Illuminating the Neurotoxic Effects of TFMPP derivatives in N27 Rat Dopaminergic Neuronal Cells through Oxidative Stress and Mitochondrial Dysfunction

by

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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 6, 2017

Keywords: designer drugs, N27 cell, oxidative stress,
mitochondrial dysfunction, neurotoxicity

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Abstract

The use of designer drugs in the United States have increased tremendously. Designer drugs are highly dangerous and most importantly the abusers don't have a clue with regard to what they are getting. Thus, it is currently casting a pall over the renaissance of scientific research into legitimate uses for psychedelic drugs. TFMPP derivatives are presently being abused and there are few reports on its neurotoxic effects. Our present study was to investigate the neurotoxic effects of the designer drug- Tri-Fluoro-Methyl-Phenyl-Piperazine derivatives (2, 3 and 4 TFMPP) in N27 dopaminergic cells. We assessed the neurotoxic effects using cell viability assay and morphological measures. Furthermore, the neurotoxic mechanisms were also elucidated. Effect of TFMPP derivatives were studied on the markers of oxidative stress and mitochondrial functions. Therefore, our results demonstrated that TFMPP derivatives (2, 3 and 4) dose-dependently induced neurotoxicity. Furthermore, they also induced oxidative stress and mitochondrial dysfunction which contributes to its neurotoxic and deleterious effects. Hence, there is an urgent need for additional studies about TFMPP derivatives to avoid the potential threat of increasing the risk for movement and mental disorders in the society.

Acknowledgements

First and above all, I praise Allah, the almighty for giving me this opportunity and granting me the ability to progress successfully. A several great people have provided assistance, support and guidance which made this thesis appear in its present form. Firstly, I would like to convey my truthful appreciation to my advisor Dr. Murali Dhanasekaran for his warm encouragement, thoughtful guidance and patience. He treated me as one of his family providing motivation and guidance whenever I needed. I could not have imagined having a better advisor for my postgraduate studies. In addition to my advisor, I would like to express my sincere thanks to the rest of my thesis committee: Dr. Randall Clark, Dr. Jack Deruiter and Dr. Vishnu Suppiramaniam for their valuable advices, support and encouragement. Moreover, I thank my lab mates for their great help, beneficial discussions as well as unlimited encouragement. I am also grateful to Saudi Arabian Cultural Mission for the financial support they provided me which made this possible. Additionally, I would like to thank my parents, my brothers and sisters for their unlimited love and support. And lastly, I would like to thank my lovely wife, Maali who moved to USA with me and lost her job because of that. Without her support and encouragement, I could not have achieved anything. Thanks for everyone for everything and may Allah give you the best in return.

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List of Abbreviations

5-HT	Serotonin
ACTH	Adrenocorticotropin
AVP	Arginine Vasopressin
BZP	N-benzylpiperazine
CAT	Catalase
CNS	Central Nervous System
CYP	Cytochrome P
DA	Dopamine
DCF-DA	2`, 7-Dichlorofluorescindiacetate
DMSO	Dimethylsulfoxide
FBS	Fetal Bovine Serum
GC/MS	Gas Chromatography/Mass Spectrometry
GSH	Glutathione
GSH-Px	Glutathione Peroxidase
H_2O_2	Hydrogen Peroxide
LC/MS	Liquid Chromatography/Mass Spectrometry
MAO	Monoamine Oxidase
mCPP	1-(3-chlorophenyl) piperazine
MDA	Malondialdehyde
MDBP	1-(3,4-methylenedioxybenzyl) piperazine
MDMA	Methylenedioxymethamphetamine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NA	Noradrenaline
NADH	Nicotinamide Adenine Dinucleotide
NO	Nitric Oxide

OPA	O-Phthalaldehyde
PBS	Phosphate Buffer Saline
PNS	Peripheral Nervous System
REM	Rapid-Eye-Movement
ROS	Reactive Oxygen Species
SCN	Suprachiasmatic Nucleus
SOD	Superoxide Dismutase
TBA	Thiobarbuturic Acid
TBARS	Thiobarbituric Acid-Reactive Substances
TCA	Trichloroacetic Acid
TFMPP	Trifluoromethylphenylpiperazine

