Elucidating the Neurotoxic Effects of Piperazine Derivatives in Human Neuroblastoma Cells through Oxidative Stress, Mitochondrial Dysfunction and Apoptosis

by

Daniel Katz

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
August 1, 2015

Keywords: piperazine designer drugs, SH-SY5Y cell, oxidative stress, mitochondrial dysfunction, apoptosis, neurotoxicity

Approved by

Muralikrishnan Dhanasekaran, Co-chair, Associate Professor of Pharmacology
Randall Clark, Co-chair, Professor of Medicinal Chemistry
Vishnu Suppiramaniam, Associate Professor of Pharmacology
Jack Deruiter, Professor of Medicinal Chemistry

Abstract

Designer drugs are synthetic structural analogues/congeners of controlled substances with slightly modified chemical structures intended to mimic the pharmacological effects of known drugs of abuse so as to evade drug classification. Benzylpiperazine (BZP), a piperazine derivative, elevates both dopamine and serotonin extracellular levels producing stimulatory and hallucinogenic effects, respectively, similar to methylenedioxymethamphetamine (MDMA). Here, piperazine derivatives were synthesized in our lab (BZP and BZOP) and the mechanisms of cellular-based neurotoxicity were elucidated in a human neuroblastoma cell line (SH-SY5Y). The principle pathways leading to cellular toxicity include oxidative stress, mitochondrial dysfunction, apoptosis, inflammation, excitotoxicity and necrosis. We evaluated the in vitro effect of benzylpiperazine and benzoylpiperzine on the generation of reactive oxygen species, lipid peroxidation, mitochondrial complex-I activity, catalase activity, superoxide dismutase activity, glutathione content, Bax, caspase-3, Bcl-2 and Tyrosine Hydroxylase expression. BZP and BZOP induced oxidative stress, inhibited complex-I activity and stimulated apoptosis. Prior studies utilizing 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), methylenedioxymethamphetamine (MDMA), methamphetamine, paraquat and corexit have established a link between oxidative stress, mitochondrial dysfunction and apoptosis with neurotoxicity. Parkinson's disease and Alzheimer's disease are associated with dopaminergic (substantia nigra) and cholinergic

(basal forebrain) neuronal cell death, respectively. We postulate that exposure to BZP or BZOP will induce destruction of these pathways resulting in movement disorders and cognitive impairment. This study provides a germinal assessment of the neurotoxic mechanisms induced by piperazine derivatives that lead to cell death.

Acknowledgments

A great many people have provided support, guidance and wisdom in my efforts to graduate with an advanced degree in Drug Discovery and Development and ultimately a career as a Pharmaceutical Scientist. I met Dr. Murali Dhanasekaran well before being accepted into the Pharmaceutical Sciences program at Auburn University. Dr. Dhanasekaran has helped me immensely with my writing skills, as we have written a previous literature review. I moved down to Auburn, from Cleveland Ohio, and Dr. Dhanasekaran has not only guided me in research and taught me coursework, but has remained my friend, as we have worked hard in unison to achieve our goals. I would like to thank Allan Valaer, the Scientific Director of Aperian Laboratory Solutions, for reaching out to me and providing me invaluable experience as a Toxicology Analyst that would help initiate my career in Pharmaceutical Sciences. Dr. Randall Clark welcomed me with open arms into the Pharmaceutical Sciences program in Spring of 2013. Dr. Clark has provided support, knowledge and most of all guidance, as I have valued his opinion throughout all aspects of my research. Dr. Jack Deruiter has given me a unique perspective toward structure activity relationships. Dr. Deruiter and Dr. Clark, alongside Dr. Dhanasekaran, Dr. Suppiramaniam and myself, has proven to be an excellent collaboration between two seemingly distinct but highly dependent branches of Drug Discovery and Development. Dr. Vishnu Suppiramaniam has pushed me to excellence. I have enjoyed Dr. Suppiramaniam's teaching because he promotes confidence, knowledge

and thoroughness that provides students with a solid background in Pharmacology/Toxicology. Thank you to my fellow lab mates, Subhrajit, Dwipayan, Jenna, Ahmad, Fatimah and Aiman who are all a function of the excellent guidance of Dr. Suppiramaniam and Dr. Dhanasekaran. I would sincerely like to thank everyone involved in my project along with all the faculty and students of the Harrison School of Pharmacy.

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List of Abbreviations

BZP	Benzylpiperazine
BZOP	Benzoylpiperazine
TFMPP	1-(3-trifluoromethylphenyl)piperazine
mCPP	1-(3-chlorophenyl)piperazine
MDMA	Methylenedioxymethamphetamine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MPP^+	1-methyl-4-phenylpyridinium
DA	Dopamine
5-HT	Serotonin
NE	Norepinephrine
H_2O_2	Hydrogen Peroxide
ROS	Reactive Oxygen Species
TBARS	Thiobarbituric Acid Reactive Substance
SOD	Superoxide Dismutase
GSH	Glutathione
TH	Tyrosine Hydroxylase
L-DOPA	L-3,4-dihydroxyphenylalanine
AAAH	Aromatic Amino Acid Hydroxylase

1. Introduction

A designer drug is defined as, *"A synthetic version of a controlled narcotic substance that has a molecular structure slightly different from that of some well-known controlled substance but whose effects are essentially the same, whereby the purpose of doing so is to avoid the drug being listed as illicit by law-enforcement organizations"* (Britannica Concise Encyclopedia, 2006). Substituted amphetamines (methamphetamine, ephedrine, cathinone, methylenedioxymethamphetamine, etc.), synthetic cathinones (methylenedioxypyrovalerone, mephedrone, methylone, etc.), synthetic cannabinoids (cannabicyclohenanol, JWH-018, JWH-073, etc.), opioids (hydrocodone, hydromorphone, oxycodone, fentanyl, tramadol, etc.) and piperazine derivatives (BZP, TFMPP, mCPP, etc.) are classes of synthetic designer drugs that structurally resemble their parent molecule and have similar pharmacological and toxicological effects. Piperazine derivatives share similarities in chemical structure and mechanism of action as compared to known stimulants (methamphetamine, amphetamine, MDMA, cocaine, etc.); therefore, one would postulate that many psychostimulants share common pathways in exerting neuronal cell death.

Oxidative stress, mitochondrial dysfunction and apoptosis are key players in the pathogenesis of neurological diseases. The designer drug benzoylpiperazine, a chemically modified analogue of benzylpiperazine, exhibits pronounced neurotoxicity via oxidative stress, mitochondrial dysfunction and apoptosis. Parkinson's disease occurs

through selective loss of dopaminergic neurons in the substantia nigra pars compacta leading to a shortage or depletion of dopamine in the striatum. Alzheimer's disease is characterized as a loss of cholinergic neurons in the basal forebrain leading to decreased cholinergic neurotransmission in the neocortex and hippocampus. Piperazine derivatives will readily cross the blood brain barrier causing long-lasting damage to key regions of the brain involved in neurological disorders. Long term use of BZP or BZOP induce destruction of these tracts resulting in movement disorders and cognitive deficits.

2. Literature Review

2.1. Introduction

Piperazine derivatives are a group of chemically modified designer drugs derived from piperazine, a six-membered ring with two oppositely positioned nitrogen atoms (Figure 2.1.a). Piperazinic derivatives are divided into two classes, benzylpiperazines and phenylpiperazines. The benzylpiperazines include N-benzylpiperazine (BZP) (Figure 2.1.b) and 1-(3,4-methylenedioxybenzyl)piperazine (MDBP), the methylenedioxy analogue. Common phenylpiperazines abused are 1-(3-chlorophenyl)piperazine (mCPP), 1-(4-methoxyphenyl)piperazine (MeOPP) and 1-(3-trifluoromethylphenyl)piperazine (TFMPP) (Figure 2.1.c). Chemical modification of piperazine compounds enable clandestine manufacturers to avoid governmental bans and promote widespread distribution under the pseudonyms “Rapture”, “Frenzy”, “Bliss”, “Charge”, “Herbal ecstasy”, “A2”, “Legal X”, and “Legal E” (Arbo et al., 2012).

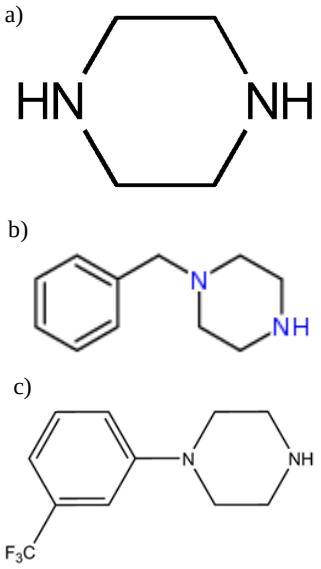

Figure 2.1.: Chemical Structures of a) Piperazine b) Benzylpiperazine c) TFMPP

Originally, benzylpiperazine was synthesized by Burroughs, Wellcome & Co. of Wellcome Research Laboratories in the United Kingdom. BZP was tested as an anti-helmintic agent for the treatment of intestinal roundworm infestations (Haroz and Greenberg, 2006), but piperazine was preferred because of greater efficacy and fewer

side effects (Gee et al., 2005; Johnstone et al., 2007). In the 1970's, benzylpiperazine was examined as a potential antidepressant, but was found inapt due to abuse potential (Bye, Munro-Faure et al, 1973). In 1999, BZP rampantly spread among New Zealand residents due to a failure of regulation. New Zealand youth popularized the legal party drug seeking its stimulatory effects (confidence, talkativeness, euphoria, vigor, activity and enhanced socialization). In 2007, an estimated 5 million BZP pills were sold in New Zealand (Gee & Fountain, 2007). The majority of epidemiological and pharmacotoxicological data including patterns of use, motivations and positive and adverse effects, pertaining to BZP use, emanates from New Zealand during 2000 to 2008 (Cohen & Butler, 2011). Students and workers, such as shift workers and truck drivers, abused the drug to increase alertness and enhance their physical and mental performance (Butler & Sheridan, 2007). BZP found its way into the performance enhancement market serving as a stimulant in horse racing (Barclay, 2003) and athletics (Tutty, 2005), but has since been prohibited. BZP also functioned as an anorectic, an appetite suppressant for young women (Wilkins et al, 2006).

On September 20, 2002 BZP was temporarily scheduled in accordance with the controlled substances act (CSA) as a schedule 1 drug, a drug with a high liability for abuse with no current medical use (DEA, 2015), following a report by the Drug Enforcement Administration (DEA) which inaccurately states that BZP was 10 to 20 times more potent than amphetamine, when actually BZP is 10 times less potent that dexamphetamine (Stargate International, 2004). Successively, on March 18, 2004 BZP was permanently placed among schedule 1 drugs. Identified BZP cases reported to federal, state and local forensic laboratories maxed out at 15,174 in 2009, while in 2013

there were 2,548 reports, according to DEA's System to Retrieve Information from Drug Evidence (STRIDE) and the National Forensic Laboratory Information System (NFLIS) (DEA, 2014).

2.2. Perception of Safety

Due to psychoactive properties, legal status in many countries, and false reputation of safety, the recreational use of piperazine derivatives have gained popularity as an alternative to amphetamine, although a plethora of experimental, clinical, and epidemiological studies have linked its use with the development of life-threatening serotonin syndrome, hepatotoxicity, neurotoxicity, psychopathology, and abuse potential (Schep et al., 2011). New Zealand users believed that legality protected the quality and purity of BZP, when manufacturers synthesized without controls! Labels gave consumers false impressions that they knew exactly what they were buying. Many users underestimated the strength of the pills and characterized the effects as moderate. Moreover, BZP-party pills were socially accepted and widely available due to a lack of legislation. BZP may entice users into using other illicit drugs ("gateway") or it may provide illicit drug users a legal alternative (Sheridan & Butler, 2010).

2.3. Patterns of Use

Administered orally in capsule, tablet, pill, powder, or liquid form (Gee et al., 2005), the piperazine designer drugs frequently appear as adulterants of ecstasy, cocaine, amphetamine and ketamine (EMCDDA, 2009). Other routes of administration include inhalation, insufflation and intravenous use for a faster onset of action. BZP's high

alkalinity deters users from administering by i.v. because of pain (Gee et al., 2005). Available as a pale, yellowish-green free-base or white hydrochloride salt (S-R Pharmaceuticals Shop, 2015; Figure 2.2.), BZP dosing ranges between 50 and 200 mg (Sheridan et al., 2007), with some pills containing up to 1,000 mg (Gee et al., 2005,2008). New Zealand "party-goers" consumed roughly 2 to 3 pills per experience, with some consuming 8 or more (Butler & Sheridan, 2007). Manufacturers recommend taking 2 pills, waiting 2 hours, then taking another two if tolerable (Thompson et al., 2006).

Figure 2.2: Benzylpiperazine Powder

Users actually preferred to consume party pills with other psychoactive substances (alcohol, ecstasy, cannabis, amphetamines and nitrous oxide) for several reasons. First, it increased stimulatory effects. Second, greater amounts of alcohol were able to be consumed because of the sobering effects of party pills. Third, the negative effects of party pills could be tolerated. For example, cannabis engendered relaxation, stimulated appetite and fostered sleep (Butler & Sheridan, 2007). Caffeine, herbal extracts, electrolyte blends and amino acids are also commonly taken with BZP. L-tyrosine, the amino acid precursor of dopamine, provides an auxiliary supply of dopamine to counteract dopamine depletion, often a consequence of overconsumption (Nikolova & Danchev, 2008; Sheridan et al., 2007).

