also directly, or indirectly via regulation of hormonal function, influences aspects of metabolism, digestion, cardiovascular function, and other physiological responses. However, since MDMA releases a large amount of serotonin, the brain loses a lot of this brain chemical, which is part of the reason people may experience adverse psychological effects for a few days after using it, including sleep disturbances, impaired attention and memory, irritability, and depression (Huffman 2021). MDMA also stimulates the production of dopamine, which increases energy levels, thus allowing one to be more active, which makes it desirable for use during parties or concerts.

Norepinephrine is a catecholamine neurotransmitter and elevated norepinephrine function increases heart rate and elevates blood pressure. This can be a risk among people with cardiac and cardiovascular dysfunction (Hysek et al. 2011).

Acute use of MDMA can have harmful effects that are commonly associated with overdose. The two most frequent harmful effects of MDMA are hyperthermia and dehydration. Hyperthermia can cause profuse sweating after physical activity, and if untreated, can be followed by seizures. Overheating of the body can cause muscle breakdown that can affect the functioning of the liver, heart, and kidney, as well as brain swelling. There are other more direct effects on these organs too. This is in fact the primary cause of death from MDMA overdose, which results from multiple organ failure. Users of MDMA have elevated body temperatures after MDMA use, particular under “binge” dosing conditions typical of recreational use (Liechti 2014; Rybarova et al. 2021; García-Montes et al. 2021), and similar effects are seen in animal models mimicking these conditions (Martins et al. 2011; Schmidt et al. 1990; Clemens et al. 2005). Dehydration and consequent imbalances in fluid and salt metabolism can result under these conditions (Bora, Yılmaz, and Bora 2016). If the body's water levels rapidly decrease and proper medical intervention is not provided, death is a possibility. Other acute effects of MDMA include insomnia, nausea, loss of appetite, vomiting, irritability, blurred vision, diarrhea, muscle cramping, and heightened senses (Kolaczynska et al. 2021).

Long-term effects of MDMA occur with repeated use. Long-term use of MDMA effects are still not clear and under investigation. However, chronic use of MDMA has shown consistent evidence of neurotoxic brain lesions that lead to neurodegeneration in serotonergic axon terminals (Bogen et al. 2003). It is known that 5-HT level elevates in the

brain when MDMA is consumed which increases microvasculature permeability of the brain and spinal Cord (Scheffel et al. 1998; Reneman et al. 2000). Chronic use of MDMA eventually results in the loss of serotonergic neurons, as evidenced by the significant reduction in cortical 5-HT levels 13 months after MDMA treatment in monkeys (Scheffel et al. 1998). These changes reduce white and grey matter density in brain structures (Cowan et al. 2003). Additionally, evidence has shown that MDMA can cause memory impairment, anxiety, depression, and cognitive problems. However, memory impairments among MDMA users are less than users of some other illicit drugs, such as cocaine and heroin (Parrott 2013; Ayllón and Ferreira-Batista 2018). MDMA use is likely to increase the risk of cardiac valvopathy since it causes activation of serotonin receptors that stimulate cardiac epigenetic and hypermethylation changes (Karch 2011). Use of MDMA has been associated with kidney, heart, and liver problems, with adverse effects among users with asthmatic and high blood pressure histories. Adverse effects diminish with time when users stop using the drug (Kolaczynska et al. 2021). Long-term adverse consequences occur with repeated use of MDMA. Long-term consequences of MDMA use are still not completely clear and under investigation. However, chronic use of MDMA has shown consistent evidence of neurotoxic brain lesions that lead to neurodegeneration in serotonergic axon terminals (Bogen et al. 2003). It is known that 5-HT levels are elevated in the brain when MDMA is consumed, which increases microvasculature permeability of the brain and spinal cord (Scheffel et al. 1998; Reneman et al. 2000). Chronic use of MDMA eventually results in the loss of serotonergic neurons, as evidenced by the significant reduction in cortical 5-HT levels 13 months after MDMA treatment in squirrel monkeys (Scheffel et al. 1998). These changes reduce white and grey matter density in brain structures (Cowan et

al. 2003). Additionally, evidence has shown that MDMA can cause memory impairment, anxiety, depression, and cognitive problems. However, memory impairments among MDMA users are less than users of some other illicit drugs, such as cocaine and heroin (Parrott 2013; Ayllón and Ferreira-Batista 2018). MDMA use also increases the risk of cardiac valvopathy since it causes activation of serotonin receptors that stimulate epigenetic and hypermethylation changes in the heart (Karch 2011). Use of MDMA has been associated with kidney, heart, and liver problems, and especially with adverse effects among users with asthmatic and high blood pressure histories. Many adverse effects do however diminish with time when users stop using the drug (Kolaczynska et al. 2021).

1.3 Methylone

3,4-methylenedioxy-*N*-methylcathinone (MDMC) is more commonly known as methylone and its action considered as is the prototypical MDMA-like cathinone (Štefková et al. 2017). Methylone belongs to a group of new psychoactive substances (NPS) called synthetic psychoactive cathinones (SPCs), that include a wide range of substances. However, not all SPCs are MDMA-like. For example, pyrovalerone and MDPV act as cocaine-like (Smith et al. 2017). Individuals use methylone most often as a replacement, either intentionally or unknowingly on the part of the user, for MDMA (Watterson et al. 2012). Chemists Alexander Shulgin and Peyton Jacob III first developed methylone in 1996 as a potential treatment for Parkinson's Disease or depression, but it was never used therapeutically. Methylone was first marketed on the illicit drug market in

the Netherlands in 2004 and quickly became used worldwide and easy to obtain (Cawrse et al. 2012; Štefková et al. 2017). When SPCs, including methylone, initially started to be used in the United States, in about 2000, they were not yet illegal. Methylone and other SPCs were initially marketed as “research chemicals”, “plant food”, or “bath salts” to avoid legal restrictions on their sale. There were warnings on the packaging of SPCs stating, "Not for human consumption" (Leonhart 2011), although it was clear from the packaging that this was exactly the intention. SPCs were often offered as mixtures of several cathinones, unlike conventional amphetamines. SPC components identified in these drugs initially included mephedrone, methylone, and MDPV. These drugs were sold at smoke shops, head shops, convenience stores, adult bookstores, and gas stations until all of these drugs were scheduled by the DEA. In 2010, these three drugs first appeared in Centers for Disease Control (CDC) reports and in reports from emergency rooms across the United States (Riley et al. 2020). The DEA placed these three SPCs on an emergency prohibition in 2011, and they were permanently categorized as Schedule 1 drugs a year later, due to the continual growth in their usage and the extent of their detrimental effects (Drug Enforcement Administration 2014a; Drug Enforcement Administration 2013; Drug Enforcement Administration 2014b). Even though SPCs are no longer available in brick-and-mortar locations, their overall use has continued to increase because SPCs can still be purchased on the dark web and mailed to the US from foreign sources. There have been many reports of acute overdose from SPCs. In one in San Diego in 2013, a healthy 19-year-old woman with a history of recreational drug use was found floating after drowning. Autopsy revealed that the woman had been exposed to high doses of methylone prior to

her death, found in her blood, stomach organs, vitreous fluid, and the liver, with the later organ showing the highest concentrations (Zaami et al. 2018).

People use methylone to experience effects similar to those of MDMA, including feelings of euphoria, increased sex drive, sociability, and energy. When people buy SPCs or "ecstasy" illicitly, there can be a great deal of uncertainty about the actual drugs consumed, which often contain a mixture of recreational synthetic drugs or adulterants. Uncertainty about the drug being used, as well as dose and purity, may increase the likelihood of adverse events and overdose. Methylone can be taken orally, through intravenous injection, sublingually, inhaled, or through rectal administration. The most common method is oral consumption of tablets or pills. Methylone users report low dose effects at doses up to 100 mg, with more moderate effects occurring between 100 mg and 200 mg, and high dose effects above 200 mg. These doses are comparable to MDMA dose ranges. The initial effects of methylone usually occur within 15-60 minutes, and moderate to severe effects usually occur between 60-90 minutes after oral administration. Typical recreational effects last for about three to five hours. In order to maximize the desired effects associated with methylone/MDMA use, drug users take a large initial dose followed by smaller maintenance doses over the course of an evening/night (Poyatos et al. 2021). Several serious adverse effects have been associated with the use of methylone, including psychosis, agitation, violent behavior, tachycardia, seizures, kidney damage and even death. Also, methylone induces high body temperatures above normal by inhibiting serotonin and/or dopamine transporters responsible for thermoregulation. This can be fatal due to dehydration that may arise due to excessive sweating (Faria et al. 2020; Hassan et al. 2017). Users of SPCs experience increased sociability, excitement, and talkativeness.

This can lead to an unrealistic feeling of cleverness, superiority, power, and sexual drive. In addition, acting aggressively, panic, and even self-injury is common in SPCs users. It is notable that methylone is metabolized by CYP2D6 in the liver and acts through a similar mechanism to MDMA. It has been reported that methylone inhibits the reuptake of norepinephrine, dopamine, and serotonin *in vitro* (Cozzi et al. 1999). However, (Nagai, Nonaka, and Kamimura 2007) have shown that methylone releases norepinephrine, dopamine, and serotonin from rat brain synaptosomes. The effects of methylone are mediated by its actions on the plasma membrane transporters for dopamine (DAT), norepinephrine (NET), and serotonin (SERT), resulting in their non-exocytotic release, although the primary subjective effects are mediated by SERT, like MDMA (Elmore et al. 2017). Some studies conducted on rats show that methylone raises the extracellular levels of DA and 5-HT in the brain, with higher selectivity for SERT causing effects on 5-HT release to be more pronounced (Schindler et al. 2016; Baumann et al. 2012). In addition, methylone increases norepinephrine, release, both a neurotransmitter and hormone. Increasing the hormone levels causes dilated pupils, irregular heart rate, high blood pressure, chest pain, and narrowing of blood vessels hence lowering blood flow (Mégarbane, et al., 2020).

Reuptake inhibition and substrate releasing effects are the main ways that SPCs impact monoamine synapses (Gregg et al. 2015; Kolanos et al. 2015; Marusich et al. 2014), although SPCs, like amphetamines, differ in their ability to act as reuptake inhibitors and releasers. Inhibition of the reuptake process increases the synaptic concentrations of neurotransmitters following normal calcium-dependent vesicular release. SPCS, like amphetamines, differ in their relative affinities for DAT, SERT, and NET, as well. The

second process is substrate release, wherein drugs reverse the direction of flow of neurotransmitters across plasma membrane transporters, resulting in non-exocytotic neurotransmitter release. Releasers also decrease vesicular uptake and elevate cytosolic monoamine levels due to inhibition of the vesicular monoamine transporter 2, or (VMAT2). In a healthy state, monoamines are transported by VMAT2 from the cytosol into synaptic vesicles via a proton gradient driven by ATPase. Ca^{++} dependent conformational changes in cytosolic proteins generate neurotransmitter vesicle binding and release (Augustine 2001). The elevation in monoamine levels in the cytosol is also essential for the neurotoxicity of monoamine releasing drugs, which is not seen in pure reuptake inhibitors that do not have releasing effects.