1.literature Review

1.1. Introduction

According to the Oxford English Dictionary, the term “addict,” refers to “attached by one's own inclination, self-addicted to a practice; devoted, given, inclined to”. Campbell's psychiatric dictionary, describes addiction as “strong dependence, both physiologic and emotional”. The term addiction has been used since the first part of the 16th century (Crocq, 2007). Currently addiction is “state of being addicted to a substance / drug or action with a compulsion and need to continue”. Addiction replaced older terms, such as habituation and inebriety. In addition, exposure to a substance can rapidly evolve from normal consumption, to abuse and then resulting in dependence. Physiologically, substances of abuse generally act on the dopaminergic neuronal tract (mesolimbic system) and glutamatergic pathway in the prefrontal cortex to induce pleasure and dependence respectively. Addiction leading to dependence and abuse has been well documented for several centuries. In Roman law, during the middle ages, addiction was the sentence pronounced against an insolvent debtor who was given over to a master to repay his debts with his work. Thus, addictus was a person enslaved because of unpaid debts. The issue of loss of control of the substance, heralding today's concept of addiction, was already being discussed in the 17th century. Substances that can have stimulatory potential leading to addiction were exploited by clerics of various religion and cultures, shamans for healing purposes and the common person for socialization. In the 18th century, opium's addictive

potential was recognized when a large number of Chinese people became addicted, and the Chinese government tried to suppress its sale and use. In Europe, the working classes were threatened by alcoholism. Benjamin Rush, an American physician in the 18th century, observed that compulsive drinking was characterized by a loss of self-control, and that the disease was primarily attributable to the drink itself and not the drinker (Gerritsen, 2000).

Drug abuse has plagued the American continent since the 1800s, when morphine, heroin and cocaine were hailed for their curative properties. The New York State Inebriate Asylum was the first hospital intended to solely treat alcoholism as a mental health condition was founded in 1864. In the late nineteenth century, several changes have occurred regarding new and exotic drugs, such as hallucinogens, amphetamines and marijuana, became more readily available. By the mid-20th century, however, the authorities tried to reduce/eliminate use of the illicit drug nationally and globally. Thus, for several centuries, people all over the world have used various substances repeatedly for their personal pleasure. During this period, there were always those who abused them, which led to full-blown addiction and the bevy of side effects that come with it. The origin of addiction medicine in modern times is sometimes credited to Calvinist theologians who offered explanations for the phenomenon of compulsive drinking, which were later accepted by physicians. Industrial revolution, international trade, were one of the reasons addiction became a global public health problem (Hübner, 1988). Illegal drug traffickers were constantly looking for potent compounds and concoct faster routes of administration, which can contribute to very high levels of abuse. This ongoing vigorous search for new substances of abuse, psychoactive substances and recreational

drugs, resulted in the concept of modern “Designer drugs”. Designer drugs are usually synthetically prepared in the clandestine labs to elicit addictive effects comparable to banned or illegal addictive substances. These drugs are usually structural analogues of a known substance of abuse / controlled substance. Synthetic designer drugs such as piperazine derivatives, synthetic cathinones and substituted amphetamines resemble their parent molecule structurally and simulate its toxicological and pharmacological actions. Because of their comparable actions and structures to their parent molecule they may have similar pathways in producing neuronal cell death. Designer drugs cause neurotoxicity through oxidative stress, mitochondrial dysfunction and apoptosis. These mechanisms are important factors in the Pathogenesis of neurological diseases. Piperazine derivatives can cross the blood brain barrier quickly affecting the areas of the brain that is responsible of neurological disorders. Initially, designer drugs were not classified under the controlled substance. 3-Trifluoromethylphenylpiperazine (TFMPP) is a well-known designer drug that is being abused throughout the world. However, there are very few reports regarding the toxic effects and the possible therapeutic strategies to overcome the abuse potential and adverse effects associated with TFMPP-induced dependence. Hence, in this study we investigated the neurotoxic effect of TFMPP derivatives using dopaminergic cells and elucidated the neurotoxic mechanisms associated with neuronal cell death.