2.4. Toxicokinetics

Piperazine derivatives are rapidly absorbed in the gastrointestinal tract (Antia et al., 2009; Schep et al., 2011) and chiefly metabolized by the liver, the phenylpiperazines

TFMPP and MeOPP more extensively metabolized than the benzylpiperazines BZP and MDBP (Maurer et al., 2004). Once ingested, BZP takes approximately two hours to take effect (Bye et al., 1973; Gee & Richardson 2005) and usually lasts between 4 and 8 hours (Nikolova & Danchev, 2008; Wikstrom et al., 2004). Pharmacokinetic data indicate detection of BZP in blood up to 30 hours after oral intake (Antia et al., 2009). Piperazine derivatives are known to readily cross the blood-brain barrier. The TFMPP brain-to-blood concentration ratio was in excess of one order of magnitude demonstrating high tissue concentrations (Schep et al., 2011). In human subjects administered a 200 mg oral BZP dose, 12.5% of BZP and its metabolites were detected in urine after 24 hours (Antia et al., 2009). Peak plasma concentrations (C_{max}) were reached after 75 minutes (T_{max}) at a concentration of 262 ng/ml. The absorption half-life ($t_{1/2}$) occurred after 6.2 minutes. The clearance was 58.3 L/h with a drug half-life of 5.5 hours (Antia et al., 2009). The two major metabolic pathways of BZP involve hydroxylation and N-dealkylation catalyzed by cytochrome P450 (CYP) enzymes (Antia et al., 2009b; Staack et al., 2002, 2003). Single or double hydroxylation of the aromatic ring, by CYP450, followed by COMT-mediated methylation to N-(4-hydroxy-4-methoxybenzyl)piperazine and subsequent phase 2 metabolism to glucuronic and/or sulphuric acid conjugates, promote BZP excretion. A benzyl carbon N-dealkylation frees the piperazine ring. Double N-dealkylation of the piperazine heterocycle yields N-benzylethylenediamine or benzylamine (Staack & Maurer, 2005). CYP450 isoenzymes that play a role in BZP and TFMPP metabolism include CYP2D6, CYP1A2 and CYP3A4. Cytochrome P450 enzymes are susceptible to genetic polymorphisms; therefore, inter-individual differences in metabolism may occur (EMCDDA, 2015). BZP and TFMPP may inhibit these

enzymes and alter the metabolism of commonly used pharmaceuticals, therefore contraindication is imperative (Antia et al., 2009b; Murphy et al., 2009).

2.5. Pharmacology

In vivo and *in vitro* studies suggest BZP raises synaptic levels of dopamine (DA), serotonin (5-HT) and norepinephrine (NE). Benzylpiperazine causes nonexocytotic substrate release of dopamine by reversing transporter flux (Baumann et al., 2004), inhibits dopamine re-uptake by blocking dopamine transporters (Meririnne et al., 2006) and acts as an agonist at postsynaptic dopamine receptor sites (Oberlander et al., 1979). As MDMA, BZP modulates both dopamine and serotonin levels in the synaptic cleft (Baumann et al., 2004). BZP inhibits serotonin transporters preventing reuptake and binds as an agonist to 5-HT_1 receptors (Tekes & Tothfalusi, 1987). Reuptake of monoamines (DA and NE, but with lesser extent 5-HT) was also inhibited in rat brain synaptosomal preparations by benzylpiperazine (Nagai et al., 2007). Monoamine-releasing activity, mostly of DA and NE, occurred after BZP administration (Nagai et al., 2007) in the same synaptosomes. The mild hallucinogenic effects experienced under high doses are due to BZP binding with the 5-HT_{2A} subtype. The 5-HT_{2B} receptor, localized in the gastrointestinal tract, is bound by BZP, inducing intense peripheral side effects, such as stomach pains and nausea. BZP-bound $5HT_3$ receptors are known for the development of migraine headaches (Nikolova & Danchev, 2008). The stimulatory effects are attributed to dopaminergic neurotransmission, while euphoria, hallucinations, alertness and sociability, all desired feelings at rave dance parties, are associated with serotonergic activation. BZP acts as an antagonist at alpha-2-adrenoreceptors, which

inhibits negative feedback and augments the release of norepinephrine in peripheral sympathetic nerve fibers in vitro (Magyar et al., 1986). Elevated levels of circulating norepinephrine bind easily with central and peripheral α- and β-adrenergic receptors provoking an increase in blood pressure and heart rate.

Yarosh et al. examined the stimulant-like behavioral patterns of BZP in mice. The head twitch response assay failed to elicit hallucinogen-like actions, a dose-dependent increase in locomotor activity was observed in the open field and BZP fully substituted for the S(+)-enantiomer (stimulant-like) of MDMA. TFMPP decreased locomotor activity, induced hallucinations in the head twitch assay, but was unsuccessful in substituting for R(-)-MDMA (hallucinogenic-like). Increases in synaptic serotonin by TFMPP yield hallucinations, while BZP/TFMPP accounts for the dopaminergic stimulatory effects (Yarosh et al, 2007).

2.6. BZP/TFMPP Combination

Typically, benzylpiperazine is taken in combination with other piperazines to enhance the positive reinforcing effects. A 2:1 combination of BZP and TFMPP is known to strongly resemble the effects of MDMA in humans (de Boer et al., 2001), although combinations up to 10:1 have been reported (Lin et al., 2011). TFMPP, a serotonin-mimicking piperazine, blocks serotonin reuptake, enhances serotonin release and is a nonselective agonist at serotonin receptors (Nikolova & Danchev, 2008). TFMPP does not possess significant dopaminergic or noradrenergic actions (Herndon et al, 1992; Fantegrossi et al., 2005), but rather binds to 5-HT_1 and 5-HT_2 receptors with

little affinity for 5-HT_3 receptors (Cunningham & Appel, 1986; Baumann et al., 2005; Robertson et al., 1992).

Baumann et al. noticed MDMA-like dopamine and 5-HT release in BZP/TFMPP administered mice (Baumann et al., 2005). They evaluated the transporter-mediated efflux of [^{3}H]MPP^+, a dopamine transporter substrate (DAT), and [^{3}H]5-HT, a serotonin transporter substrate (SERT), in rat synaptosomes. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a meperidine anologue, is a highly lipophilic neurotoxin that readily crosses the blood-brain barrier. MPTP becomes oxidized to 1-methyl-4-phenylpyridinium (MPP^+) via monoamine oxidase B (MAO-B) (Ramsay et al., 1986). BZP caused a DAT-dependent release of [^{3}H]MPP^+, but unlike TFMPP, BZP showed no effect on [^{3}H]5-HT release by SERT (Baumann et al., 2005). Remarkably, the BZP/TFMPP blend reacted synergistically, resulting in an increase in endogenously released dopamine and serotonin, similar to MDMA (Yeap et al., 2010; Baumann et al., 2004, 2005). Cumulative release of DA and 5-HT was greater in combination than when drugs were given individually (Hashimoto, 1993). TFMPP was 3-fold less potent than MDMA at elevating extracellular 5-HT levels (Baumann et al., 2005). Rats experience seizures and ataxia at higher level doses of BZP/TFMPP (Baumann et al., 2004, 2005).

2.7. Health Effects

BZP adverse side effects include insomnia, mild to severe hangover, dilated pupils, dryness of mouth, extreme alertness, pruritus,

insomnia	palpitations	urine retention
hangover	dizziness	metabolic acidosis
dilated pupils	anxiety	respiratory acidosis
xerostoma	insomnia	hyponatremia
alertness	vomiting	seizures
pruritus	chest pain	acute paranoid psychosis
confusion	collapse	dissociative states
agitation	hypertension	rhabdomyolysis
tremor	tachycardia	intravascular coagulation
dystonia	hyperventilation	acute renal injury
headache	hyperthermia	multiple organ failure

Table 2.1: Adverse Side Effects of Benzylpiperazine

confusion, agitation, tremor, dystonia, headache, dizziness, anxiety, insomnia, vomiting, chest pain, tachycardia, hypertension, palpitations, collapse, hyperventilation, hyperthermia and urine retention (Nikolova & Danchev, 2008) (Table 2.1.), reflective of a sympathomimetic toxidrome. The adrenergic effects, in the peripheral nervous system, have serious cardiovascular effects; vasoconstriction, ischemia, tachycardia and arrhythmia. Over activation leads to hypertension and potential stroke or myocardial infarct (Arbo et al., 2014). In women, a 200 mg single administration of BZP elevated systolic and diastolic blood pressure and heart rate when compared to placebo (Lin et al., 2009). Single administration of BZP/TFMPP has shown the same cardiovascular effects (Thompson et al., 2010; Lin et al., 2011).

Serious toxic effects include metabolic and respiratory acidosis, hyponatremia, seizures, acute paranoid psychosis, dissociative states, worsening of mental illnesses, rhabdomyolysis, disseminated intravascular coagulation, acute renal injury and multiple organ failure (Gee et al., 2005, 2008; Wood et al., 2007,2008; Austin & Monasterio, 2004; Alansari & Hamilton, 2006). The environment of a rave dance party, consisting of intense heat, increased physical activity, sleep deprivation, dehydration and excessive fluid intake, contributes to BZP-party pill toxicity (Berney-Meyer et al., 2012). After effects, occurring hours to days after BZP ingestion, may include insomnia, lack of appetite, tiredness, dehydration, headache, xerostoma, aching/shaking body, depressed mood, tension and anxiety (Butler & Sheridan, 2007; Nicholson, 2006; Thompson et al., 2009)

BZP and TFMPP have serotonergic effects that may lead to serotonin toxicity (Baumann et al., 2005). Combination of BZP and/or TFMPP with other recreational

drugs (MDMA) or therapeutic serotonergic agents, selective serotonin reuptake inhibitors (SSRI's), may increase the risk of developing serotonin syndrome. Treatment of serotonin toxicity centers on controlling agitation by managing symptoms with the benzodiazepines, cyproheptadine and chlorpromazine (Schep et al. 2011). Death due to lone BZP consumption is rare. A 23-year-old woman ingested BZP, MDMA and a large quantity of water resulting in hyponatremia, cerebral edema and ultimately death (Balmelli et al., 2001). In most BZP-related fatality cases, BZP was detected along with other drugs of abuse, such as MDMA (Hartung et al., 2002). Therefore, it is difficult to establish the role, if any, that BZP plays in morbidity.

2.8. Addictive Properties

Previous users of amphetamine were unable to discriminate between equipotent intravenous doses of BZP and dexamphetamine (Campbell et al., 1973). The acute effects of BZP are similar to methamphetamine in rats, although methamphetamine displays greater potency, indicating potential abuse and drug dependency in BZP users (Herbert & Hughes, 2009). Dose-dependent hyperactivity and stereotypy were observed in Sprague-Dawley rats co-administered with BZP and methamphetamine. Behavioral sensitization, a phenomenon in which repeated doses of drug causes a long-lasting and progressive increase in effect, was noticed upon 5 day repeated exposure of BZP and methamphetamine. Furthermore, administration of a low dose of BZP in methamphetamine pre-treated rats, upon a 2 day withdrawal period, evoked cross-sensitization between BZP and methamphetamine (Brennan et al., 2007). Cross-sensitization occurs when sensitivity to one drug, in this case methamphetamine,

predisposes the user to sensitivity to another drug because of similarities in their chemical structures.

BZP-administered rats repeatedly returned to the location of administration, dose-dependently spending more time in the apartment associated with the drug, indicating a conditioned place-preference. The D_1 receptor antagonist SCH and the 5-HT_3 receptor antagonist MDL attenuated conditioned place-preference in BZP treated rats (Meririnne et al., 2006), suggesting the compound has rewarding properties and therefore abuse potential. Also, primates (rhesus monkeys) displayed repeated intravenous self-administration, a reinforcing activity of BZP. TFMPP alone did not maintain self-administration. Appropriately, the BZP/TFMPP combination proved to be less effective reinforcers than BZP alone (Fantegrossi et al., 2005). Heroin, cocaine and methamphetamine also exhibit conditioned place-preference and self-administration properties. A 1:1 BZP/TFMPP cocktail elevated DA and 5-HT in the nucleus accumbens (Baumann et al., 2005), the "pleasure center" in the mesocorticolimbic pathway. Dopaminergic input extending from the ventral tegmental area to the nucleus accumbens regulates rewarding experiences, such as drug addiction.

2.9. Detection and Quantification

Granted that BZP has been classified as a schedule 1 controlled substance, availability over the internet and underground drug trafficking has necessitated accurate and sensitive forensic toxicological testing techniques. Immunoassays and colorimetric tests are used to screen biological specimens in order to rapidly detect drugs (Arbo et al., 2012). Colorimetric assays lack specificity and sensitivity because they target specific

functional groups on a molecule as opposed to the molecule as a whole. Therefore, colorimetric assays frequently produce false positives and negative results (Elie et al., 2012; Flanagan et al., 2007). Immunoassays utilize antigen-antibody specific reactions and are prone to cross-reactivity, particularly with amphetamines. Chromatographic techniques are the preferred method for identification and quantification of BZP and its hydroxylated metabolites, 3-OH-BZP and 4-OH-BZP (Tsutsumi et al., 2006). Gas and liquid chromatography coupled with mass spectrometry is the preferred system for detection, but diode array detector, fluorescence and ultraviolet detection have been employed. Sample preparation, including hydrolysis (acid or enzymatic), extraction (solid phase or liquid-liquid) and derivitization, along with internal standard are all considered for development of protocol.