1.4 Alcohol and Nicotine Co-use

It is common for recreational drug users to use multiple drugs at one time, especially at concerts and or nightclub environments, including alcohol, tobacco, and other stimulants. Many cases have been reported where people have been discovered driving cars, lying down unconscious or even dead after polydrug use; so, it is likely that co-use contributes to adverse events and overdose (Knoy, Peterson, and Couper 2014; Matthai et al. 1996; McIntyre et al. 2013; Mueller et al. 2016). These studies found multiple drugs in the system in *post mortem* analyses after overdose, including alcohol, MDMA, some SPCs, including methylone, and others. The influence of nicotine on the lethal and toxic effects of illicit drug use is often overlooked in analyses. The tobacco plant (*Nicotiana tabacum*) contains between 0.6 - 3 percent nicotine, which is consumed largely for its mild stimulant,

anxiolytic, and stress-relieving effects, but is highly addictive (Hoffmann and Hoffmann 1998). The central and peripheral nervous systems are targeted by nicotine in addition to activation of the sympathetic nervous system. Nicotine increases cardiac rate by 10-20 beats/minute and increases blood pressure 5-10 mmHg (Benowitz 1988; Benowitz et al. 1988; Benowitz 1991). In severe poisoning from nicotine, there are tremors, prostration, cyanosis, dyspnea, convulsion, collapse, and coma. Death may result from respiratory paralysis or central respiratory failure (Landoni 1991).

MDMA consumption with other substances, such as alcohol, nicotine, cocaine, or other amphetamines, may have additive effects, as these drugs may pharmacologically and pharmacokinetically interact with MDMA (Mueller et al. 2016). In the United Kingdom and in the United States more than 70% of MDMA users also consume high amounts of alcohol (Winstock, Griffiths, and Stewart 2001; Strote, Lee, and Wechsler 2002). In mammals, ethanol (EtOH) is converted to acetaldehyde (ACe) mainly by alcohol dehydrogenase (ADH) and, to a lesser degree, by cytochrome P450 2E1. ACe is further oxidized to acetate by enzymes of the aldehyde dehydrogenase family (ALDHs) (Lieber 2004; Rashkovetsky, Maret, and Klyosov 1994). A study shows that following MDMA ingestion, numerous liver mitochondrial proteins including ALDHs such as aldehyde dehydrogenase 2 (ALDH2), undergo oxidative alteration and become inactive (Moon et al. 2008). Moreover, the same study found that when MDMA and EtOH are administered together, MDMA suppresses mitochondrial ALDH2 activity and cytosolic ALDH1 in treated rats, resulting in ACe buildup and hepatotoxicity (Upreti et al. 2009). However, a clinical study has demonstrated that when cocaine, d-amphetamine, or MDMA is combined with EtOH, their plasma concentrations increase by 13%. The combination resulted in a

longer-lasting feeling of euphoria and well-being than either MDMA or alcohol alone, and MDMA reversed the subjective sedation induced by alcohol but did not reduce feelings of drunkenness or sluggishness (Hernández-López et al. 2002). Furthermore, it has been shown that MDMA combined with alcohol dissociates subjective from objective sedation: subjects might feel euphoric and less sedated, and may feel as if they are performing better, but in actuality the effect of alcohol continues to impede performance (Mohamed et al. 2011). Co-consumption of alcohol and MDMA leads to greater activation of the cardiac sympathetic system, cardiac cellular stress, and toxicity. Decreasing cardiovascular function more than occurs from either drug alone (Althobaiti and Sari 2016). The co-administration of MDMA and EtOH also showed decreased learning and memory, decreased sedative effects of alcohol, stimulation of anxiety behavior, and depletion of serotonin and dopamine in rats were also mentioned (Althobaiti and Sari 2016).

Approximately 70% of tobacco users also consume other substances. It has been reported that 64% of college students who use MDMA use nicotine at the same time (Mohamed et al. 2011). Tobacco products are not the only way one can consume nicotine now. Synthetic nicotine was invented in the 1960s (Jordt 2021). Among youth electronic cigarettes (e-cigarettes) are increasing in popularity, and newer e-cigarettes contain synthetic nicotine salts, that allows for higher nicotine intake (Choi et al. 2021). It is widely established that nicotine in tobacco interacts with nicotinic acetylcholine receptors (nAChRs). nAChRs are widely spread in the central nervous system (CNS) and stimulate the release of DA, NA, and 5-HT, as well as acetylcholine (ACh). Adolescents who use nicotine can alter the function of areas of the brain that regulate attention, learning, emotion, and impulse control (Taylor et al. 2014), and some of these effects persist in the

still-developing adolescent brain, in particular. MDMA is a partial agonist of α7 nAChR (Pubill et al. 2011), in addition to its more well-known serotonin releasing effects. Interestingly, MDMA also acts as an antagonist on α4β2 nAChRs (Garcia-Ratés et al., 2010), whereas nicotine stimulates both α7 and α4β2 types nAChRs in the brain. The impact of these nicotinic effects on MDMA, and whether nicotinic receptors play a role in the lethal and toxic effects of MDMA is not known.

1.5 Zebrafish

Several factors led to the choice of Zebrafish (*Danio rerio*) as the model species for this research, such as its high throughput, low cost, and similarity of molecular and cellular processes that accurately model many aspects of human physiology. Zebrafish have about 70% of human genes (Freeman et al. 2007). There are likely to be despite the substantial evolutionary distance between the two copies of many genes in humans in zebrafish, and zebrafish are likely to have two copies of many human chromosome segments (Postlethwait et al. 1998). Primary aspects of physiology and anatomy have broad similarities across the vertebrate lineage that connects zebrafish and humans: including two eyes, a mouth, brain, spinal cord, intestine, pancreas, liver, bile ducts, kidney, esophagus, heart, ear, nose, muscle, blood, bone, cartilage, and teeth (Burke 2016). Zebrafish also have the advantage of having large and optically transparent embryos and high fecundity (number of eggs per fish per spawn), which facilitates experimental analysis (Vliegenthart et al. 2014). In the wild, it is common to find zebrafish in slow-moving bodies of water in parts of southern and central Asia, like India, Pakistan, Myanmar, and Nepal. Zebrafish are

usually around 40 mm in length as adults. Male zebrafish usually have darker stripes than females, although this is more prominent in dominant males due to their morphology, which is characterized by blue stripes along their bodies (Reed and Jennings 2011). Zebrafish can breed all year in warm and shallow water, and one adult female can produce up to a hundred eggs in one mating. Zebrafish embryos are raised externally without support from the mother (Kimmel et al. 1995). The zebrafish lifecycle is defined by the following phases: the embryonic phase, that is defined as 0-72 hours post fertilization (hpf); early larval phase is from 72 hpf to 13 days post fertilization (dpf); mid-larval phase is from 14 to 29 dpf; and then from 30 dpf fish are considered juveniles, until at 3-4 months post fertilization fish reach sexual maturity and are considered adult fish. When fish reach sexual maturity, they are considered adults. Environmental and genetic factors affect the timing of development and of these phases, reducing or increasing the rate of development (Reed and Jennings 2011).

In recent years, zebrafish have gained popularity in toxicity screening for a variety of reasons. They are a low-cost and high-throughput approach to screening compounds for potential drug candidates. Many drugs that are successful *in vitro* fail in further preclinical studies with animal models, likely because some forms of toxicity occur at a physiological level, rather than a cellular level, and thus zebrafish are increasingly used for toxicity testing because they provide a fully functional physiology that can be assessed in a high throughput format (Chen 2021). Zebrafish assays are generally performed to measure cardiac, gastrointestinal, liver, and central nervous system functions as well as for developmental toxicity assessment (Vliegenthart et al. 2014). In addition, zebrafish can be used to identify new drug targets and be beneficial in preclinical drug development.

Furthermore, replacing higher-order animals with lower-order zebrafish (particularly, zebrafish embryos) is in line with the reduce, refine, and replace principle underlying ethical animal use for scientific purposes (Rothenbücher et al. 2019).

1.6 Objective and Hypothesis

MDMA or methylone in combination with alcohol or nicotine were studied to determine whether lethality was increased by co-administration, modeling their combined use in humans. Lethal concentrations were determined by finding the LC_{50} values using 5 dpf wild-type larval zebrafish after immersion for 5 hrs. This co-use model was chosen due to the high numbers of MDMA and methylone overdose reports globally, in which nicotine and ethanol are very often taken as well. One of the shortcomings of clinical reports is that they are by their nature uncontrolled, with numerous co-variants, so that it is difficult to assess the consequences of drug combinations on outcomes. Preclinical studies, in which these factors can be precisely controlled, allow for these questions to be addressed.

Chapter 2

Methods and Materials

2.1 Zebrafish Husbandry

20 wildtype (AB) adult zebrafish consisting of 10 male and 10 female fish were initially obtained from the Zebrafish International Resource Center (ZIRC, University of Oregon, Eugene, Oregon, USA) and housed at the University of Toledo Center for Drug Design and Development Zebrafish Core Facility (Toledo, Ohio). Zebrafish in this facility are kept in a zebrafish rack with a temperature, pH, and conductivity-controlled water recirculation system (Tecniplast®, Buguggiate, Italy). The temperature in the room is maintained between 26° C and 28° C. The temperature of the system water is maintained between 26° C and 28° C, pH 7.6 - 7.8, and 500 - 550 µS. When not being bred, fish are kept in separate tanks of 10 females and 10 males. Fish used to breed zebrafish larvae for experiments were aged between 6 months post fertilization and 18 months post fertilization. Males are added to the female group after placement in breeding tanks (shown in Figure. 2.1.). The room's light cycle is 14 hours on and 10 hours off.

Figure 2.1. Breeding tank.

The experiments described here use 5 dpf embryos. To produce embryos, male and female fish are placed together for 15 hours to breed in customized tanks called breeding tanks, beginning after 3:00 PM. Breeding takes place in a 1.7-liter tank which is a two-part tank that allows the fish to breed in the upper area while the embryos fall to the bottom and are subsequently harvested (see Figure2.1.). In breeding groups, the male:female ratio is 1:2 allowing maximum breeding potential. The embryos are collected the next morning. The adult fish are first removed from the breeding tanks and returned to their home tanks. To ensure that no embryos are lost, the water is filtered through a small porous mesh filter. "Embryo water" or "Egg water" is autoclaved system water made from distilled water sterilized with 60 mg/L sea salt added back to provide electrolytes. To remove the embryos from the mesh filter, embryo water is used to transfer them onto a small dish. More embryo water is poured onto the plate, and disposable pipettes are used to remove any non-embryonic materials (e.g., scales, feces, dead embryos, and other debris). To eliminate any non-embryo debris that remains, a vacuum system is applied to remove all the water from

the dish. This procedure is repeated two to four times until all non-embryo debris is eliminated and no more bubbles form on the surface of the water in the dish. This cleaning process is repeated every day from 0 dpf to 5 dpf.