1.2. Piperazines

Piperazine is heterocyclic molecule that consist of two opposite nitrogens and four carbons disseminated among them (Figure1.1). Piperazine designer drugs can be classified into:

- ✓ The benzylpiperazines such as N-benzylpiperazine (BZP) and 1-(3,4-methylenedioxybenzyl) piperazine (MDBP).
- ✓ The phenylpiperazines such as 1-(3-chlorophenyl) piperazine (mCPP), 1-(3-trifluoromethylphenyl) piperazine (TFMPP) and 1-(4-methoxyphenyl) piperazine (Yeap, Bian, Fahmi, & Abdullah, 2010).

  Piperazine are known by different nicknames such as party pills, herbal/natural highs, A2, Legal X, Pep X, Frenzy or Nemesis when it is used as recreational drug (M. D. Arbo, Bastos, & Carmo, 2012). The most frequently abused piperazines are 1-benzylpiperazine (BZP) and 1-(3-trifluoromethylphenyl) piperazine (TFMPP) they were promoted as safe replacements to 3,4-methylenedioxymethamphetamine (MDMA) and amphetamines.

BZP and TFMPP are usually used in combination to gain synergistic effect. TFMPP primarily affects serotonin pathway and demonstrate variable affinity to the different Serotonin (5-HT) receptor subtypes. While BZP affects dopamine pathway.

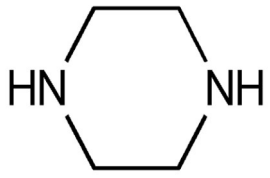


Figure 1.1. Piperazine chemical structure

1.3. Patterns of Use

Piperazine designer drugs can be consumed orally as capsule, tablet, pill, powder, or liquid form. Also, for quicker onset of action Piperazine designer drugs can be inhaled. However, because of its high alkalinity, abuser usually avoid to take it intravenously to avert the pain that it can cause (Gee, Richardson, Woltersdorf, & Moore, 2005).

Piperazine designer drugs are available as a pale, yellowish-green free-base or white hydrochloride salt (Drugs-forum, 2009; Figure 1.2.), its concentration within its different forms varies with range from 50 to 200 mg (Sheridan & Butler, 2007), while some pills can contain a dose up to 1,000 mg (Gee et al., 2008, 2005). (Sheridan & Butler, 2007) reported that New Zealand abusers administer 2 to 3 pills at the same time, with some heavy abuser that consume 8 pills or more. Piperazine Producers suggest consuming 2 pills, then consuming another 2 pills after 2 hours if the first pills where tolerated (Imogen Thompson et al., 2006).

Figure 1.2.: TFMPP powder

Party pills consumers usually take it with other psychostimulant such as alcohol, ecstasy, cannabis, amphetamines and nitrous oxide to gain synergistic effect besides its allow them

to consume more alcohol. Furthermore, taking party pills along with other psychostimulant can mask party pills negative effects and make it more bearable.

Piperazine designer drugs abusers may also combine it with other substances for instance caffeine, herbal extracts, electrolyte blends and amino acids. Overconsumption of piperazine designer drugs can led to dopamine diminution, therefore taking amino acid precursor of dopamine, L-tyrosine grants supplementary source of dopamine (Nikolova & Danchev, 2008; Sheridan & Butler, 2007).