2.10. Conclusion

Piperazine-derived designer drugs have gained popularity worldwide because of widespread distribution, legality and a false reputation of safety. Piperazine derivatives exert their stimulatory and hallucinogenic effects by elevating synaptic monoamine concentrations, similar to amphetamine, cocaine and MDMA. Concomitant use of BZP and TFMPP, usually in cocktails or blends, amplify the amount of endogenously released dopamine and serotonin, resulting in powerful effects. Adverse effects of BZP are mostly adrenergic, leading to profound cardiovascular effects. BZP can also cause detrimental psychological, neurological and systemic effects, including severe serotonin syndrome and multiple organ failure. Human and animal studies reveal BZP and other piperazine derivatives have reinforcing effects and are therefore susceptible to addiction.

A lack of existing literature and data on piperazine derivatives prompts further investigations into all aspects of drug development, formulation, mechanism of action, toxicity and detection.

3. Materials and Methods

3.1. Chemicals and Reagents

Thiazolyl Blue Tetrazolium Bromide (MTT) was purchased from Tokyo Chemical Industry America. Dulbecco's Modified Eagle Medium (DMEM), Trypsin-EDTA solution, Fetal Bovine Serum (FBS) and Penicillin-Streptomycin Solution were purchased from ATCC. Phosphate buffer saline (PBS), Dimethylsulfoxide (DMSO), Nicotinamide adenine dinucleotide (NADH), 2`, 7-dichlorofluorescindiacetate (DCF-DA), Pyrogallol, Hydrogen Peroxide (H_2O_2), Phosphoric acid, o-phthalaldehyde (OPA), L-Glutathione reduced, Trichloroacetic acid, Thiobarbituric acid and Phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma Aldrich (St. Louis, MO). Cell lysis buffer, secondary anti-rabbit antibody conjugated with fluorophore DyLight 550, Bax, Bcl-2, caspase-3 and β-actin primary antibodies were purchased from Cell Signaling Technologies (Cell Signaling Technology, Inc., Danvers, MA). A Thermo Scientific Pierce 660 nm Protein Assay reagent kit was purchased (Pierce, Rockford, IL) for protein quantification.

3.2. Human Neuroblastoma Cells (SH-SY5Y)

The parental line SK-N-SH was subcloned three times producing the SH-SY5Y subline. Derived from the bone marrow of 4 year old females with neuroblastoma, SH-

SY5Y are of mixed phenotype exhibiting both adherent and floating cells. Cells with the neuroblast-like morphology exhibit tyrosine hydroxylase and dopamine-β-hydroxylase activity (Kovalevich & Langford, 2013). SH-SY5Y cells were cultured in DMEM supplemented with Fetal Bovine Serum (10%) and Penicillin-Streptomycin Solution (1%). For the MTT assay, cells were propagated into 75 cm^2 flasks, removed by trypsinization after reaching 80% confluency (3-4 days) and plated into 96 well plates at a density of 1 x 10^5 cells/well. Cells were incubated at 37°C and supplemented with 5% CO_2. Cultures were used within 20 passages after the cells were received.

3.3. Synthesis of Benzylpiperazine and Benzoylpiperazine

A mixture of benzaldehyde (1 g, 0.01 mol) and piperazine (1.43 g, 0.0165 mol) in methanol was stirred for half an hour. Then sodium cyanoborohydride (2.1 g, 0.033 mol) was added and the mixture was allowed to stir for half an hour. The reaction was quenched by adding ice/water mixture and stirring the mixture for 20 minutes followed by extracting the final product using dichloromethane (3x30 ml). The combined organic extract was dried with anhydrous magnesium sulfate, filtered and evaporated to yield yellow oil. The oil was dissolved in anhydrous diethyl ether, and hydrochloric acid gas was added to form the hydrochloride salt. White crystals of BZP (Figure 3.1.a) were obtained by filtration. MS, molecular weight 176, m/z 91 [100%].

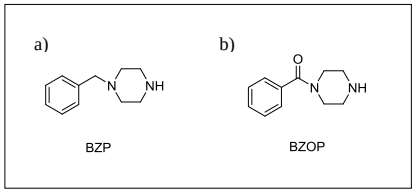

Figure 3.1.: Chemical Structures of a) Benzylpiperazine and b) Benzoylpiperazine

A sample of piperazine (2.6 g, 0.03 mol) was dissolved in 50 ml of dichloromethane in a round bottom flask and the flask was placed in an ice bath for 15

minutes. Benzoyl chloride (1.4 g, 0.01 mol) was dissolved in dichloromethane and the solution was dripped slowly over the piperazine solution over 10 minutes. The mixture was allowed to stir for 15 minutes. The solution was evaporated under reduced pressure to yield light yellow oil. The oil was dissolved in anhydrous diethyl ether, and hydrochloric acid gas was added to form the hydrochloride salt. White crystals of the BZOP (Figure 3.1.b) product was obtained by filtration. MS, molecular weight 190, m/z 105 [100%].

3.4. Treatment Design

Benzylpiperazine and benzoylpiperazine were initially dissolved in methanol (500 mM) and then diluted in Phosphate Buffered Saline to a 10 mM stock solution. For cytotoxicity testing, different concentrations of benzylpiperazine and benzoylpiperazine (62.5, 125, 250, 500, 1,000, 1,500, 2,000 uM) were achieved by serial dilution with PBS followed by further dilution in serum-enriched fresh culture medium. Test concentrations were exposed to the cell line for 48 hours representing relatively long exposure of drug based toxicity testing. For the collection of cell homogenate, drug concentrations (1 mM and 500 uM) were achieved by further dilution in serum-enriched fresh culture medium. Cells were exposed to drug for 24 hours before extensive cell death occurred in order to elucidate the neurotoxic mechanisms leading to cell death. All stock solutions were stored at -20º C and freshly diluted on the day of the experiment.

3.5. Cytotoxicity Assay

For the assessment of cytotoxicity, an MTT reduction assay was performed following the Vybrant MTT Cell Proliferation Assay Kit protocol. The MTT cell viability assay colorimetrically measures succinate dehydrogenases reduction of the yellow colored water soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to an insoluble crystal forming dark purple formazan by live mitochondria of viable cells. After 48 hour incubation with Benzylpiperazine and Benzoylpiperazine, a 12 mM MTT stock solution was added to each well along with fresh culture medium. Following a 2 hour incubation at 37° C the medium was removed and 100 ul of DMSO was added to solubilize the insoluble formazan crystals. After a quick incubation at 37° C for 10 minutes the absorbance was measured using a microtiter plate reader (Synergy HT, Bio-Tek Instruments Inc., Winooski, VT, USA) at 540 nm. Results were reported graphically and expressed as % viability vs. concentration (uM). Hydrogen peroxide induced cell death after 8 hours of exposure served as a positive control. Cells were imaged using an Axiovert 25 inverted microscope equipped with a Nikon Coolpix 4500 camera.

3.6. Quantifying Reactive Oxygen Species

The generation of reactive oxygen species by benzylpiperazine and benzoylpiperazine in SH-SY5Y cells was spectrofluorometrically detected, at an excitation wavelength of 492 nm and emission wavelength of 527 nm, via reaction of a nonfluorescent chloromethyl-DCF-DA (2`, 7-dichlorofluorescin diacetate) with ROS to produce fluorescent DCF. A reaction mixture composed of 0.05% w/v DCF-DA in

ethanol, phosphate buffer and supernatant of tissue was incubated at 37°C for 1 hour. Readings were taken by a Thermo Scientific Varioskan Flash Spectral Scanning Multimode Reader. Results were expressed as percentage change from the control (Dhanasekaran et al., 2008).

3.7. Lipid Peroxide Content

Lipid peroxidation is a chain reaction mechanism in which reactive oxygen species attack polyunsaturated fatty acids resulting in the oxidative degradation of lipids. Lipid peroxide content was estimated by measuring the amount of thiobarbituric acid-reactive substances (Ohkawa et al., 1979). Cell homogenate was combined with Trichloroacetic acid (20 % w/v), Thiobarbuturic acid (0.5 % w/v) and deionized water. The assay mixture was heated in a water bath for 15 minutes at 80°C then cooled on ice. Following centrifugation at 10,000 rpm for 5 minutes at 4°C samples were placed in a 96-well plate and absorbance was read at 532 nm with a plate reader. Results were expressed as percentage change from the control.

3.8. Superoxide Dismutase Activity

The autoxidation of pyrogallol in an alkaline environment results in the generation of superoxide anion radicals. Superoxide dismutase, a free radical scavenging antioxidant enzyme, rapidly dismutases superoxide into hydrogen peroxide and water. Superoxide dismutase catalyzed pyrogallol autoxidation inhibition can rapidly and conveniently be determined spectrophotometrically with visible light at 420 nm for 3 minutes by combining 2 mM pyrogallol solution, 50 mM Tris buffer pH 8.2, and cell

homogenate (Marklund & Marklund, 1974). Superoxide dismutase activity was measured as an increase in absorbance at 420 nm and expressed as percentage change from the control.

3.9. Catalase Activity

Catalase, an antioxidant enzyme, catalyzes the conversion of hydrogen peroxide to water and oxygen. A reaction mixture containing 50 mM PBS at pH 7.0, supernatant of tissue and 30 mM hydrogen peroxide was spectrophotometrically analyzed under ultraviolet light at 240 nm for 2 minutes to determine hydrogen peroxide decomposition (Aebi, 1984). The hydrogen peroxide contributes approximately 0.5 absorbance. A standard curve was prepared from commercially obtained H_2O_2. Results were reported as percentage change from the control.

3.10. Glutathione Content

Glutathione peroxidase (GPx) catalyzes the conversion of hydrogen peroxide to water in the presence of glutathione (GSH). Spectrofluorometric measurement of GSH utilizes a condensation reaction between GSH and o-phthalaldehyde (OPA) generating a fluorescent product at pH 8.0 (Cohn & Lyle, 1966). The reaction mixture was comprised of supernatant of tissue, 0.1 M phosphoric acid to precipitate protein, 0.1 % OPA solution in methanol and 0.01 M PBS. Ensuing a 20 minute dark incubation at room temperature, fluorometric readings were taken at an excitation wavelength of 340 nm and an emission wavelength of 420 nm. A GSH standard curve was prepared from commercially purchased GSH. Results were expressed as percentage change from the control.

3.11. Mitochondrial Complex-I Activity

Mitochondrial complex-I (NADH dehydrogenase) catalyzes the oxidation of NADH to NAD^+. The supernatant of tissue was added to a reaction mixture comprised of phosphate buffered saline and NADH. NADH dehydrogenase activity was measured with a spectrophotometer at 340 nm. A standard curve was constructed from commercially available NADH. The amount of NADH oxidized was measured as a decrease in absorbance at 340 nm and reported as percentage change from the control (Ramsay et al., 1986).

3.12. Expression of Bax, Caspase-3, Bcl-2 and Tyrosine Hydroxylase

Cell lysis buffer, supplemented with protease inhibitor, was used to isolate and collect total protein for Western Blot analysis. A Thermo Scientific Pierce 660 nm Protein Assay reagent kit, for the determination of protein concentration, allowed equal loading of protein in each well. Samples were mixed thoroughly with 6x Laemmli buffer, boiled at 90°C for 10 minutes, then loaded onto a 10% gel and separated by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis. Proteins were then electrophoretically transferred onto PVDF membranes (0.2 um) through a wet transfer technique. 5% defatted milk in Tris-buffered saline with 0.1% Tween-20 (TBST) at pH 7.4 blocked all non-specific binding sites. Membranes were washed with TBST and incubated overnight at 4°C with primary antibodies for Bax, Caspase-3, Bcl-2 and Tyrosine Hydroxylase (1:1000). Primary antibodies were probed with secondary anti-rabbit antibody (1:10,000) conjugated with fluorophore DyLight 550 for 4 hours at room

temperature. Membranes were scanned at excitation wavelength of λ_{532} nm and emission wavelength of λ_{570} nm then visualized using a FluorChem Q imager with a 532 nM green laser and an LPG filter set. B-actin was used as a loading control. Band intensity was calculated by densitometric analysis using AlphaView software, normalized to β-actin and reported as percentage change from the control.

3.13. Statistical Analysis

Data was expressed as means ± SEM. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Dunnet's multiple comparisons test ($P < 0.05$ was considered to be statistically significant). Statistical analysis was performed using Prism-V software (La Jolla, CA, USA).

4. Results

4.1. Benzylpiperazine and Benzoylpiperazine Elicit Dose-Dependent Cell Death

Different doses of BZP and BZOP (62.5, 125, 250, 500, 1,000, 2,000 uM) were incubated with undifferentiated SH-SY5Y cells for 48 hours. Controls were grown under the same conditions without exposure to BZP and BZOP. BZP and BZOP significantly decreased cell viability in a dose-dependent manner when compared to control ($n=12$, $p<0.05$; Figure 4.1.a). BZP induced cell death by nearly 7% and 37% at 1,000 and 2,000 µM, respectively, while at the same doses BZOP induced 57% and 87% cell death. Hydrogen peroxide induced cell death is shown for comparison as positive control ($n=12$, $p<0.05$; Figure 4.1.b). The morphological changes upon SH-SY5Y cells exposed to BZP and BZOP (500 uM and 1000 uM) after 48 hours are visualized by microscope (Figure 4.2.). Increasing doses of BZP and BZOP induced deformation of the cell structure, neuronal cell shrinkage and decreased cell density leading to cell death.