Adult fish are fed once a day, between 11 a.m. and 1 p.m. On Mondays, Wednesdays, and Fridays, with brine shrimp are provided. Tuesdays, Thursdays, Saturdays, and Sundays fish pellets (500-800 microns) are provided. Pellet food is Golden Pearl Reef and Larval Fish Food (Brine Shrimp Direct). Brine shrimp are prepared fresh at every feeding time and hatched in-house in a brine shrimp cone. The cone is filled with 10 liters of deionized water, and each liter receives one 30 ml beaker full of Instant Ocean Sea Salt. When the salt is completely dissolved, one scoop of brine shrimp cysts is added per liter one day before they are harvested.

2.2 Materials

As a model for drug overdose, lethality and toxicity of MDMA or methylone, in combination with alcohol or nicotine, were examined in larval zebrafish. Five dpf larval zebrafish were chosen as the test animals in each experiment. At this stage of development, the sex of zebrafish cannot be distinguished. Each experiment was carried out with a separate set of zebrafish. MultiScreen-Mesh Filter Plates with 96-Well Receiver Plates (Figure 2.2.) were used to move larval zebrafish between water and solutions containing the drugs of interest (Figure 2.3.). For each experiment, two transport receiver plates were utilized. These plates have a mesh at the bottom of each well that allows for rinsing and transfer. One plate contains embryo water and the other holds various drug concentrations.

Figure 2.2. MultiScreen-Mesh ® Filter Plates with 96-Well Receiver Plates.

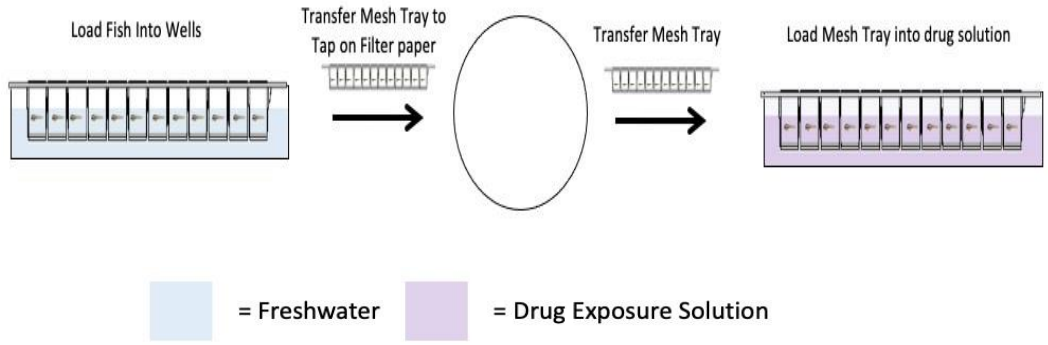

Figure 2.3. An experiment's fish transfer workflow (Heeren 2021).

2.2.1 Drugs

In this study MDMA, methylone, EtOH, and nicotine were used. MDMA, and methylone were synthesized in Dr. Isaac Schiefer's laboratory at the University of Toledo College of Pharmacy and Pharmaceutical Sciences. 95% EtOH was purchased from Sigma Chemical Co. (St. Louis, MO, USA), and nicotine was purchased from Acros Organics Co. (Morris Plains, New Jersey, USA). 100 mM MDMA, methylone and nicotine stock solutions were made. The different stock solutions were then diluted to the following

concentrations: 0.1 mM, 0.3 mM, 1 mM, 3 mM, 10 mM, 30 mM, and 50 mM. 1548.5 mM; (95%) stock solutions of EtOH were made using 95% EtOH. Different working solutions were then diluted from the stock solution providing the following concentrations for the studies: 1.63mM (0.1%), 4.89 mM (0.3%), 16.3 mM (1%), 48.9 mM (3%), 163 mM (10%), 489 mM (30%), and 815 mM (50%). All stock solutions and dilutions were prepared using embryo water. Stock solutions were stored at -20 °C until needed. The drug concentration range used for each drug was the same for each experiment and was determined based on preliminary studies. The working solutions were prepared in egg water. After the maximum non-lethal concentration (MNLC) of each substance alone was determined, the MNLC of each substance was added to the required concentration of the other drug, e.g. the MNLC for nicotine was added to a series of concentrations of MDMA and methylone, the MNLC for EtOH was added to a series of concentrations of MDMA and methylone, the MNLC for MDMA was added to a series of concentrations of EtOH and nicotine, and the MNLC for methylone was added to a series of concentrations for EtOH and nicotine (see Figure 2.4.).

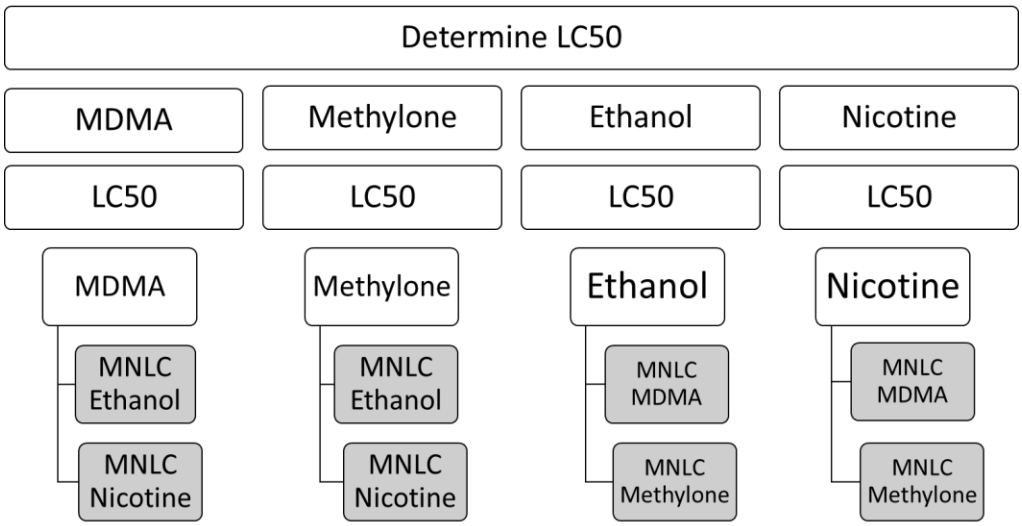

Figure 2.4. Workflow of drug LC50 determinations for drug combinations. There are two stages to the experimental design: (1) LC50 determination of the substances alone; (2) LC50 determinations of combinations of substances.

2.3 LC_{50} Determinations

After adding embryo water to a 96-well receiver plate, a 96-well transfer mesh filter plate was placed on the receiver plate. 48 of 5 dpf larval zebrafish were loaded into each of two 96-well filter plates with one fish per well, as shown in figure 2.5. where the red represents where no fish are placed so that evaporation at the edges of the plate do not confound the results (Walzl et al. 2012). The blue line in figure 2.5. represents where fish are placed in egg water. In each experiment, 6 zebrafish were utilized for each concentration. The drug concentrations were prepared on a second receiving plate with a control group exposed to embryo water alone with no drug treatments. 300 µL of the drug solutions were loaded into the wells, as shown in figure 2.6. where the red line represents where no fish are placed around the outside of the plate. The different shaded blue lines in figure 2.6. represent different concentrations of test drugs in egg water, including a control group of just egg water alone. This procedure prevents the wells from overflowing into neighboring wells as the filter plate is initially placed in the drug solutions. 500 µL is the maximum capacity that a well can contain. Prior to placement in the drugs solutions the filter plate from the egg water plate is removed, placed on filter paper to drain extra fluid, and moved to the receiver plate containing the drug solutions. 100 µL more of each drug solution are then added to the corresponding wells to ensure the fish can swim in the solution and the surface tension has been broken. The plate is then placed into an incubator for 5 hr at 28.0 ± 0.2 °C after a labelled lid is placed on top of the plate.

Separate plates of zebrafish for each drug are tested. After the 5-hour exposure is completed, the lethality at each concentration is determined and used to calculate the LC_{50} value, as well as the MNLC value. Behavioral and morphological changes are also observed after the 5-hour exposure. Death is defined by the absence of a heartbeat.

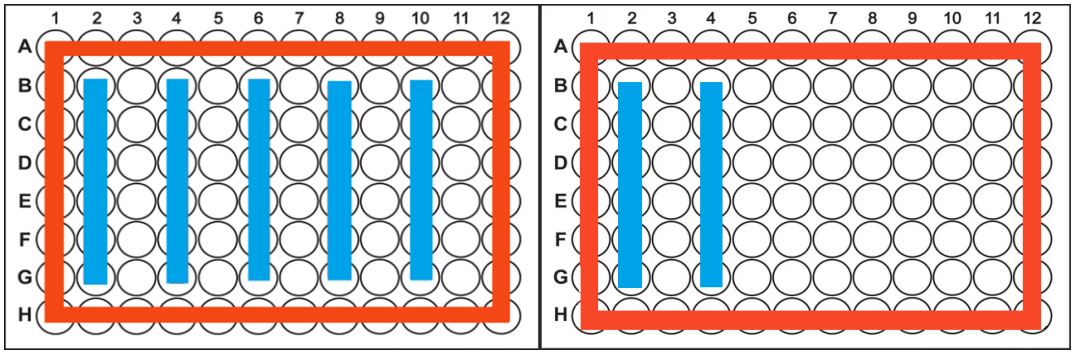

Figure 2.5. Plate layout with water only.

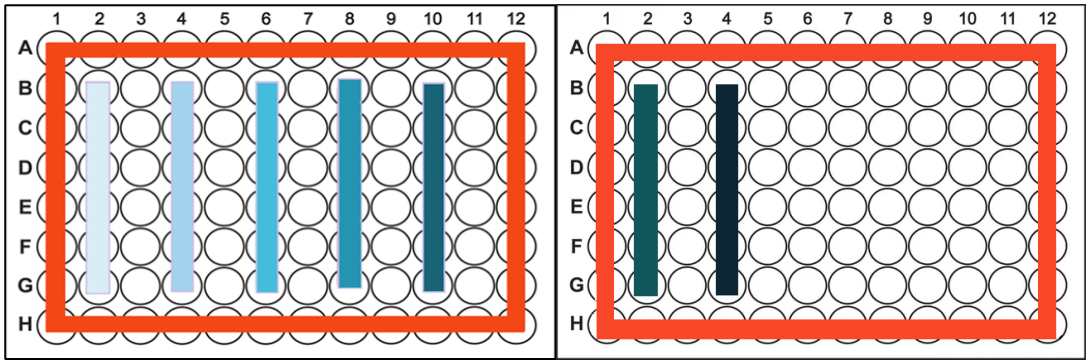

Figure 2.6. Plate layout with different drug solutions represented by the blue gradient.

2.4 Euthanasia

Zebrafish that are not used for experiments that day or that display symptoms of sickness or illness are euthanized using the hypothermic shock method (Wallace et al. 2018; Strykowski and Schech 2015). This procedure involves placing a plastic dish with a mesh bottom halfway into an ice bath, ensuring there is no ice in the dish for euthanasia. The fish that require euthanasia are then transferred into the plastic dish's ice-filled

compartment. Death is confirmed by that absence of a heartbeat, which occurs within 10 to 15 min.