1.4. Perception of Safety

Multiple factors led to the spread of piperazines use as substitution to amphetamine. These factors include psychostimulant activity, legal system weakness and untruthful reputation of safety, even though many studies have demonstrated that piperazines use has been associated with hepatotoxicity, cognitive disorders, mood disorders and abuse potential (Schep, Slaughter, Vale, Beasley, & Gee, 2011). It is believed that stimulant use can affect the ability of its user to normally response to stimulus or inhibitor (Curley et al., 2015). The potency of piperazines was undervalued among its consumers. In New Zealand, piperazines were largely accessible and socially accepted due to failure in legal system to list piperazines as illegitimate drug in addition to the false believe that because some of the piperazines was legal its quality and safety is guaranteed (Sheridan & Butler, 2010).

1.5. TFMPP

Trifluoromethylphenylpiperazine (TFMPP) is a scheduled I controlled substance of abuse which is a member of the piperazine chemical class of designer drugs. Piperazine designer

drugs emerged in the drug market for recreational purposes with psychoactive properties. Substituting different functional groups onto the basic piperazine structure creates derivatives such as benzylpiperazine (BZP or 1-benzylpiperazine) and trifluoromethylphenyl-piperazine (TFMPP). BZP is a benzyl substituted piperazine, while TFMPP is a substituted phenyl amine (Figure 1.3). TFMPP is most commonly consumed with benzylpiperazine or ecstasy to give psychostimulatory effects similar to illegal or banned drugs such as morphine, heroine, methamphetamine, MDMA, ecstasy. TFMPP has been associated with various street names including "X4" and numerous brand names relating to its availability as a perceived legal "Ecstasy" alternative (e.g. "PEP", "Twisted", "Flying Angel" and "Wicked High").

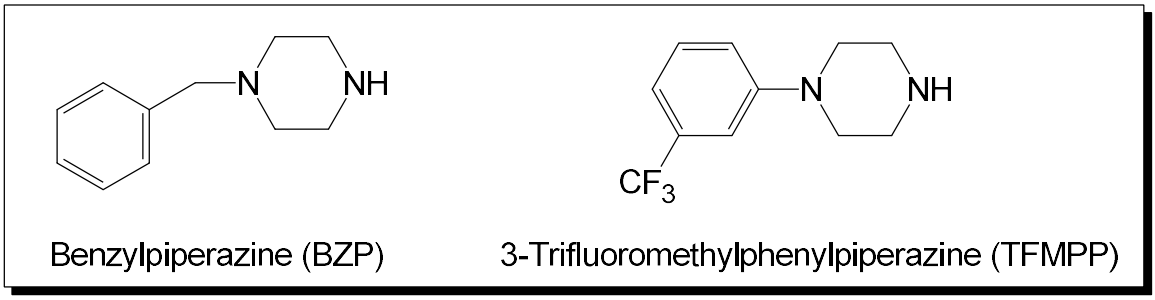


Figure 1.3. Chemical structure of BZP and 3-TFMPP

Initially, piperazine derivatives were designed to be used as anti-helminthic. In 1999, Japanese scientists discovered N-benzylpiperazine to stimulate the production of acetylcholine. Increased cholinergic neurotransmission in the Central Nervous System (CNS) is associated with enhanced learning and memory. This conceptual scientific intervention provided in part the rationale for the design and synthesis of Donepezil, a substituted piperidine derivative of BZP (Figure 1.4.) that inhibits acetylcholinesterase. Donepezil is the current first line of therapy in the treatment of Alzheimer's disease and

other age-related dementias, or brain diseases associated with progressive loss of memory, learning, and thinking ability.

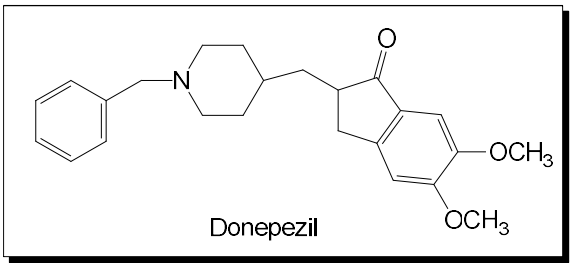


Figure 1.4. Chemical structure of Donepezil

In the 1970s, TFMPP was found to be a metabolite of antrafenine, an analgesic anti-inflammatory medicine comparable to naproxen. During metabolism studies, it was suggested that due to its serotonergic effects, TFMPP may be partly responsible for its activity (Figure 1.5.). However, while there have been a number of studies investigating the therapeutic potential of TFMPP as well as BZP, these drugs have not demonstrated significant efficacy or safety in the treatment of disease. Currently TFMPP is not listed on the WHO Model List of Essential Medicines and has never been marketed as a drug.