4.2. Benzylpiperazine and Benzoylpiperazine Induce Oxidative Stress

Oxidative stress spurred on by ROS generation causes damage to biological molecules including lipids, proteins and DNA (Freeman & Crapo, 1982). Damage brought about by ROS is implicated in many human diseases including atherosclerosis, pulmonary fibrosis, cancer, neurodegenerative diseases and aging (Cross et al., 1987; Halliwell & Gutteridge, 1992). Antioxidants serve to detoxify ROS effectively

counteracting the deleterious effects of oxygen derived species. We tested the activities of the antioxidants catalase and superoxide dismutase, along with glutathione content to determine the cell response to BZP and BZOP treatment. BZP and BZOP dose-dependently increased the production of ROS in SH-SY5Y cells ($n=6$, $p<0.05$; Figure 4.3.). BZP increased the generation of ROS by 21% at 1,000 µM, while the slightly modified molecule BZOP increased ROS generation by 33% at 1,000 uM, when compared to controls. Free radicals are known to interact with lipids and increase the formation of lipid peroxides. To assess the oxidation of lipids, we employed a lipid peroxidation assay, in which the amount of thiobarbituric acid-reactive substances (TBARS) was measured, indicating membrane damage. BZP and BZOP (both at 1,000 uM) significantly increased the levels of lipid peroxide by more than two and three times, respectively, when compared to control ($n=6$, $p<0.05$; Figure 4.4.). Superoxide dismutase protects the cell by catalyzing the conversion of superoxide anion to the less harmful non-radical hydrogen peroxide and water. Higher doses of BZOP (1,000 uM) increased the SOD activity by 1.5 fold as compared to controls ($n=6$, $p<0.05$; Figure 4.5.). In response to high levels of hydrogen peroxide, the cells enhanced their catalase activity to counteract the oxidative stress, effectively breaking down hydrogen peroxide into the byproducts water and molecular oxygen. BZP and BZOP both increased the activity of catalase above control ($n=6$, $p<0.05$; Figure 4.6.). BZP and BZOP had no effect on glutathione content ($n=6$, $p<0.05$; Figure 4.7.).

4.3. Benzylpiperazine and Benzoylpiperazine Inhibit Mitochondrial Complex-I Activity

As the powerhouse of the cell, the mitochondria are key players in regulating cell survival and death. Therefore, mitochondrial complex-I deficits are implicated in the aging process and several neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, Amyotrophic lateral sclerosis and Huntington's disease (Lin & Beal, 2006). BZOP significantly inhibited Complex-I activity of the mitochondrial electron transport chain in a dose-dependent manner ($n=6$, $p<0.05$; Figure 4.8.). However, BZP did not display comparable complex-1 inhibitory effects. BZOP (1,000 µM) had more than seven times the inhibitory effects when compared to control. Mitochondrial complex-I inhibitors can induce mitochondrial dysfunction (drop in ATP production), excessive production of reactive oxygen species, apoptosis and inflammation.

4.4. Benzylpiperazine and Benzoylpiperazine Stimulate Apoptosis

Autophagy, necrosis and apoptosis are different types of cell death. Apoptosis (programmed cell death) has specific morphological features including rounding of the cell, reduction of cellular volume (pyknosis), chromatin condensation, nuclear fragmentation (karyorrhexis) and plasma membrane blebbing (Hengartner MO., 2000; Nagata S., 2000). Bax and Bcl-2, both members of the Bcl-2 family, are pro-apoptotic and anti-apoptotic, respectively. Caspase-3, a zymogen, is a member of the cysteine-aspartic acid protease family and mediates the execution phase of apoptosis. Bax expression has been related to apoptosis (Porter & Janicke, 1999). Bax, caspase-3 and Bcl-2 expression was quantified by immunoblotting to determine BZP and BZOP

apoptotic stimulation. BZOP (1,000 uM) significantly increased the expression of Bax (N=3, P<0.05; Figure 4.9.) and cleaved caspase-3 (N=3, P<0.05; Figure 4.10.), while Bcl-2 expression significantly decreased (N=3, P<0.05; Figure 4.11.). BZP did not exhibit the same robust effects on apoptotic markers, but rather middling changes in the expression of Bax, cleaved caspase-3 and Bcl-2 upon 24 hours exposure.

4.5. Benzoylpiperazine Reduces Tyrosine Hydroxylase Expression

Tyrosine hydroxylase, an aromatic amino acid hydroxylase (AAAH), catalyzes the conversion of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) using molecular oxygen (O_2), iron (Fe^{2+}) and tetrahydrobiopterin (BH_4) as cofactors. L-DOPA is a precursor of dopamine, norepinephrine and epinephrine; therefore, tyrosine hydroxylase catalyzes the rate limiting step in the synthesis of these catecholamines. SH-SY5Y cells express tyrosine hydroxylase. BZOP (1,000 µM) significantly decreased tyrosine hydroxylase expression, while BZP (1,000 µM) had no effect (N=3, P<0.05; Figure 4.12.).

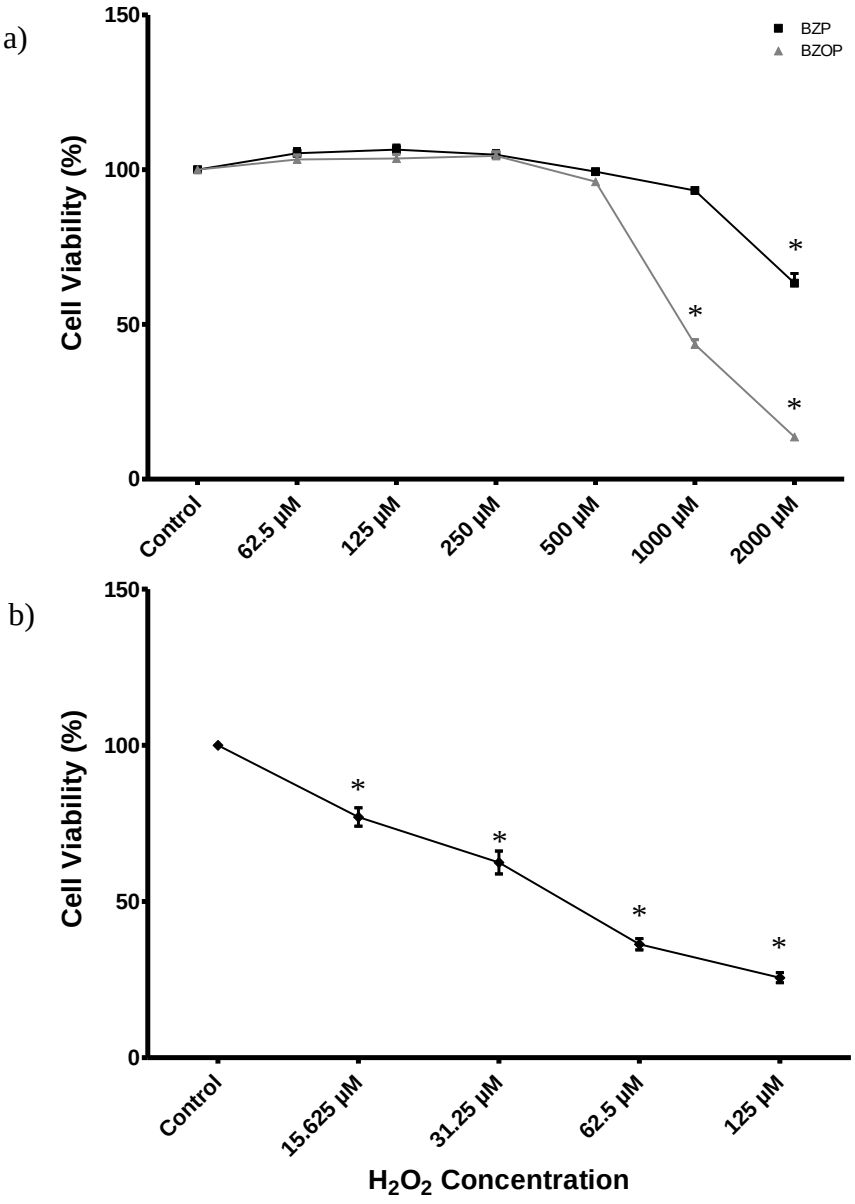

Figure 4.1. Concentration-response (cell viability) curves in SH-SY5Y cells

a) Cells were treated with different doses of BZP and BZOP for 48 hours at 37°C. b) Cells treated with different doses of hydrogen peroxide (H_2O_2) for 8 hours at 37 °C. Cell viability was evaluated through the MTT reduction assay. The data was expressed as mean ± SEM. Statistical comparisons were made using one-way ANOVA/Dunnet's multiple comparison test, (*) indicates a statistically significant difference compared to controls (n=12, $p < 0.05$).

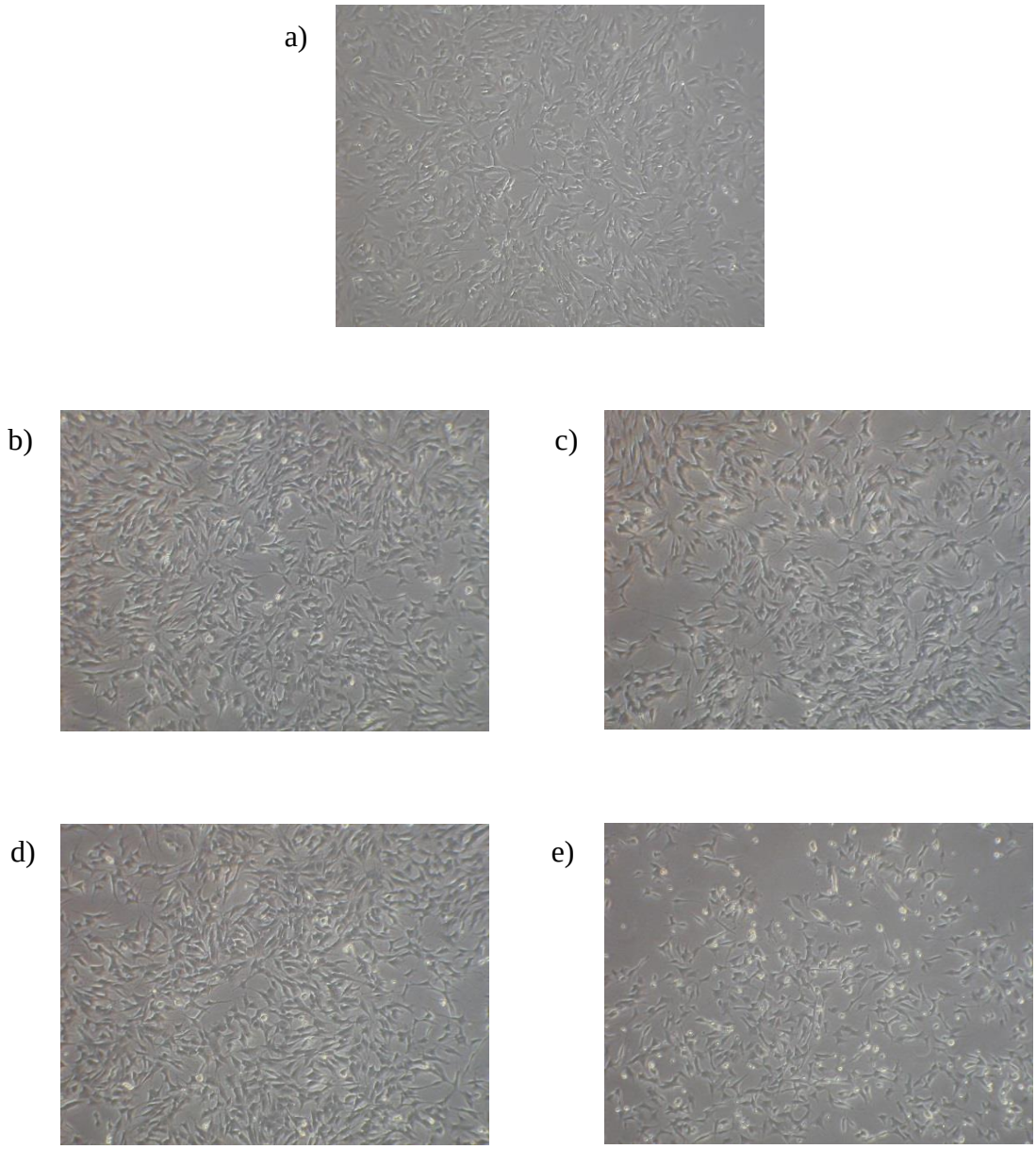

Figure 4.2. Morphological characterization of neuronal cells (SH-SY5Y) treated with BZP and BZOP

Cells were treated with different concentrations of BZP and BZOP for 48 hours. After incubation the cells were washed with PBS and visualized under microscope (magnification 10x). a) Control b) BZP 500 µM c) BZP 1,000 µM d) BZOP 500 µM e) BZOP 1,000 µM