2.5 Data Analysis

The LC_{50} values of the drugs under different conditions were determined using GraphPad Prism 5.0 (San Diego, California, USA). At first, the LC_{50} values were calculated by non-linear regression (log(inhibitor) vs. response -- variable slope, four parameters) and defined by the concentration which caused 50% mortality after 5 hr. exposure. Then, comparisons were made between curves (e.g., MDMA alone vs. MDMA plus nicotine) using dose-response - EC50 shift method. The model used was <A>LogEC=LogEC50Control and <~A>LogEC=LogEC50Control + log(EC50Ratio). The model equation was Y=Bottom + (Top-Bottom)/(1+10^((LogEC-X)*HillSlope)). All experiments were performed in at least 3 independent trials, with 6 animals per concentration per trial (N = 6, total N = 18); data were combined for the final analysis. All data were expressed as mean ± SEM. The level of significance was set at $p < 0.05$ for all analyses.

Chapter 3

Results

3.1 Lethality

5 dpf zebrafish larvae were exposed to different concentrations of MDMA, methylone, EtOH, and nicotine for 5 hrs. at 28.0 ± 0.2 °C to establish LC_{50} values for each drug alone, as well as MNLC values to be used in subsequent studies (Figure 3.1.). LC_{50} and MNLC values were calculated for each compound (Table 3.1.). Nicotine had the lowest LC_{50} (0.817 mM) among drugs that were used in this study. MDMA had the next lowest value with an LC_{50} of 3.032 mM, and then methylone with an LC_{50} value of 7.311 mM. Finally, EtOH had the highest LC_{50} value of 119.2 mM (7.311 % vol/vol).

Consistent with the stimulant actions of these drugs at low doses, somewhat greater activity at the 5-hour timepoint for MDMA, methylone, EtOH, and nicotine were observed at some drug concentrations compared to animals in egg water, although this was based solely on casual observations. In MDMA and methylone, similar activity was observed. Larval fish exposed to 0.1 mM MDMA and methylone were highly active but decreased in heart rate was observed compared to the control. As the concentrations increased, activity decreased substantially. 1 mM of MDMA or methylone produced pronounced reductions in activity in larval fish and lower heart rate was observed, whereas at concentrations of 10 mM and higher larval fish were dead, which was defined by no observable heartbeat. As

observed previously for these drugs (Chen 2021), distinct bending of the whole body and tail deflection, along with immobility and paralysis were observed in larval zebrafish treated with MDMA or methylone. After EtOH treatment (1.63 mM; 0.1%), faster movement was noted on larval fish in comparison to the control group. At 16.3 mM (1%), larvae fish were inactive and had lost the ability to maintain an upright position. At 48.9 mM (3%) larvae fish were largely immobile, and bradycardia was noted compared to control group. At 163 mM (10%) and higher larval fish were dead, with zero heartbeat per ten seconds. Erratic swimming, paralysis, pectoral fins movement without swimming, and immobility were observed. The larval fish in the nicotine treatment group (0.1 mM) showed normal movement patterns in comparison to the control group. Whereas at 0.3 mM larvae fish were less active with some seizure-like activity observed compared to the control group. At 1 mM and higher, larval fish were dead with zero heartbeats per ten second.

Table 3.1.

LC_{50} and MNLC values for MDMA, Methylone, Ethanol, and Nicotine

Drugs	**Calculated LC_{50}**	**Maximal non-lethal concentration (MNLC)**
MDMA	3.032 mM	1 mM
Methylone	7.311 mM	3 mM
EtOH	119.2 mM (7.311%)	48.9 mM (3%)
Nicotine	0.817 mM	0.3 mM

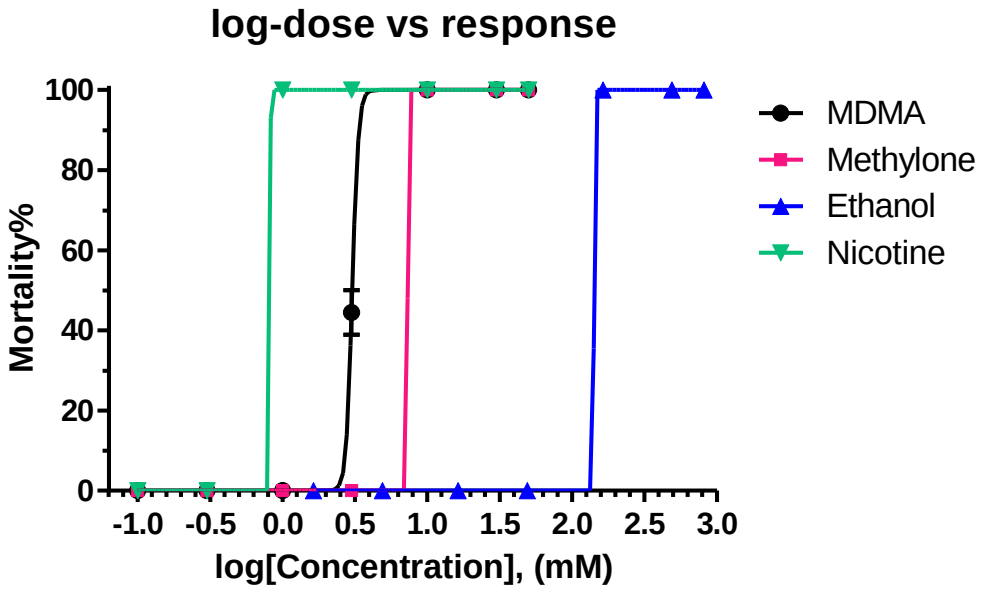

Figure 3.1. Dose-response curves for MDMA, methylone, EtOH, and nicotine for 5 hr. exposure.

3.1.1 Lethality of MDMA combined with Alcohol

The MNLC for EtOH (3%; 48.9 mM) was combined with a range of concentrations of MDMA, and the MNLC for MDMA (1 mM) was combined with a range of concentrations of EtOH in zebrafish larvae for 5 hrs. at 28.0 ± 0.2 °C to determine LC_{50} values compared to MDMA and EtOH alone. The LC_{50} data after treatment with MDMA + EtOH (3%; 48.9 mM) is shown compared to MDMA alone in figure 3.2., the LC_{50} for EtOH + 1 mM MDMA compared to EtOH alone is shown in figure 3.3. Both combinations shifted the lethality curve significantly toward lower doses compared to each drug alone (MDMA + 48.9 mM EtOH: $F(1,36)=173.1$, $p<0.0001$; and EtOH + 1 mM MDMA: $F(1,37)=256.5$, $p<0.0001$). The LC_{50} for MDMA + 48.9 mM EtOH was 0.368 mM, 8.2-fold less than MDMA alone (3.032 mM). The LC_{50} for EtOH + 1 mM MDMA was 18.6 mM, 6.4-fold less than EtOH alone (119.2 mM).

Casual observation of the fish in this experiment revealed similar behaviors to those observed for the drugs alone, albeit with some alteration in the doses at which those behaviors were observed, in accordance with the shifts in LC_{50} values. Some differences in activity were observed at the 5-hour time point for MDMA in combination with EtOH. For example, at the MNLC of EtOH (48.9 mM; 3%) + 0.1 mM MDMA larval fish were observed to be less active compared to the control group. The larval fish in the 48.9mM EtOH + 0.3 mM MDMA condition were less active compared to the control group. 16 out of 18 larvae fish in total were dead in the 48.9 mM EtOH + 0.3 mM MDMA group, and those fish that were alive were less active, whereas at the 48.9 mM EtOH + MDMA (3 mM) condition and higher, larval fish were dead with zero heartbeats per ten seconds. Pectoral fin movement, tail deflection, and seizure-like activity were also observed in the EtOH MNLC (48.9mM) + MDMA combination. Moreover, similar changes were observed in larval zebrafish treated with the MNLC for MDMA (1 mM) + 1.63 mM or 4.89 mM EtOH. Larval fish were less active compared to the control group under most conditions. In the 1 mM MDMA + 16.3 mM EtOH condition larval fish were inactive or dead. 7 out of 18 fish in total were dead. When 1 mM MDMA was combined with 48.9 mM EtOH or higher, larval fish were all dead with zero heartbeats per ten seconds. Bends of the body or tail due to sustained muscular contractions, signs of seizure, and immobility were also seen in larval zebrafish treated with the MNLC of MDMA + EtOH combinations.

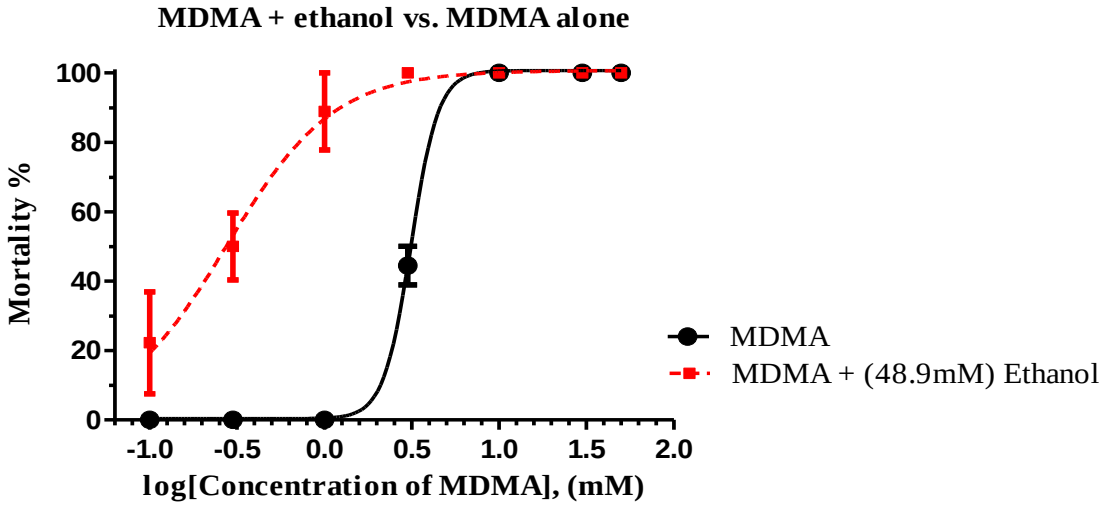

Figure 3.2. MDMA + ethanol vs. MDMA alone. LC50 curve shift for MDMA when combined with the MNLC of EtOH (3%; 48.9 mM). The LC50 for MDMA is 3.032 mM and the MNLC is 1 mM. The LC50 for EtOH combined with MDMA is 0.368 mM, 8.2-fold less than MDMA alone. The shift in the LC_{50} value was highly significant ($F(1,36)=173.1$, $p<0.0001$). Data are expressed as mean ± SEM.