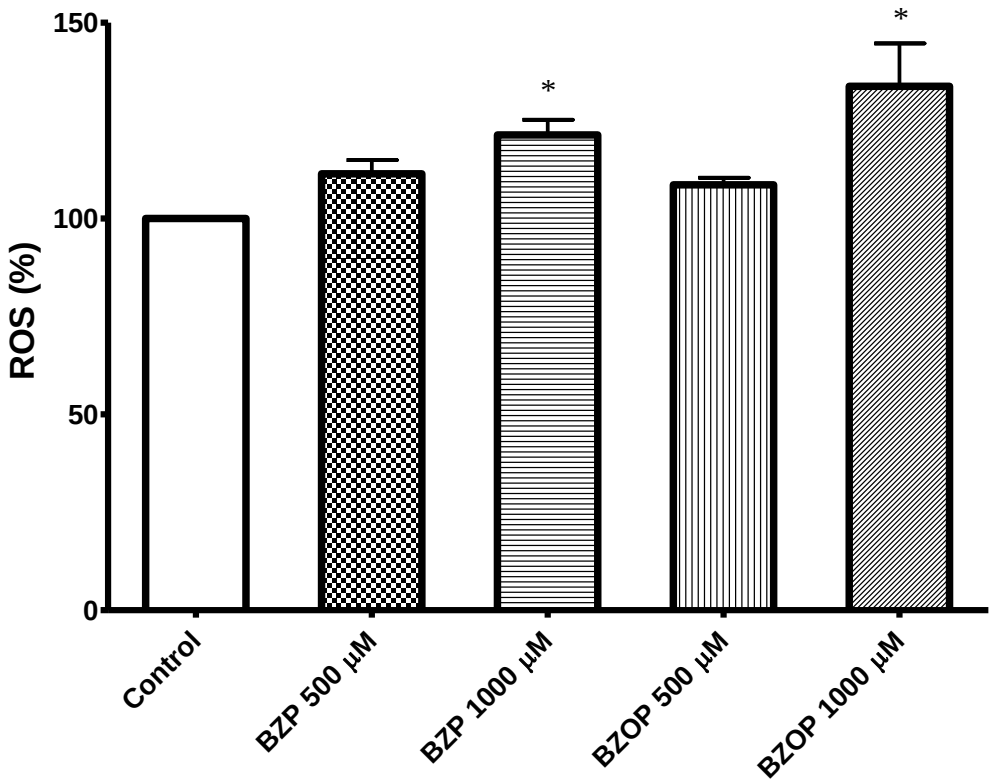

Figure 4.3. Effect of BZP and BZOP on ROS generation in SH-SY5Y cells

BZP and BZOP induce oxidative stress as seen by increased reactive oxygen species generation in SH-SY5Y cells after 24 hours. The fluorescent product DCF was measured spectrofluorometrically. BZP and BZOP (1000 µM) showed a significant increase in ROS generation ($p < 0.05$, n=6). Results are expressed as percentage control ± SEM. Statistical comparisons were made using one-way ANOVA/Dunnet's multiple comparison test. Note (*) indicates a statistically significant difference when compared to controls.

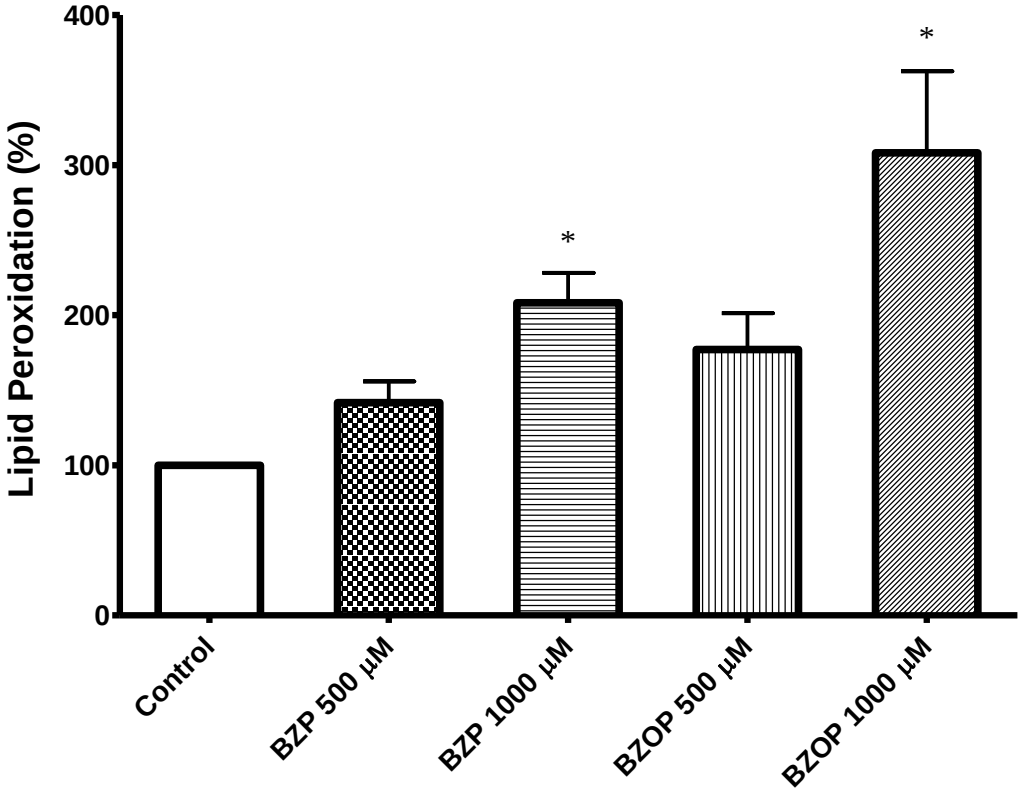

Figure 4.4. Effect of BZP and BZOP on lipid peroxide content in SH-SY5Y cells

BZP and BZOP (1000 µM) significantly and dose-dependently increased lipid peroxidation (n=6, $p < 0.05$) in SH-SY5Y cells after 24 hours. Lipid peroxidation was measured colorimetrically as TBARS (thiobarbituric acid reactive substances), an indicator of cellular membrane damage. Results are expressed as percentage control ± SEM. Statistical comparisons were made using one-way ANOVA/Dunnet's multiple comparison test. Note (*) indicates a statistically significant difference when compared to controls.

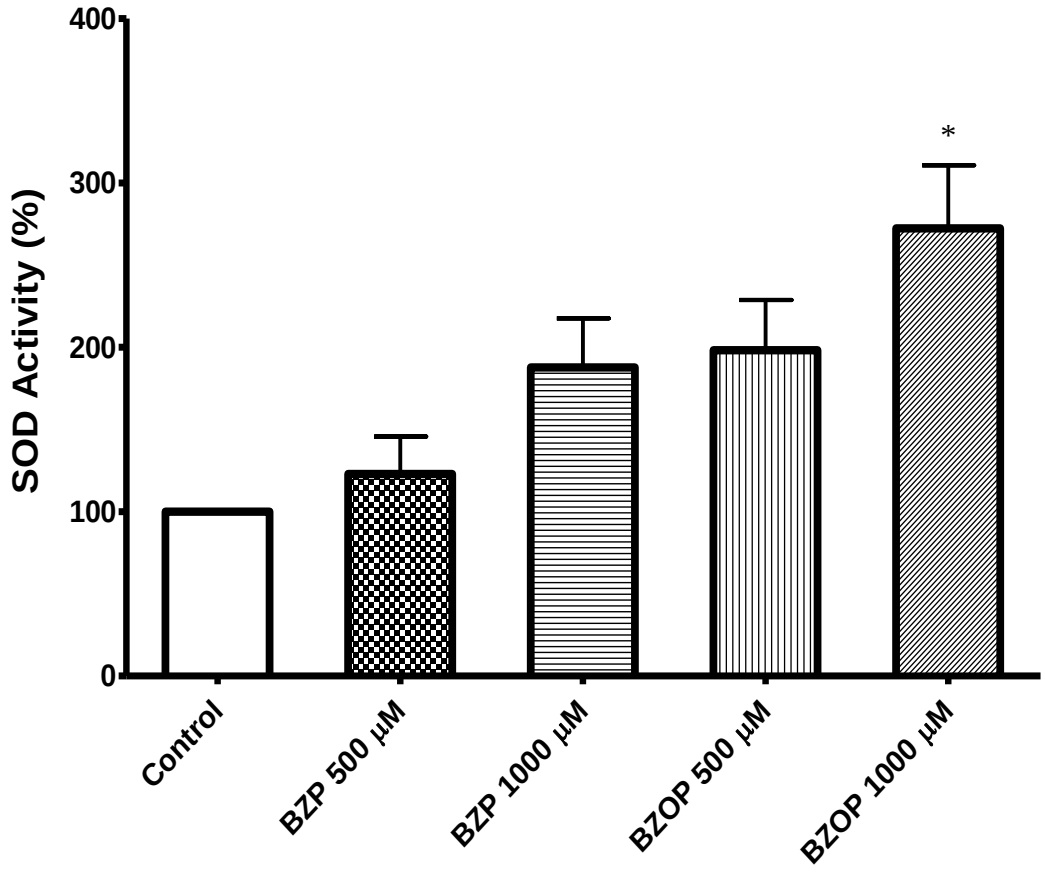

Figure 4.5. Effect of BZP and BZOP on superoxide dismutase activity in SH-SY5Y cells

BZOP (1000 µM) significantly and dose-dependently increased SOD activity (n=6, $p < 0.05$) in SH-SY5Y cells after 24 hours. Results are expressed as percentage control ± SEM. Statistical comparisons were made using one-way ANOVA/Dunnet's multiple comparison test. Note (*) indicates a statistically significant difference when compared to controls.

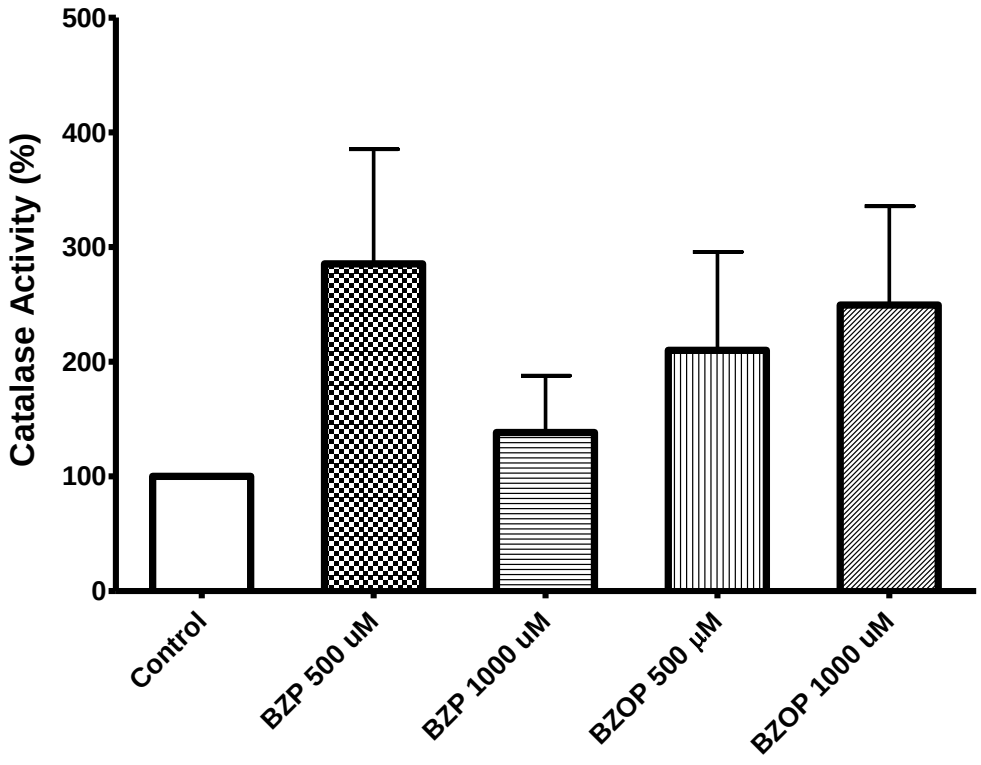

Figure 4.6. Effect of BZP and BZOP on catalase activity in SH-SY5Y cells

BZP and BZOP increased catalase activity (n=6, $p < 0.05$) in SH-SY5Y cells after 24 hours. Results are expressed as percentage control ± SEM. Statistical comparisons were made using one-way ANOVA/Dunnet's multiple comparison test.

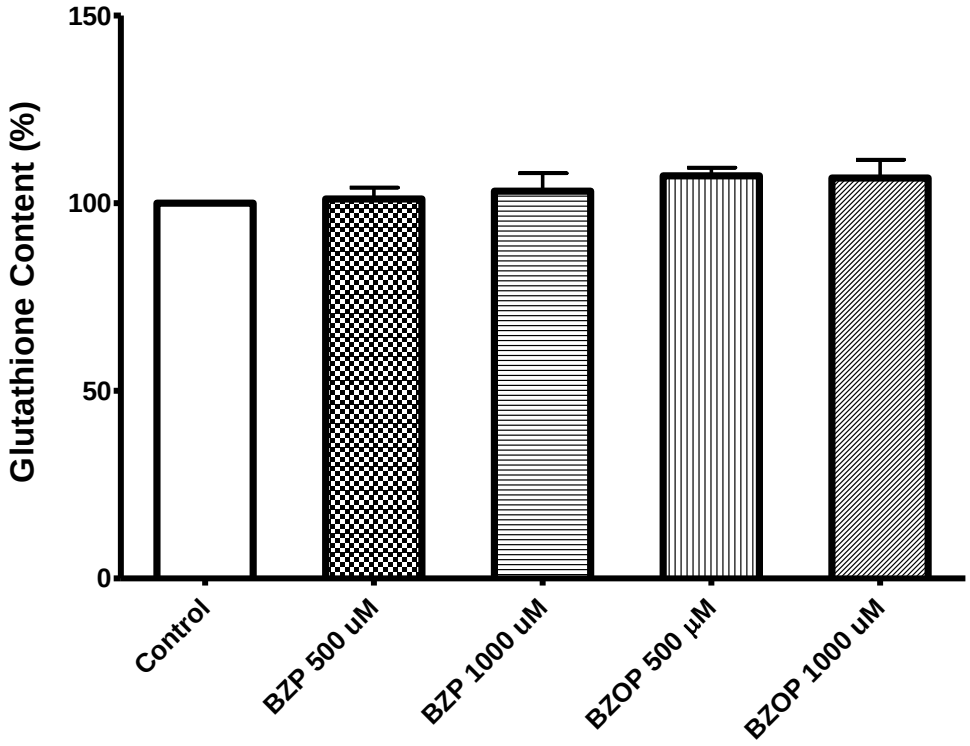

Figure 4.7. Effect of BZP and BZOP on glutathione content in SH-SY5Y cells

GSH content did not significantly change in drug treated cells when compared to control (N=6, $p < 0.05$) after 24 hours. Results are expressed as percentage control ± SEM. Statistical comparisons were made using one-way ANOVA/Dunnet's multiple comparison test.

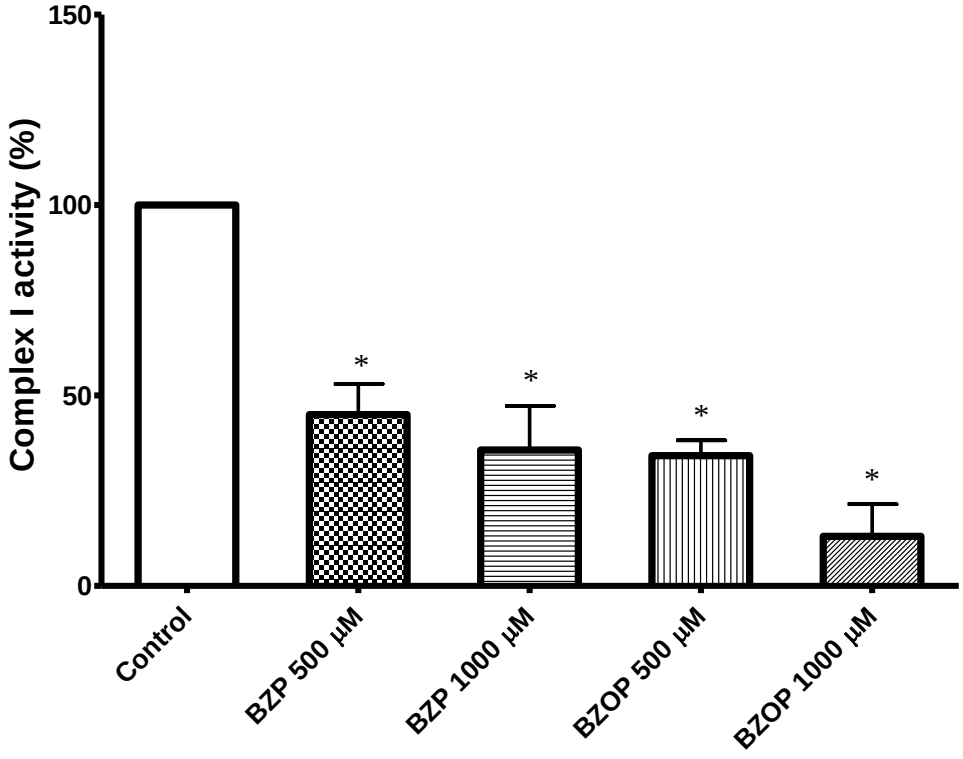

Figure 4.8. Effect of BZP and BZOP on complex I activity in SH-SY5Y cells

BZP and BZOP significantly and dose-dependently decreased complex I activity (n=6, $p < 0.05$) in SH-SY5Y cells after 24 hours. BZP and BZOP exhibit neurotoxicity by inhibiting mitochondrial respiration and generating superoxide dismutase. Mitochondrial complex-1 activity was measured spectrophotometrically. Results are expressed as percentage control ± SEM. Statistical comparisons were made using one-way ANOVA/Dunnet's multiple comparison test. Note (*) indicates a statistically significant difference when compared to controls.

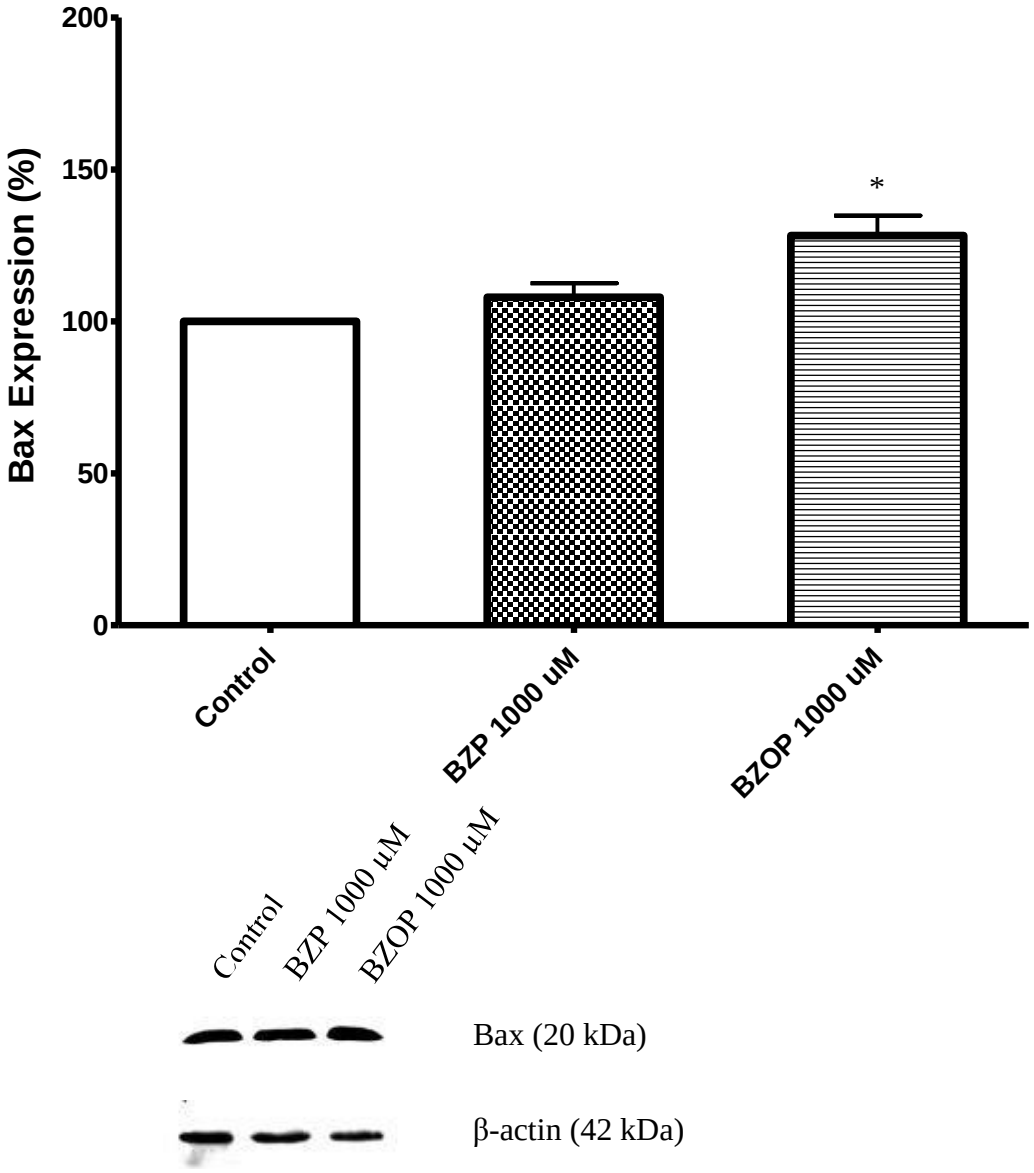

Figure 4.9. Expression of Bax in BZP and BZOP-treated SH-SY5Y cells BZOP (1,000 µM) significantly increased Bax expression in SH-SY5Y cells after 24 hours. Blots were developed using 1:1,000 dilution for Bax. β-actin (1:1,000) was used as a loading control. Densitometric Analysis was performed with AlphaView software and results are expressed as percentage control ± SEM. Statistical comparisons were made using one-way ANOVA/Dunnet's multiple comparison test (N=3, $p<0.05$). Note (*) indicates a statistically significant difference when compared to controls.

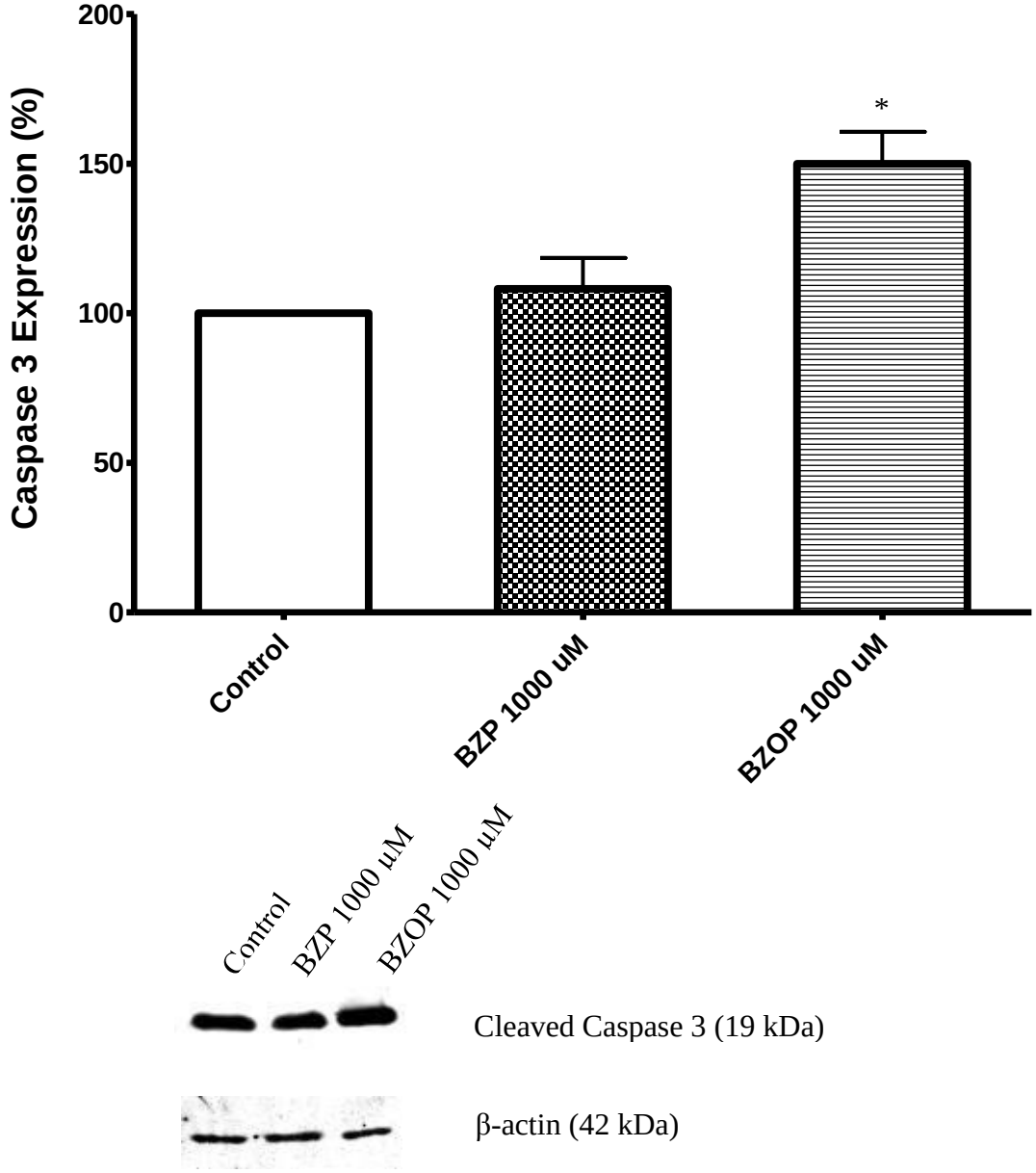

Figure 4.10. Expression of Cleaved Caspase 3 in BZP and BZOP-treated SH-SY5Y cells

BZOP (1,000 µM) significantly increased cleaved caspase 3 expression in SH-SY5Y cells after 24 hours. Blots were developed using 1:1,000 dilution for caspase 3. β-actin (1:1,000) was used as a loading control. Densitometric Analysis was performed with AlphaView software and results are expressed as percentage control ± SEM. Statistical comparisons were made using one-way ANOVA/Dunnet's multiple comparison test (N=3, $p<0.05$). Note (*) indicates a statistically significant difference when compared to controls.