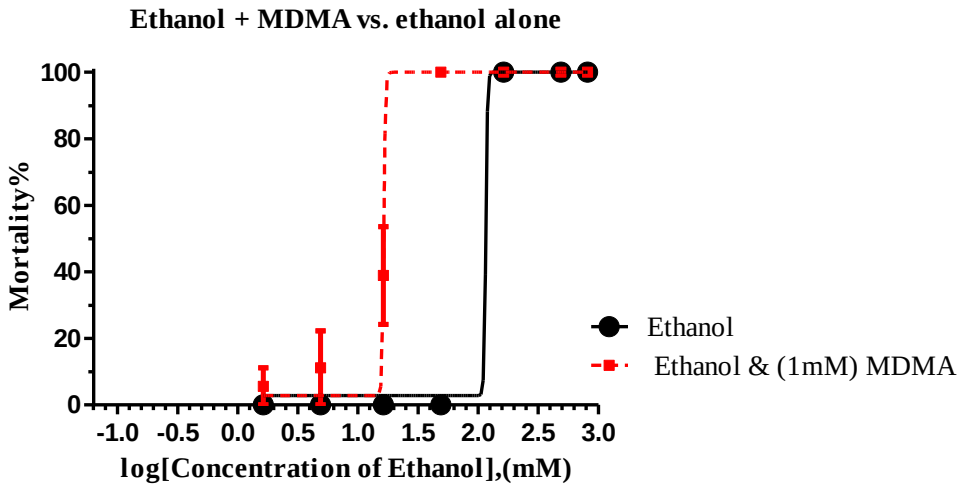

Figure 3.3. Ethanol + MDMA vs. Ethanol alone. LC50 curve shift for 1 mM MDMA combined with EtOH. The LC50 for EtOH alone is 119.2 mM and the MNLC is 48.9 mM. The LC50 for MDMA combined with EtOH is 18.6 mM, 6.4-fold less than EtOH alone. The shift in the LC50 value was highly significant ($F(1,37)=256.5$, $p<0.0001$). Data are expressed as mean ± SEM.

3.1.2 Lethality of MDMA combined with Nicotine

The MNLC for nicotine (0.3 mM) was combined with a range of concentrations of MDMA to determine the shift in the LC_{50} value compared to MDMA alone. Similarly, MNLC for MDMA (1 mM) was combined with a range of concentrations of nicotine to determine the magnitude of the shift in the LC_{50} value compared to nicotine alone. As before, testing used zebrafish larvae for 5 hrs. at 28.0 ± 0.2 °C. The LC_{50} data after treatment with MDMA + 0.3 mM nicotine compared to the control MDMA alone condition is shown in Figure 3.4. It shows that nicotine caused a significant shift in the lethality curve toward higher doses ($F(1,37)=191.4$, $p<0.0001$). Larval zebrafish were observed to be alive in the 0.3 mM nicotine + 10 mM MDMA condition, protecting against the lethal effects of MDMA with an LC_{50} value of 12.06 mM, 4-fold higher compared to the 3.032 mM value of MDMA alone. In contrast, the LC_{50} data after treatment with different concentrations of nicotine + 1 mM MDMA relative to nicotine alone is shown in Figure 3.5. This data shows that the addition of MDMA to nicotine produced a significant shift in the lethality curve toward lower doses ($F(1,37)=9.250$, $p<0.0043$), slightly potentiating the lethal effects of nicotine alone. The LC_{50} for nicotine + 1 mM MDMA was 0.568 mM, 1.4-fold less compared to nicotine alone (0.817 mM).

Differences in activity were also observed at the 5-hour time point for MDMA in combination with nicotine. In the 300 µM nicotine + MDMA (100µM, 300 µM, and 1mM) conditions, larval fish were observed to be more active than the control group. In the 0.3 mM nicotine + 3 mM MDMA condition larval fish had similar activity to the control group. Upon exposing larval fish to 0.3 mM nicotine + 10 mM MDMA, some larvae were less

active and swam upside down, while other surviving fish were almost completely inactive, and 4 out of 18 fish were dead. Larval fish exposed to 0.3 mM nicotine with 30 mM MDMA or higher were dead, with 0 heartbeats per ten seconds. Other behavior, like elevated movement of pectoral fins, and sustained muscle contractions consistent with seizure-like activity were also observed when the MNLC of nicotine MDMA was combined with different concentrations of MDMA. Differences in activity in larval zebrafish treated with the MNLC of MDMA (1mM) + different concentrations of nicotine were observed. Larval fish were less active upon exposure to 1 mM MDMA + 0.1 mM nicotine compared to the control group. When treated with 1 mM MDMA + 0.3 mM nicotine, larval fish were more active compared to the control group. When larval fish were exposed to 1 mM MDMA + 1 mM nicotine or higher, larval fish were all found dead. At doses just below this there was evidence of sustained muscle contractions, indicative of seizure, including bends on the body and tail, as well as hyperkinetic movement of the pectoral fins.

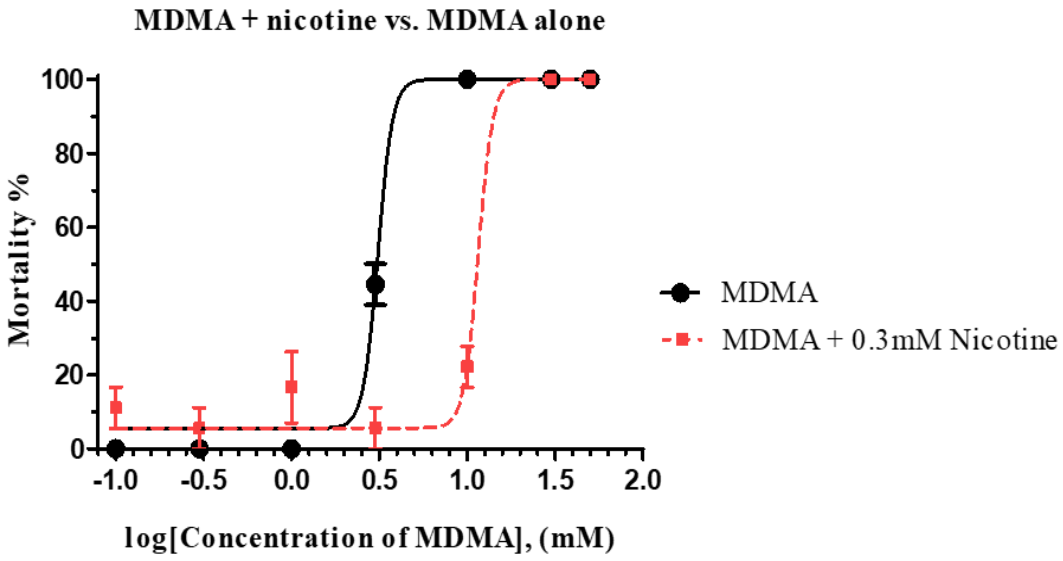

Figure 3.4. MDMA + nicotine vs. MDMA alone. LC50 curve shift for MDMA combined with 0.3 mM nicotine. The LC_{50} for MDMA is 3.032 mM and the MNLC is 1 mM. The

LC_{50} for MDMA combined with 0.3 mM nicotine is 12.06 mM, 4-fold higher than MDMA alone. The addition of nicotine significantly shifted the LC_{50} for MDMA to the right ($F(1,37)=191.4$, $p<0.0001$). Data are expressed as mean ± SEM.

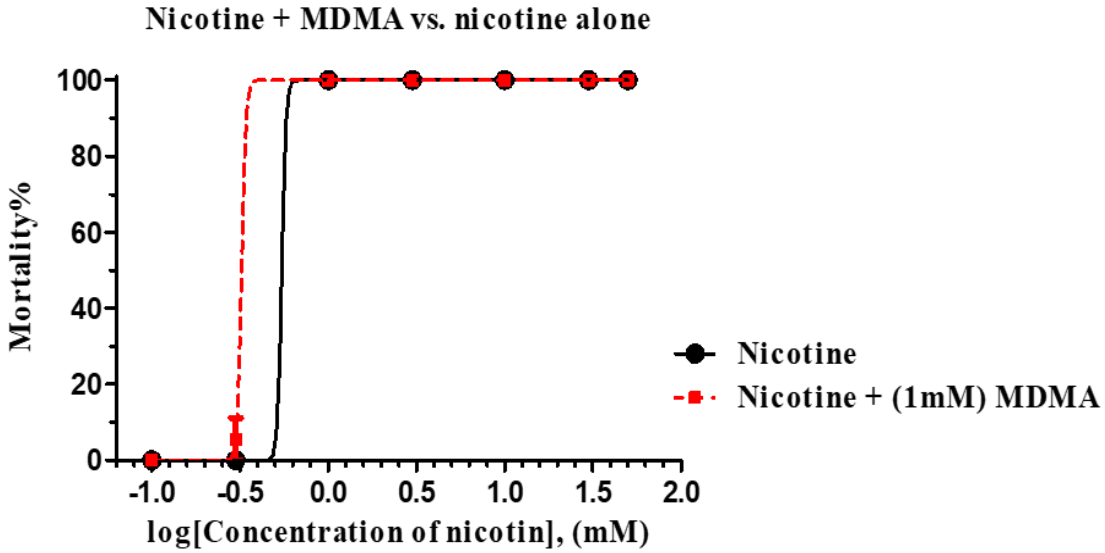

Figure 3.5. Nicotine + MDMA vs. Nicotine alone. LC50 curve shift for nicotine when combined with 1 mM MDMA. The LC_{50} for nicotine is 0.817 mM and the MNLC is 0.3 mM. The LC_{50} for nicotine when combined with 1 mM MDMA is 0.568 mM,1.4-fold less than nicotine alone. The LC_{50} for nicotine shifted significantly to the left ($F(1,37)= 9.250$, $p<0.0043$. Data are expressed as mean ± SEM.

3.1.3 Lethality of methylone combined with alcohol

The MNLC for EtOH (3%; 48.9 mM) was combined with a range of concentrations of methylone, and the MCNL for methylone (3 mM) was combined with a range of concentrations of EtOH in zebrafish larvae for 5 hr. at 28.0 ± 0.2 °C to determine LC_{50} values compared to methylone and EtOH alone. The LC_{50} data after treatment with methylone + EtOH (3%; 48.9 mM) compared to methylone alone are shown in Figure 3.6. The data for EtOH combined with 3 mM methylone compared to EtOH alone are shown in Figure 3.7. Both combinations shifted the lethality curves significantly toward lower concentrations (methylone + 48.9 mM EtOH ($F(1,37) = 97.14$, $P<0.0001$; and EtOH + 3

mM methylone ($F(1,37)=2098$, $P<0.0001$) compared to each drug alone. The LC_{50} for methylone + 48.9 mM EtOH was 0.586 mM, 12.5-fold less compared to methylone alone (7.311 mM). The LC_{50} for EtOH + 3 mM methylone was 37.474 mM, 3.2-fold less compared to EtOH alone (119.2 mM).

Some differences in behavior were noticed by casual observation at the 5-hour time point for methylone in combination with EtOH. In the 48.9 mM EtOH + 0.1 mM methylone condition larval fish were less active compared to the control group. In the 48.9 mM EtOH + methylone (0.1 mM and 1mM) conditions larval fish were less active, whereas in the 48.9 mM EtOH + 1 mM methylone condition 11 out of 18 fish were found dead. When 48.9 mM EtOH was combined with 3 mM methylone or higher all fish were found dead at 5 hrs. In addition, sustained muscle contractions indicative of seizure, with bends of the body or tail deflection, and immobility, were observed in larval zebrafish treated with the MNLC for EtOH + methylone. There were similar observations for 3 mM methylone combined with different concentrations of EtOH. For fish treated with 3 mM methylone + EtOH (1.63 mM, 4.89 mM, or 16.3 mM), larval fish were inactive. Decreased heart rate was observed in fish treated with 3 mM methylone + EtOH (4.89 mM and 16. 3 mM) in comparison to the control group. Upon exposing larval fish to 3 mM methylone + 48.9 mM or higher EtOH, all fish died. At lower doses, sustained muscle contractions produced distinct bending of the body or tail, and immobility was seen.

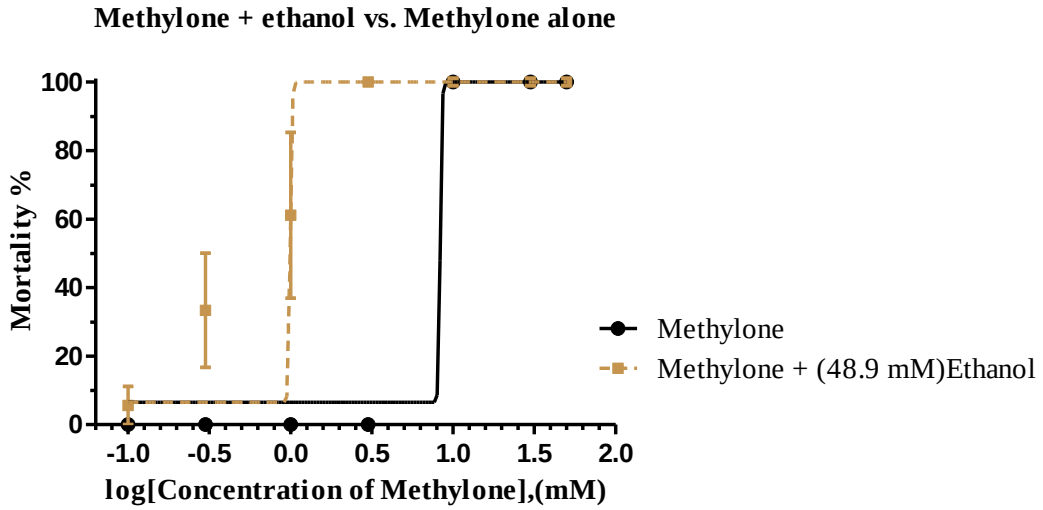

Figure 3.6. Methylone + ethanol vs. Methylone alone. LC_{50} curve shift for methylone when combined with 48.9 mM EtOH. The LC_{50} for methylone alone is 7.311mM and the MNLC is 3 mM. The LC_{50} value for methylone combined with 48.9 mM EtOH is 0.586 mM, 12.5-fold less than methylone alone. LC_{50} was shifted significantly to the left ($F(1,37)=97.14$, $p<0.0001$). Data are expressed as mean ± SEM.

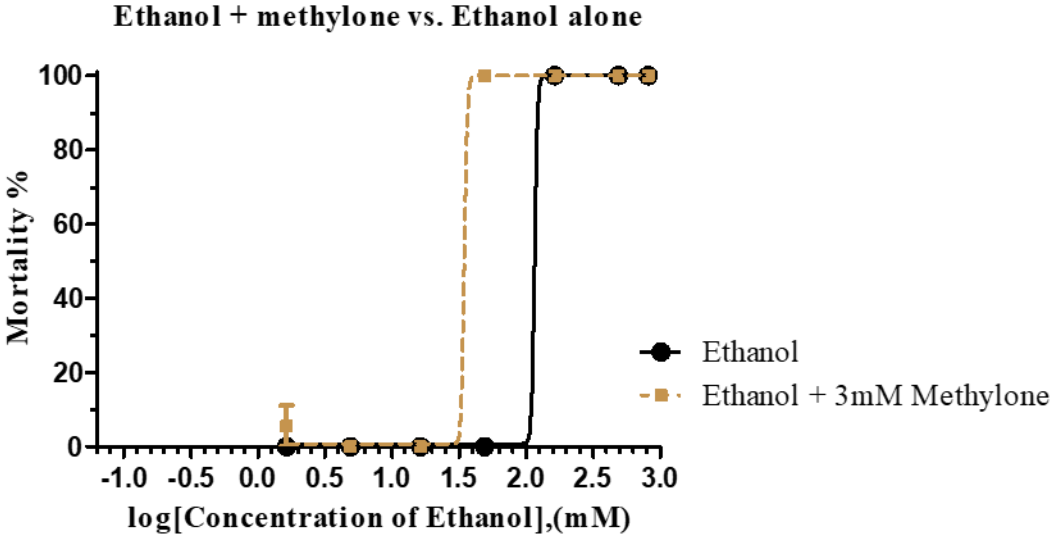

Figure 3.7. Ethanol + methylone vs. Ethanol alone. LC_{50} curve shift for EtOH combined with 3 mM methylone. The LC_{50} for EtOH alone was 119.2 mM and the MNLC is 48.9 mM. The LC_{50} for EtOH combined with 3 mM methylone was 37.47mM, 3.2-fold less than EtOH alone. The LC_{50} value was significantly shifted to the left ($F=(1,37)=2098$, $p<0.0001$). Data are expressed as mean ± SEM.

3.1.4 Lethality of methylone combined with nicotine

The MNLC for nicotine (300 µM) was combined with a range of concentrations of methylone to determine the shift in the LC_{50} value compared to methylone alone. Likewise, MNLC for methylone (3 mM) was combined with a range of concentrations of nicotine to determine the magnitude of the shift in the LC_{50} value compared to nicotine alone. Zebrafish larvae were used in this experiment and exposed to the drugs for 5 hrs. at 28.0 ± 0.2 °C. The comparison of the LC_{50} data after treatment with 300 µM nicotine + methylone compared to methylone alone is shown in Figure 3.8. It shows that nicotine caused a significant shift in the LC_{50} curve toward higher doses ($F(1,37)=1.825 \times 10^{36}$, $p<0.0001$), with an LC_{50} of 22.17 mM, 3-fold higher compared to methylone alone (7.311 mM), protecting against the lethal effects of methylone. Figure 3.9. shows the LC_{50} for 3 mM methylone + nicotine compared to nicotine alone. The addition of methylone to nicotine did not produce any shift in the lethality curve. The LC_{50} for 3 mM methylone + nicotine was 0.817 mM compared to 0.817 mM for nicotine alone.

Some differences in behavior were at apparent by casual observation for methylone in combination with nicotine, similar to those observed previously. 0.3 mM nicotine + methylone (0.1 mM and 0.3 mM) had similar activity compared to the control group, but 0.3 mM nicotine + higher methylone doses (1mM, 3mM, and 10mM) decreased activity and heart rate was noticeably reduced in fish treated with 0.3 mM nicotine + 3 mM or 10 mM methylone in comparison to the control group. Larval fish were found dead in the groups treated with 0.3 mM nicotine in combination with 30 mM methylone or higher. There was again clear observational evidence of seizure activity, in terms of sustained muscle contractions, producing sustained bending of the body and tail, as well as some

hyperkinetic movements of the pectoral fins in larval zebrafish treated with the MNLC of nicotine + methylone. Differences in activity were also observed in larval zebrafish treated with 3 mM methylone + nicotine. For the 3 mM methylone + nicotine (0.1 mM and 0.3 mM) groups, larval fish were inactive and decreases in heart rate were observed in comparison to the control group. In groups treated with 3 mM methylone + 1 mM nicotine or higher, all larval fish died. There was again evidence of seizure, i.e. sustained muscle contractions shown by sustained curvature of the body and tail deflection, and consequent immobility.

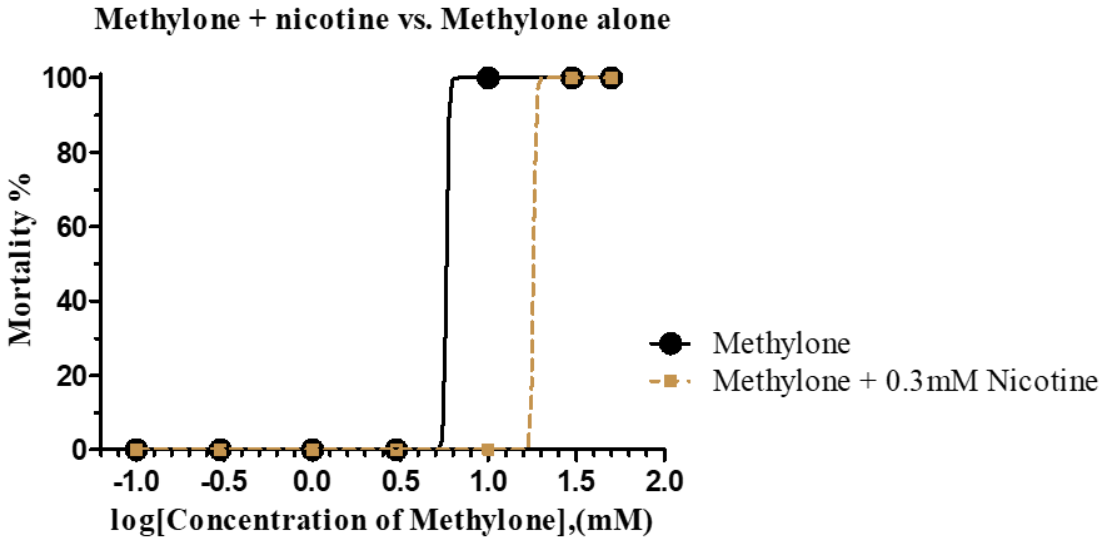

Figure 3.8. Methylone + nicotine vs. Methylone alone. LC_{50} curve shift for methylone in combination with 0.3 mM nicotine. The LC_{50} for methylone is 7.311 mM and the MNLC is 3 mM. The LC_{50} for 0.3 mM nicotine combined with methylone is 22.17 mM, 3-fold higher than methylone alone. The LC_{50} value shifted significantly to the right ($F(1,37)=1.825\times10^{36}$, $p<0.0001$). Data are expressed as mean ± SEM.

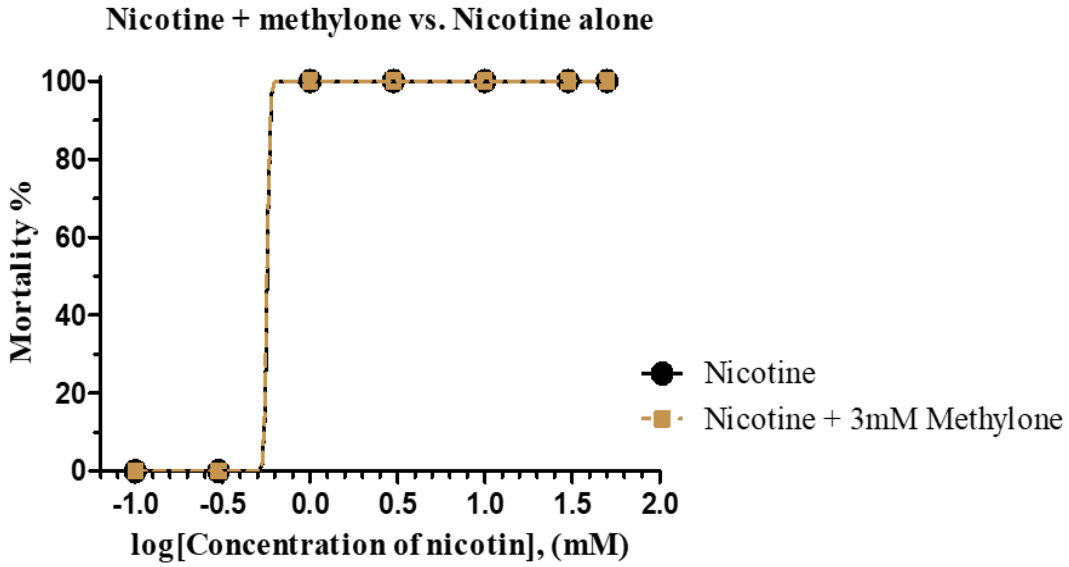

Figure 3.9. Nicotine + methylone vs. Nicotine alone. LC_{50} curve shift for nicotine in combination with 3 mM methylone. The LC_{50} for nicotine is 0.817 mM and the MNLC is 0.3 mM. The LC_{50} for 3 mM methylone combined nicotine is 0.817 mM. Data are expressed as mean ± SEM. There was no significant shift under these conditions.