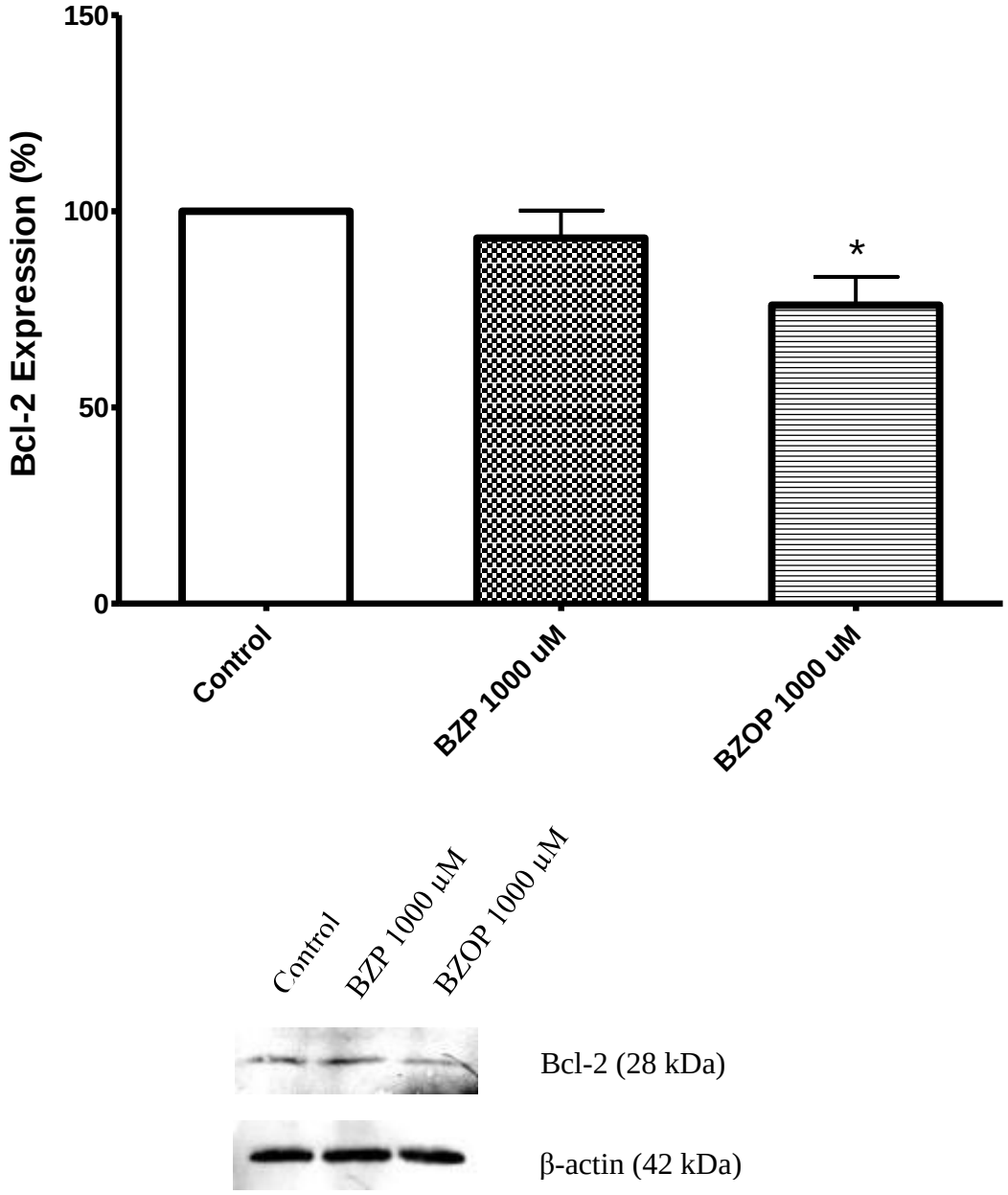

Figure 4.11. Expression of Bcl-2 in BZP and BZOP-treated SH-SY5Y cells

BZOP (1,000 µM) significantly decreased Bcl-2 expression in SH-SY5Y cells after 24 hours. Blots were developed using 1:1,000 dilution for Bcl-2. β-actin (1:1,000) was used as a loading control. Densitometric Analysis was performed with AlphaView software and results are expressed as percentage control ± SEM. Statistical comparisons were made using one-way ANOVA/Dunnet's multiple comparison test (N=3, $p<0.05$). Note (*) indicates a statistically significant difference when compared to controls.

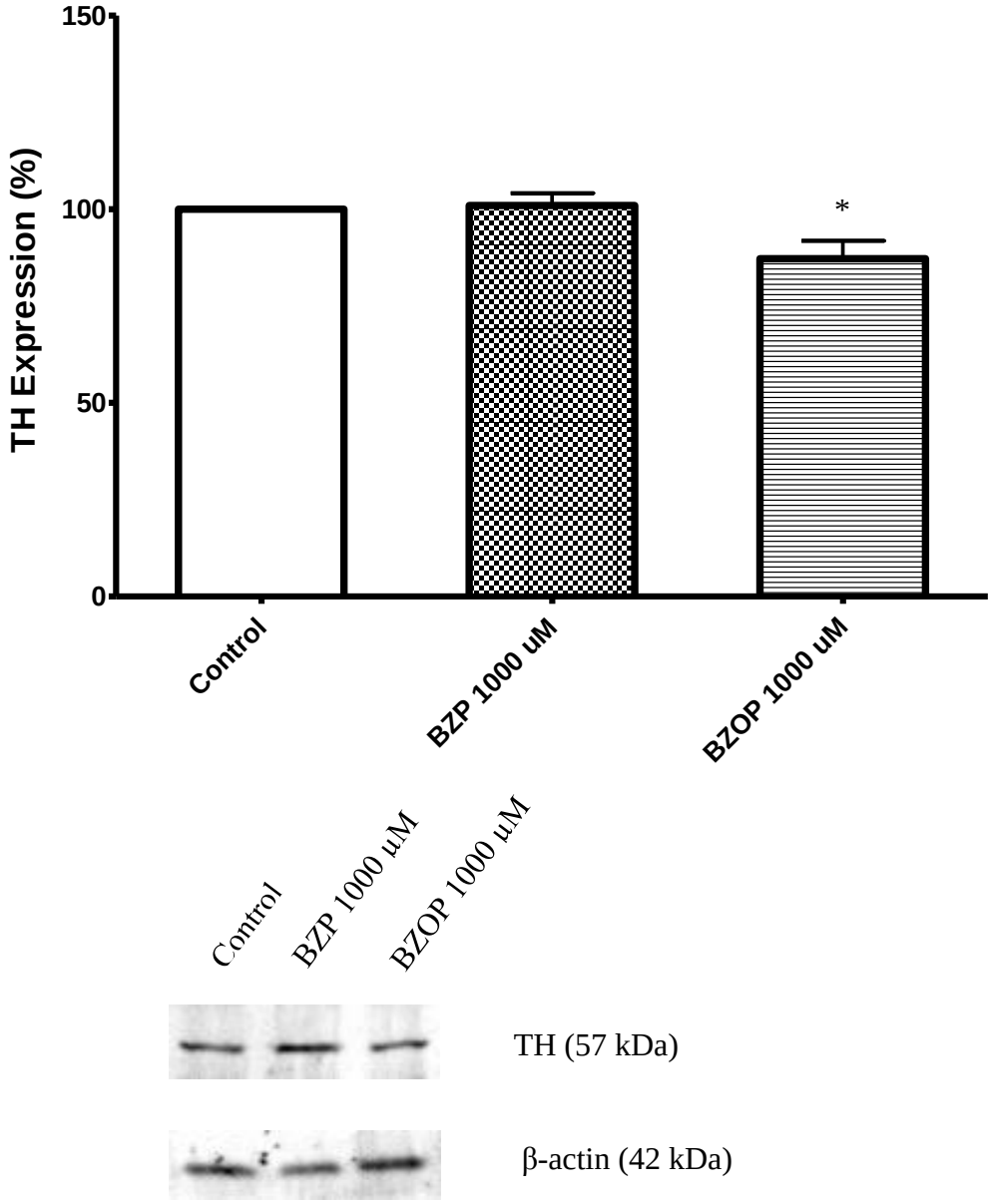

Figure 4.12. Expression of Tyrosine Hydroxylase in BZP and BZOP-treated SH-SY5Y cells

BZOP (1,000 µM) decreased tyrosine hydroxylase expression in SH-SY5Y cells after 24 hours. Blots were developed using 1:1,000 dilution for TH. β-actin (1:1,000) was used as a loading control. Densitometric Analysis was performed with AlphaView software and results are expressed as percentage control ± SEM. Statistical comparisons were made using one-way ANOVA/Dunnet's multiple comparison test (N=3, $p<0.05$).

5. Discussion

The principle pathways leading to cellular neurotoxicity include oxidative stress, mitochondrial dysfunction, apoptosis, inflammation, excitotoxicity and necrosis (Mattson, 2000). Prior studies utilizing 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Dhanasekaran et al., 2006), methylenedioxymethamphetamine (MDMA) (Karuppagounder et al., 2014), methamphetamine (Thrash et al., 2010), paraquat (Thrash et al., 2007) and corexit (Zheng et al., 2014) have evaluated the roles oxidative stress, mitochondrial dysfunction and apoptosis play in neurotoxicity. Recent toxicological investigations suggest that BZP is highly cardio, nephro and hepatotoxic in heart, kidney and liver cell lines, respectively (Arbo et al., 2012, 2014, 2015; Monteiro et al., 2013). Similarly, we demonstrate the in vitro neurotoxic effects of BZP and BZOP in SH-SY5Y cells, a human neuroblastoma cell line. SH-SY5Y cells are highly dopaminergic (but also cholinergic, glutamatergic and adenisonergic) and suitable for assessing the cytotoxicity of stimulants. These cells express a number of dopaminergic neuronal markers including tyrosine hydroxylase, the dopamine transporter (DAT) and dopamine receptor subtypes (D2R and D3R); therefore, they provide an exemplary in vitro model to study neurotoxicity elicited by drugs of abuse. Also, cancerous cell lines are ideal for testing the potential toxicity of new drugs and chemicals. First, they are highly-specialized cells that continuously grow by division. Second, due to high turnover rate, these cells are more susceptible to toxin-induced cellular damage, thereby providing a

sensitive, rapid and accurate model for assessing cellular toxicity (Ekwall et al., 1990). Cancer cell lines have previously been used to evaluate the cytotoxic effects of other drugs of abuse, including amphetamines (Dias-da-Silva et al., 2015; Wu et al., 2007).

Under physiological conditions, there is a delicately maintained balance between pro-oxidants (ROS and RNS) and antioxidants (glutathione, catalase and superoxide dismutase) to prevent "oxidative stress". Antioxidants combat the noxious effects of toxic radicals and prevent cell death. Exposure to atmospheric pollutants, cigarette smoking, ultraviolet rays, radiation, toxic chemicals or advanced glycation end products introduces an imbalance of homeostatic mechanisms shifting toward a pro-oxidative state. Hydroxyl ($OH^{\cdot}$), alkoxyl ($RO^{\cdot}$) and peroxyl ($ROO^{\cdot}$) radicals are short lived diffusible species, while superoxide ($O_2^{\cdot}$) and nitroxyl ($NO^{\cdot}$) radicals have medium lifespans. The non-radicals hydrogen peroxide (H_2O_2), organic hydroperoxides (ROOH) and hypochlorous acid (HClO) have relatively longer lifespans as compared to other ROS. The neurotoxins paraquat (Thrash et al., 2007) and MPTP (Muralikrishnan & Mohanakumar, 1998) induced significant reactive oxygen species generation. Also, MDMA (Colado et al., 1997), MDMA-analogues (Karuppagounder et al., 2014) and methamphetamine (Abane & Mezger, 2010; Giovanni et al., 1995; Fleckenstein et al., 1997; Thrash et al., 2010; Yamamoto & Zhu, 1998) have shown increased free radical production. Stimulants release dopamine by reversing dopamine transporter flux. Dopamine can undergo auto-oxidation, via the Fenton reaction using iron as a co-factor, producing ROS (Graham, 1978). Intracellular dopamine can also be oxidized to quinones and semiquinones (Graham, 1978; Kalyanaraman et al., 1985) and transformed, by redox cycling, into superoxide and nitrogen radicals resulting in further oxidative stress and

damage to proteins, lipids, nucleic acids, and membrane components (Fisher & Gutierrez, 1991; Halliwell & Gutteridge, 1985). Wu et al. observed increases of intracellular ROS levels in methamphetamine-treated SH-SY5Y cells. 48 hours post-methamphetamine exposure, treated cells exhibited a 2.5-fold increase in ROS generation when compared to controls (Wu et al., 2007). BZP, significantly and dose-dependently, increased the formation of ROS in primary rat hepatocytes after a 24 hour incubation (Arbo et al., 2015). Likewise, we show that the piperazine derivatives BZP and BZOP induced reactive oxygen species generation in SH-SY5Y cells.