3.1.5 Summary Comparisons

Lethal concentration values and the shift of the lethality curve for nicotine, EtOH, MDMA, and MDMA in combination with alcohol or nicotine are shown in Figure 3.10. It is quite clear that there are substantial differences in toxicity across each of the substances alone (LC_{50} values were: nicotine < MDMA < ethanol), moreover, when combined there are substantial changes in toxicity (LC_{50} values were: MDMA + 48.9 mM EtOH < Nicotine + 1 mM MDMA < EtOH + 1 mM MDMA < MDMA+ 0.3 mM Nicotine). Zebrafish larvae were exposed to different concentrations of MDMA, EtOH, nicotine, MDMA + (48.9 mM; 3%) EtOH, EtOH + 1 mM MDMA, MDMA + 0.3 mM nicotine, and nicotine + 1 mM MDMA for 5 hrs. LC_{50} and MNLC values were calculated for each compound shown in Table 3.2., which are shown now to facilitate comparisons between these conditions.

MDMA in combination with alcohol or nicotine led to increased lethality and toxicity in zebrafish compared to their controls except when MDMA and 0.3 mM nicotine were combined, which decreased lethality and toxicity.

Table 3.2.

Summary of LC_{50} determinations of MDMA in Combination with Alcohol and Nicotine

Drugs	**MNLC**	**LC_{50}**	**Fold change of LC_{50}**
MDMA	1mM	3.032 mM	
EtOH	48.9mM (3%)	119.2 mM (7.311%)	
Nicotine	0.3 mM	0.817 mM	
MDMA + 48.9 mM EtOH	N/A	0.368 mM	8.2-fold compared to MDMA alone
EtOH + 1mM MDMA	N/A	18.6 mM (1.145%)	6.4-fold compared to EtOH alone
Nicotine + 1mM MDMA	0.1 mM	0.568 mM	1.4-fold compared to nicotine alone
MDMA+ 300 µM Nicotine	N/A	12.06 mM	4-fold compared to MDMA alone

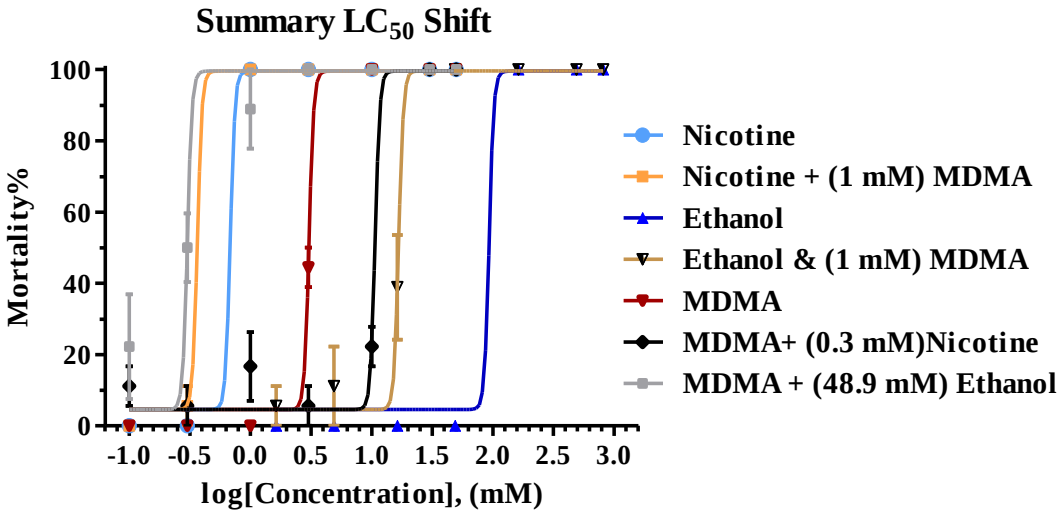

Figure 3.10. Summary of lethality curves for nicotine, alcohol, MDMA, and MDMA in combination with alcohol and nicotine.

Figure 3.11. below shows lethal concentration values for nicotine, alcohol, methylone and methylone in combination with alcohol or nicotine. Clearly, each of the substances differs in toxicity (LC_{50} values were nicotine < methylone < ethanol), moreover, when combined there are substantial changes in toxicity (LC_{50} values were: Methylone + 48.9 mM EtOH < Nicotine + 3 mM methylone < Methylone + 0.3 mM Nicotine < EtOH + 3 mM methylone). Zebrafish larvae were exposed to different concentrations of methylone, EtOH, nicotine, methylone + 48.9 mM EtOH, EtOH + 3 mM methylone, methylone + 0.3 mM nicotine, and nicotine + 3 mM methylone for 5 hrs. LC_{50} and MNLC values for each compound are listed in Table 3.3., which are shown now to facilitate comparisons between these conditions. The combinations of methylone and alcohol resulted in an increase in lethality and toxicity in zebrafish compared to their controls. Furthermore, the combinations of Methylone and nicotine decreased lethality and

toxicity in zebrafish compared to methylone alone except for nicotine + 3 mM methylone did not alter lethality and toxicity in zebrafish compared to nicotine alone.

Table 3.3.

Summary of LC_{50} determinations of Methylone in Combination with Alcohol and Nicotine

Drugs	**MNLC**	**LC_{50}**	**Fold change of LC_{50}**
Methylone	3mM	7.311mM	
EtOH	48.9mM (3%)	119.2mM (7.311%)	
Nicotine	0.3 mM	0.817 mM	
Methylone + 48.9 mM EtOH	N/A	0.586 mM	12.4-fold compared to methylone alone.
EtOH + 3mM methylone	16.3 mM (1%)	37.474 mM (2.299%)	3.2-fold compared to EtOH alone.
Nicotine + 3mM methylone	0.3 mM	0.817 mM	1-fold compared to nicotine alone.
Methylone +300 μM Nicotine	10 mM	22.17 mM	3-fold compared to methylone alone.

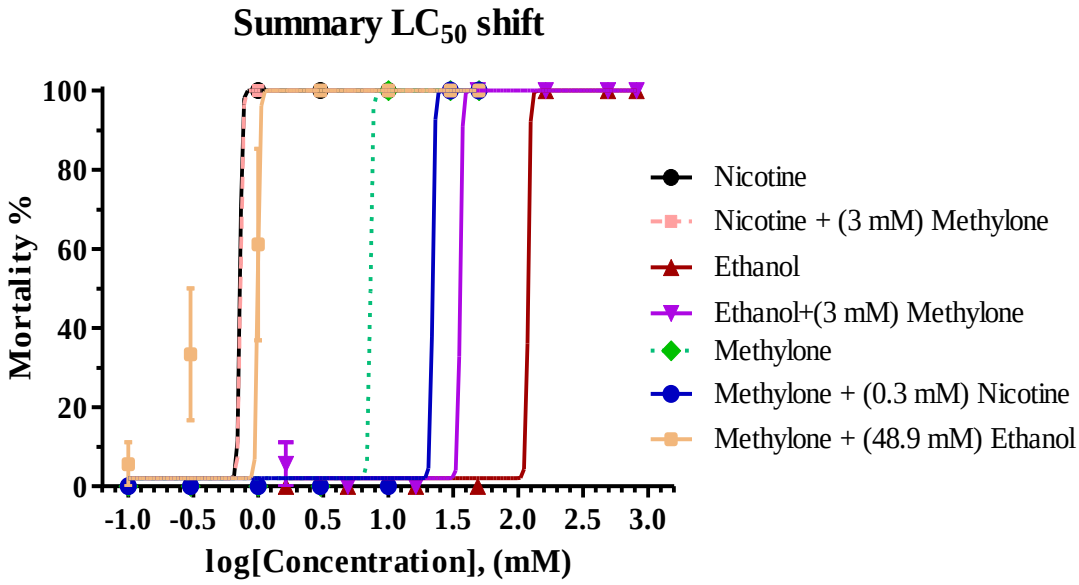

Figure 3.11. Summary of lethality curves for nicotine, alcohol, methylone and methylone in combination with alcohol or nicotine.

Chapter 4

Discussion

In this study, we examined the lethal effects of synthetic phenethylamines MDMA and methylone in combination with alcohol or nicotine as a model for overdose using zebrafish larvae. It was thought that the combination of MDMA and methylone with alcohol or nicotine would induce greater toxic and lethal effects. Although this hypothesis was partially confirmed, the results showed different effects among different combinations, including both potentiation of lethal effects and protection against some lethal effects. These interactions may involve several underlying mechanisms.

The combination of the MNLC of MDMA or methylone with EtOH potentiated the lethal effect of EtOH (e.g., reduced LC_{50} values), and similarly the combination of MNLC of EtOH with MDMA or methylone potentiated their lethal effects (e.g., reduced LC_{50} values). The increased lethal effects of combining these drugs with alcohol confirmed the initial hypothesis. Although the mechanisms underlying the lethal toxic effects of most of these drugs are surprisingly poorly understood alone, and little work has investigated their interactions, one study did show that EtOH added to MDMA potentiated DA release leading to hyperlocomotion behavior following neurotoxicity in rats' brain (Riegert et al. 2008). MDMA or methylone co-consumption with alcohol is thought to significantly affect neurotransmitters in the brain, which raises a more serious health risk than taking them

separately. MDMA and alcohol combinations increase of dopamine and serotonin levels in the brain, resulting in serotonin syndrome (Althobaiti and Sari 2016), which is a range of symptoms that can be life-threatening without treatment. The combination of synthetic phenethylamines with alcohol can end in excessive use of either drug, especially alcohol, which can ultimately end in an overdose. MDMA (Althobaiti and Sari 2016) and methylone (López-Arnau et al. 2014) cause changes in the dopamine reward pathways, and the addition of alcohol leads to an increase in alcohol consumption (Izco et al. 2007). This could result in dehydration, depression, or low mood. Long-term alcohol consumption with MDMA caused serotonin depletion and may contribute to psychopathological disturbances observed in MDMA and alcohol co-users (Cassel et al. 2005). In the context of acute overdose, modeled here, due to the liver's metabolism of these drugs, the combination of alcohol with MDMA or methylone may alter the metabolism of the drugs. This may result in higher toxic levels of these drugs' metabolites in the blood, leading to serious side effects or stronger adverse reactions. Two studies reported changes in metabolism upon co-exposure to MDMA and EtOH. In rats, decreased activity of ALDH1 was observed (Upreti et al. 2009). Presumably, this could increase ACe levels and enhance hepatotoxic effects. In addition, a higher rate of MDMA metabolites was also seen in primary rat hepatocytes (Pontes et al. 2010). Some of these metabolites are potentially more toxic than MDMA itself (Monks et al. 2004) and therefore may increase hepatotoxicity. However, the metabolism of EtOH and MDMA combination remains unclear as both studies used high MDMA doses or concentrations (10 mg/kg and 1.6 mM). Neurotoxicity occurs at high amphetamine concentrations (Berman et al. 2008), although the potential for SPCs to induce neurotoxicity appears to be much less (Anneken et al. 2017; Anneken, Angoa-Pérez,

and Kuhn 2015). Our results did show that MDMA is slightly more lethal than methylone, the LC_{50} for MDMA was 3.032 mM while LC_{50} for methylone was 7.311mM. The results in this study also pointed out that lethality occurs as MDMA's or methylone's concentration is increased in combination with EtOH or EtOH's concentration is increased in combination with MDMA or Methylone in zebrafish larvae.

Neuroinflammation can also influence neurotoxic outcomes. In CNS, microglia and astrocytes are the primary mediators of inflammation due to their roles in the immune response, blood–brain barrier (BBB) maintenance, and synaptic support (for review see Lecuyer, Kebir, and Prat (2016)). Disturbance of these functions might be likely to result in a change in sensitivity to toxic effects of drugs in the brain. A review of the literature suggests alcohol abnormally activates glial cells through the secretion of proinflammatory mediators such as cytokines, chemokines, and reactive oxygen species (ROS). These mediators are known to potentiate the inflammatory cascade and damage neuronal tissues as oxidative stress and cell death pathways are activated (Yang et al. 2014).

Another reason MDMA and methylone in combination with alcohol may increase the lethal effects is physiological, through hyperthermia and dehydration. Some studies have shown that MDMA and methylone cause hyperthymia in rats' brains (Kiyatkin and Ren 2016). Increasing the brain's temperature would disrupt BBB permeability because microglia and astrocytes are affected (Sharma and Ali 2008). Like mammals, zebrafish have the capacity to experience hyperthermia and emotional fever (Rey et al. 2015). When the temperature increases in zebrafish, the fish migrate toward warmer water (Rey et al. 2015). Severe dehydration involving a significant loss of body fluids, or a disruption in fluid balance within the body, can be fatal when MDMA or methylone (Kiyatkin and Ren

2016). Alcohol would be expected to make this worse. When hyperthermia occurs, the body compensates (in mammals) by sweating. In this case, alcohol inhibits the body's release of the hormone vasopressin leading to a significant loss of body fluid. In addition, alcohol functions as a vasodilator at low concentrations and a vasoconstrictor at higher levels (Kiyatkin and Ren 2016; van Amsterdam et al. 2021; Harper et al. 2018). Although the mechanisms by which ethanol might affect fluid (and electrolyte) balance in fish are not known, this is still a likely mechanism that might contribute to the effects observed here.

The effects of MDMA or methylone combined with nicotine in this study had quite different effects on lethality when compared MDMA along. 1) The combination of the MNLC of nicotine with MDMA or methylone significantly increased LC_{50} values compared to the LC_{50} values for each drug alone, meaning that there was a reduction of the lethal effects produced by adding the MNLC of nicotine to lethal concentrations of the synthetic phenethylamines MDMA and methylone. These results are in agreement with recent findings that were done on male rats, suggesting that nicotine reduces the neurotoxicity induced by MDMA in hippocampal neurons (Kowsari et al. 2021; Rostami et al. 2017). Moreover, Kowsari et al. (2021) showed that the greatest reduction and protection against neurotoxicity induced by MDMA is by the therapeutic combination of nicotine in combination with modafinil. This may be associated with the protective effects of nicotine and modafinil inhibition of apoptosis observed in the hippocampal CA1 region (Kowsari et al. 2021). MDMA-treated rats exhibited increased levels of brain-derived neurotrophic factor and tyrosin kinase receptor B in hippocampal neurons following nicotine administration, which has been associated with neuroprotection and elevation of

antioxidant capacity (Arevalo and Wu 2006; Kowsari et al. 2021). MDMA is also toxic to astrocytes (Alvaro et al. 2008), in addition to its neuronal toxicity. A non-lethal concentration of nicotine alone had a beneficial effect on astrocytes through effects at α7 nAChRs (Aryal et al. 2021). One study found that nicotine binding to astrocytes rapidly increased the number of processes during short-term exposure, and then extended those processes with increased cell volume and increased calcium activity during long-term exposure in mice (Aryal et al. 2021). Additionally, nicotine protects astrocytes from apoptosis and prevents reactive astrocytosis caused by lipopolysaccharide and interleukin 1 cytokine by acting as an anti-inflammatory agent (Revathikumar et al. 2016; Liu et al. 2015).

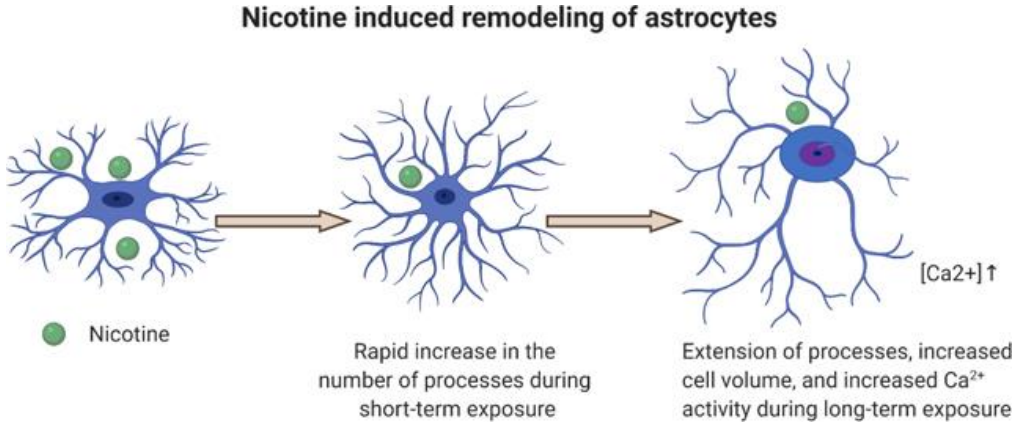

Figure 4.1. Changing in astrocytes morphology induced by nicotine (Aryal et al. 2021).

The protective effects on the treated fish of nicotine on MDMA and methylone induced lethality may suggest an approach for treating drug overdose. 2) However, it should be noted that the combination of the MNLC of MDMA with nicotine lowered the LC_{50} value compared to LC_{50} value of nicotine alone, e.g., a potentiation of its toxic effects, so it is not clear that this combination of drugs is always protective. Moreover, to model

the clinical circumstance, a treatment for overdose would need to be given after the pre-exposure to the toxic drug. This will require further experimentation to determine if this will work. MDMA-induced toxicity and production of reactive oxygen species (ROS) result in oxidative stress (Budzynska et al. 2018; Gonçalves, Baptista, and Silva 2014). A study on behavior showed that by preventing DA reuptake and stimulating release in the brain, MDMA has some effects resulting from elevated dopaminergic neurotransmission. The co-administration of MDMA and nicotine increased the oxidative stress level and altered the cortex structure in mice's brains due to overproduction of DA. (Budzynska et al. 2018).

The CNS is stimulated by small doses of nicotine but depressed by large doses (Solarino et al. 2010), so the effects of nicotine could very well be dose dependent, explaining the differences between the two studies. In other words, the mechanism of toxicity for nicotine may be different than for MDMA, such that sublethal doses of nicotine protect against MDMA toxicity, but not the other way around. Furthermore, the decreased activity observed after nicotine exposure at high concentrations may be the result of nAChR desensitization leading to greater MDMA effects (Aryal et al. 2021). 3) Our results showed that the MNLC of methylone did not change the lethal effects of nicotine. This observation may suggest that methylone is not as harmful as MDMA, and least with respect to potentiating the mechanisms that underlie nicotine toxicity. This will require further explanation, although it is already clear that MDMA and methylone differ substantially in their ability to induce neurotoxicity ((Anneken, Angoa-Pérez, and Kuhn 2015; Angoa-Pérez et al. 2014)), although the reasons for that remain unclear.

Chapter 5

Conclusions

In this study, MDMA and methylone were combined with alcohol and nicotine as a model for co-use to assess toxicity and lethality using LC_{50} assays in larval zebrafish. Our studies showed that the combination of alcohol with either MDMA or methylone potentiates the toxic and lethal effects of either drug by itself. By contrast, nicotine was shown to be protective against MDMA and methylone induced lethality, although the opposite was not the case – MDMA and methylone did not protect against nicotine induced lethality.

These findings therefore have two important implications for the treatment of MDMA and methylone overdose: (1) consumption of ethanol with these drugs may make overdose more likely to occur, or rather to occur at lower doses of MDMA or methylone; and (2) nicotine may be protective against MDMA and methylone induced lethality. This may partially explain why nicotine is so often used in combination with these drugs. Users may perceive on some level that nicotine mitigates the adverse effects of these drugs, even prior to doses sufficient for overdose. Although this is speculative, it is worth investigation. It is also quite possible that nicotine potentiates desirable effects of these drugs, particularly at low doses. Regardless of whether this is the case, the protective activity of nicotine could

be used as a potential therapeutic target for recreational psychoactive drug overdoses, for which there are no current treatments other than symptomatic care.

The limitations of our studies include that our observations were limited to one-time point, since they were primarily intended to identify LC_{50} values. More time points and chronic exposures should be investigated to better understand the potential of these drugs for overdose, as well as to seek interventions to reduce lethality and other adverse outcomes. This study was done on one type of lethality testing (LC_{50}), and more lethality and toxicity studies needed to be done. Indeed, there has been very little work that has examined MDMA or methylone in combination with alcohol or nicotine, either as this may relate to their abuse, or to overdose. This lack of understanding extends to the pharmacodynamic mechanisms underlying the combined reinforcing effects of these drugs in addition to their toxic effects, which may have different mechanisms, as well as the pharmacokinetic properties, in any species, not just zebrafish. I should also be noted that these studies were done on young fish that are still developing and age may affect the toxicity of these drugs; a wider range of ages should be studied.

In future studies it will be important to examine MDMA and methylone in combination with alcohol and nicotine at a wider range of concentrations in larval zebrafish, particularly at more behaviorally relevant doses, as well as to conduct confirmatory studies in mice. Moreover, further studies need to be done to have a better understanding of the mechanisms underlying MDMA and methylone toxicity, as well as the protective effects of nicotine, such as fluorescence nano-thermometry to better determine individual organs producing heat, as well as other measures of cellular toxicity in specific organs or cell types. These assays would focus on the cell death signaling

pathways involved, cardiotoxicity (which other work has shown to occur), gene expression pathway, and liver toxicity, in order to gain a better understanding of psychostimulant drug overdose.

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