Lipid peroxidation is initiated by a hydrogen abstraction, in which free radicals attack methylene bridges on polyunsaturated fatty acids, effectively "stealing" a single electron forming a carbon-centered radical. Molecular oxygen reacts with the lipid radical to form a lipid peroxyl radical. The lipid peroxyl radical is then capable of abstracting a hydrogen from another polyunsaturated fatty acid forming another lipid radical, resulting in a chain reaction. The lipid hydroperoxide is the first stable product that degrades over time to aldehydes and ketones. Lipid peroxidation is terminated when the concentration of radical species is high enough for there to be a high probability for collision of two radicals to produce a non-radical species. Chain-breaking antioxidants, such as vitamin E and superoxide dismutase can also cause termination (Halliwell & Gutteridge, 1984). The presence of ROS is imperative for lipid peroxidation and a clear indicator of cellular membrane damage. Excessive hydrogen peroxide formation will result in rapid degradation to hydroxyl radicals. The harmful hydroxyl radical attacks cellular membranes (mitochondria, microsomes, peroxisomes and plasma membrane) and initiates lipid peroxidation that results in DNA cleavage and protein degradation. Lipid

peroxides inflict damage on the mitochondrial membrane, altering the membrane potential established by the electron transport chain and diminishing the formation of ATP. Perforation of the mitochondrial membrane can also cause leakage of cytochrome-c, a critical initiator of apoptosis. Cells treated with BZOP (1,000 uM) displayed considerable lipid peroxidation suggesting degradation of the cellular membranes and subsequent cell death.

Elevated superoxide anion levels, due to complex-I inhibition, causes an increase in SOD activity. Glutathione peroxidase (GPx) activity has been proven to increase with elevated SOD activity, an adaptive response (Ceballos et al., 1988). A study in a Down's syndrome fetal brain has shown that elevated SOD activity does not always increase with GPx activity, accompanied by lipid peroxidation (Brooksbank et al., 1984). Acute and chronic repeated administration of methamphetamine increased SOD activity and TBARS levels in the striatum of rats, but GPx activity was not affected (Acikgoz et al., 1998). Interestingly, we showed similar increases in TBARS levels and SOD activity with no change in glutathione content upon BZP and BZOP exposure in a human neuroblastoma cell line.

The electron transport chain provides eukaryotes with ATP by means of oxidative phosphorylation and this occurs in the inner mitochondrial membrane. Mitochondrial complex-I inhibitors, such as rotenone (Ferrante et al., 1997) and MPP+ (Nicklas et al., 1985), prevent the transfer of electrons from iron-sulphur clusters of NADH dehydrogenase to ubiquinone (coenzyme Q). A pool of electrons is created within the mitochondrial matrix (approximately 1-5% of electrons in the ETC) leading to reduction of molecular oxygen, the final electron acceptor, and the generation of superoxide

radicals. Superoxide radicals readily react with protons to form hydrogen peroxide and hydroxyl radicals. Under basal physiological conditions, the principle source of intracellular reactive oxygen species originates from the mitochondria (Chance et al., 1979). Complex-I inhibition also prevents the buildup of hydronium ions (from the matrix to the intermembrane space) that produce a pH gradient to power ATP synthase. Loss of the mitochondrial membrane potential ($\Delta\psi m$) will interfere with oxidative phosphorylation, diminishing the cell of energy, causing cell death. Decreased activity of complex-I leads to dysfunction of the mitochondria, generation of ROS (Ide et al., 1999) and the induction of apoptosis (Hartley et al., 1994). Mitochondrial complex-1 inhibition is a key player in Parkinson's disease pathogenesis (Schapira et al., 1990), as levels of coenzyme Q_{10} were found to be reduced in Parkinson's disease patients (Thrash et al., 2010). Rotenone and MPP^+ induce apoptosis of nigral dopaminergic neurons (Greenamyre et al., 2001). Methamphetamine exhibited complex-I activity deficits and in turn raised ROS levels (Thrash et al., 2010; Burrows et al., 2000). Also, MDMA-analogs dose dependently inhibited mitochondrial complex-I activity (Karuppagounder et al., 2014). Dias-da-Silva et al. reveal significant depolarization of the mitochondria (loss of $\Delta\psi m$) and ATP depletion in primary rat hepatocytes exposed to BZP for 24 hours (Dias-da-Silva et al., 2015). Additionally, 1 and 2 mM doses of BZP induced 23% and 45% decreases in ATP levels when compared to controls in H9c2 rat cardiomyoblasts (Arbo et al., 2014). Our results show significant and dose-dependent decreases in complex-I activity in SH-SY5Y cells treated with BZP and BZOP, indicating extensive mitochondrial dysfunction and generation of ROS.

In the mitochondrial-mediated pathway, bax migrates to the surface of the mitochondria and inhibits Bcl-2 protective effects by perforating the mitochondrial membrane (loss of $\Delta\psi m$) and releasing cytochrome-c. Cytochrome-c and Apaf-1 (apoptotic protease activating factor-1) form apoptosomes (ATP-dependent) which bind to and activate caspase-9, an initiator (activator) caspase. Caspase-9 cleaves activating caspase-3, an effector (executioner) caspase. Caspase-3 executes apoptosis by creating an expanding cascade of proteolytic activity that digests structural proteins in the cytoplasm, degrades chromosomal DNA and neighboring phagocytosis of the cell. Methamphetamine was found to set off apoptosis in mouse brain neurons (Deng et al., 2001), immortalized rat striatal cells (Deng et al, 2002) and rat neocortical neurons (Stumm et al., 1999). In mouse neocortex, methamphetamine provoked an increase in the pro-apoptotic proteins BAX, BAD and BID, while the anti-apoptotic proteins Bcl-2 and Bcl-X_L decreased (Jayanthi et al., 2001). Jimenez et al. noted caspase activation by amphetamine derivatives in cerebellar granule cells (Jimenez et al., 2004). Also, Cocaine promotes apoptotic cell death in mouse cortical neurons (Nassogne et al., 1998). Caspase-3 activity significantly increased after 24 hour incubation of BZP in primary rat hepatocytes (Dias-da-silva et al., 2015). We assessed the expression of apoptotic markers (BAX, Caspase-3 and Bcl-2) in BZP and BZOP treated SH-SY5Y cells and found that BZOP significantly increased expression of BAX and Caspase-3, while decreasing expression of Bcl-2, similar to methamphetamine.

Dopamine plays a critical role in drug-induced neurotoxicity. Dopamine metabolism causes oxidative stress by generating reactive oxygen species in the form of hydrogen peroxide, hydroxyl radicals, semiquinones and superoxide anion. Experiments

that reduce dopamine production or block dopamine release have ameliorated the toxic effects of methamphetamine. Methamphetamine engenders the loss of dopaminergic and serotonergic neuronal markers including tyrosine and tryptophan hydroxylase (Thrash et al., 2009). The decrease in tyrosine hydroxylase expression in BZOP (1,000 μM) treated cells when compared to the BZP (1,000 μM) treated cells confirms that BZOP is more neurotoxic, as cells under insult lack the enzymatic machinery to synthesize the same levels of dopamine when compared to a healthy cell. Parkinson's disease can be considered a TH-deficiency syndrome of the striatum due to degeneration of dopaminergic neurons in the substantia nigra (Haavik & Toska, 1998).

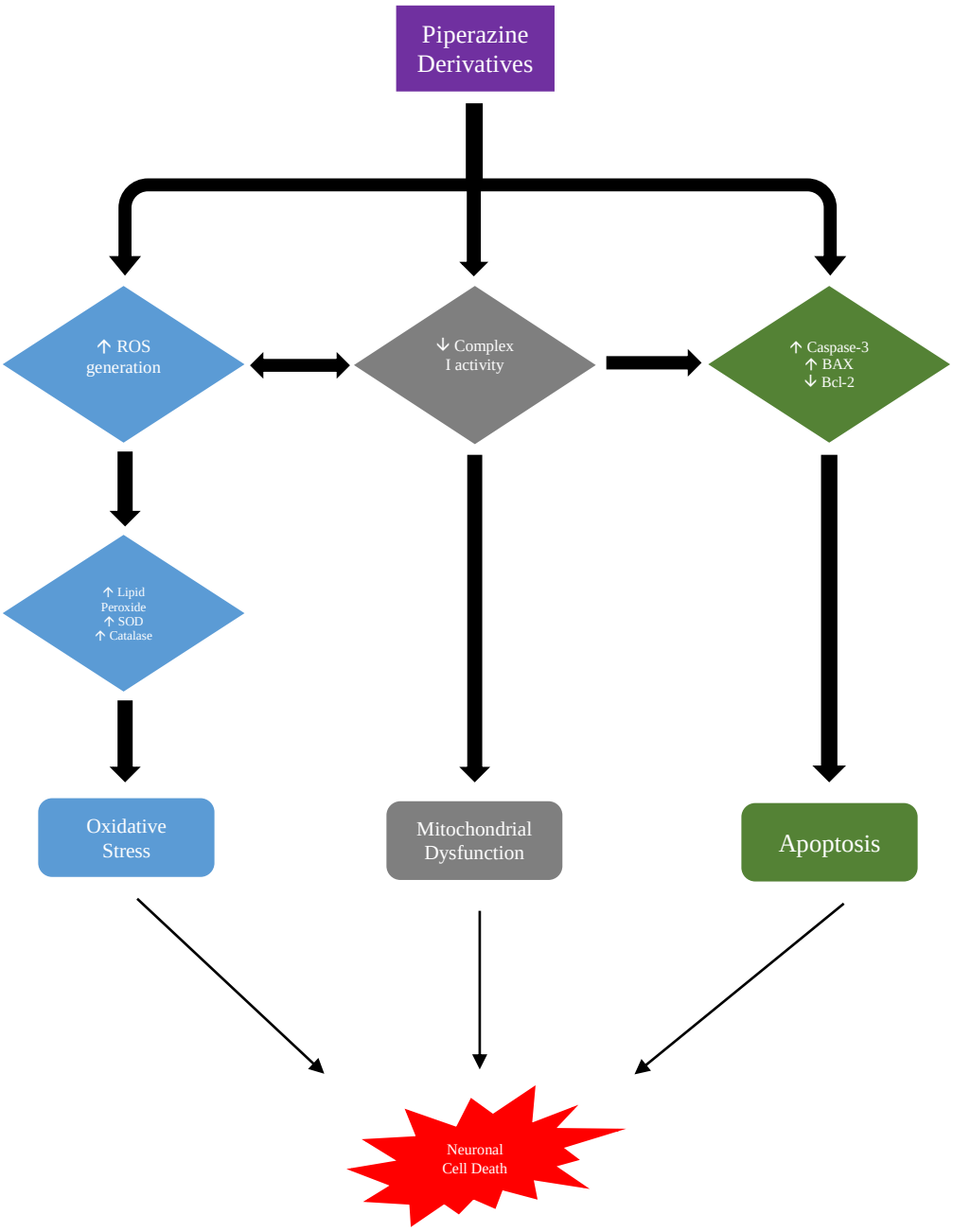

Figure 5.1. Schematic representation of Piperazine derivative induced neurotoxicity

6. Conclusion

We have demonstrated the *in vitro* neurotoxic effects of BZP and BZOP in human neuroblastoma cells, SH-SY5Y. The MTT cytotoxicity assay determined the doses at which a distinct difference in cell death occurred between the piperazine derivatives. Microscopic visualization confirms that cell death was most evident in BZOP-treated cells. We conclude that BZOP displays greater neuronal cytotoxicity due to more pronounced ROS generation, lipid peroxidation, complex-I inhibition, increased BAX and Caspase-3 expression, decreased Bcl-2 expression and decreased Tyrosine Hydroxylase expression. Piperazine derivatives induce oxidative stress, mitochondrial dysfunction and apoptosis (Figure 5.1.). A significant amount of interplay occur between these neurotoxic mechanisms, for instance, complex-I inhibition generates reactive oxygen species, which may contribute to the formation of lipid peroxides, causing cytochrome-c leakage from the mitochondria, and subsequent signaling for intrinsic apoptosis. Therefore, one must consider, in totality, all of the neurotoxic mechanisms contributing to cell death. Chronic use of piperazine derivatives can induce neurodegeneration of specific pathways in the brain, resulting in the development of movement disorders and cognitive deficits displayed in Parkinson's and Alzheimer's disease, respectively. Additional mechanisms of toxicity may contribute to the neuronal cell death observed in this study; including but not limited to neuroinflammation, excitotoxicity, impaired ubiquitination, lack of neurotrophic factors and necrosis. Animal

studies would logically follow in order to determine the toxic effects of piperazinic derivatives in a whole living organism in which organ systems contribute to the development of potential disease states. Blood brain barrier permeability and hepatic drug metabolism affect drug induced toxicity in the animal body. Designer drugs are synthesized by underground chemists to deliver greater potency to the user but often carry more deleterious side effects. The quick turnaround of designer drug development and subsequent banning by the DEA requires accelerated pharmacological and toxicological investigations so as to gain a better understanding of the toxic mechanisms involved in disease progression and for the development of appropriate pharmacotherapies that will alleviate harmful side effects and provide medical care.

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