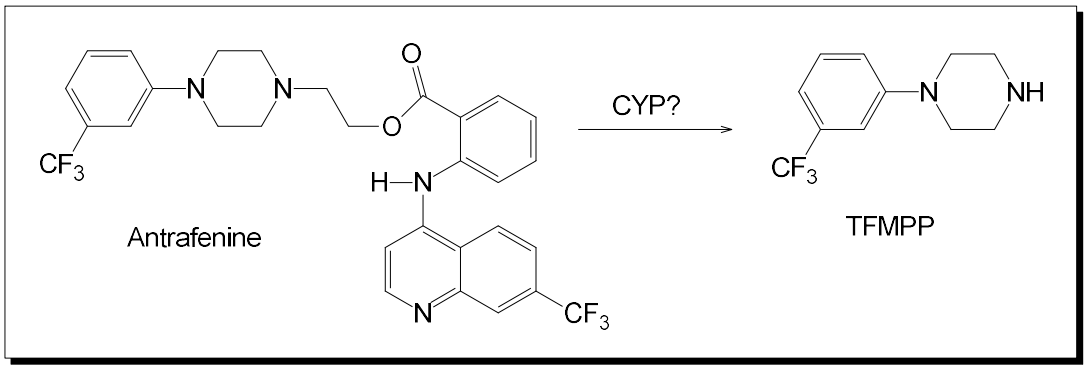


Figure 1.5. Metabolism of Antrafenine

In fact, there are serious adverse effects due to the consumption of TFMPP as described in the sections that follow. As of 2002, two deaths have reported due to toxic effects of

TFMPP and many more cases of non-fatal intoxication. In the United States, TFMPP was temporarily classified under Schedule I due to concerns about toxicity and abuse potential, along with the lack of clear medical application. However, in 2002, based on the scientific and medical evaluation conducted by the Food and Drug Administration (FDA) and the National Institute on Drug Abuse (NIDA), the Department of Health and Human Services (DHHS) did not recommend further control, leaving TFMPP as a federally uncontrolled substance after March of 2004. Since then there has been an escalation in the abuse of TFMPP in the United States as evidenced by the increasing encounters of this substance by law enforcement officials in various states. This prompted some states such as Florida that have banned the drug in their criminal statutes making its possession a felony. In New Zealand, BZP and TFMPP were initially classified under Schedule IV of the Misuse of Drugs Amendment Act 2005 (Amendment to the Misuse of Drugs Act 1975) as restricted compounds, available for legal sale to any person aged over 18 years. The sale of TFMPP is also controlled Canada, China, Denmark, Japan, Sweden, Belgium, Greece the United Kingdom and Australia. There are numerous studies over the past two decades that has revealed the abuse of TFMPP around the world (M. D. Arbo et al., 2012; C. Chen, Kostakis, Irvine, & White, 2013; de Boer et al., 2001; Elliott & Evans, 2014; Elliott & Smith, 2008; Gao, Qi, & Zhang, 2017; Maciów-Głąb, Rojek, Kula, & Kłys, n.d.; Maskell, Paoli, Seetohul, & Pounder, 2011; Poon, Lai, Lui, Chan, & Mak, 2010; Sheridan, Dong, Butler, & Barnes, 2013; Tang et al., 2015; Tscharke, Chen, Gerber, & White, 2016; Wilkins & Sweetsur, 2010; Wilkins, Sweetsur, & Girling, 2008; World Health Organization, 2012; Young et al., 2013; Zuba & Byrska, 2013). Prior to the abuse of TFMPP, it was used by

numerous scientists as a valid pharmacological tool for research purpose. TFMPP was used for the following research purposes:

- ✓ Evaluate the role of monoamines in Addiction (J. C. Lin, Jan, Lee, et al., 2011; J. C. Lin, Jan, Kydd, & Russell, 2011)

- ✓ Study of Aggressive actions (Oliver, Klocek, & Wells, 1995; Sánchez, Arnt, & Moltzen, 1996)
- ✓ Role of monoamines and hormones in Anorexia (Rowland, Marshall, & Roth, 2000; Rowland, Robertson, Lo, & Rema, 2001)

- ✓ Anxiogenic mechanisms (Tokumo, Tamura, Hirai, & Nishio, 2006)

- ✓ Establish the effect of monoamines on various General Behavior:
    - Chewing (Liminga, Johnson, Andrén, & Gunne, 1993; Stewart, Jenner, & Marsden, 1989)
    - Discriminative stimulus (Fantegrossi, Winger, Woods, Woolverton, & Coop, 2005; Yarosh, Katz, Coop, & Fantegrossi, 2007) Exploratory activities (Chojnacka-Wójcik, 1992; Maj et al., 1996)
    - Head twitch effects (Darmani, Martin, & Glennon, 1990; Vickers et al., 2001)
    - Learning abilities (Grant & Colombo, 1993; Herndon, Pierson, & Glennon, 1992; Kant et al., 1996)
    - Locomotory ability (movement) (Lucki, 1998)
    - Memory formation (Meneses, 2002)
    - Operant behavior (De Vry, Schreiber, Daschke, & Jentzsch, 2003; McKearney, 1990)

- Psychoactive behavior (Elliott & Evans, 2014)
- Social behavior (Frances, Monier, & Debray, 1994; Lucion, De Almeida, & De Marques, 1994)
- Stimulatory effect (Mørk & Geisler, 1990)

- ✓ Regulation of monoamine in Body temperature (Francis, Palmer, Snape, & Wilcock, 1999; Lecci et al., 1990)

- ✓ Role of serotonin in regulating Cardiovascular function

- ✓ Cell signaling pathway (Mørk & Geisler, 1990)

- ✓ Serotonergic mechanisms in Circadian rhythm (Pickard, Weber, Scott, Riberdy, & Rea, 1996)
- ✓ Influence of monoamine in Convulsion (Hernandez, Williams, & Dudek, 2002; Przegaliński, Baran, & Siwanowicz, 1994)

- ✓ Consequences of monoamines and hormones in Depression (Cohen, Fuller, & Kurz, 1983; Crick, Manuel, & Wallis, 1994)

- ✓ Understand Drug-Receptor ligand binding (Brown, Kilpatrick, Martin, & Spedding, 1988; McKenney & Glennon, 1986)

- ✓ Role of monoamine in Emesis (Schep et al., 2011; I Thompson et al., 2010)

- ✓ Establish Endocrine function (Glucagon, Glucose, Insulin, Neuropeptide-Y, Somatostatin, Androgen, pituitary hormone) (Di Sciullo et al., 1990; Rouru, Pesonen, Isaksson, Huupponen, & Koulu, 1993)

- ✓ Feeding behavior (Kennett, Whitton, Shah, & Curzon, 1989; Kitchener & Dourish, 1994)
- ✓ Hypersensitivity reactions (Roudebush & Bryant, 1993)
- ✓ Lordosis (Aiello-Zaldivar, Luine, & Frankfurt, 1992)
- ✓ Meiosis reinitiation (Krantic, Robitaille, & Quirion, 1992)
- ✓ Melatonin production (Rea & Pickard, 2000)
- ✓ Mechanisms involved in Neuronal firing (Heidenreich & Napier, 2000)
- ✓ Nociception mechanisms (Sawynok & Reid, 1996)
- ✓ Understand Pain pathway (J. C. Lin, Jan, Kydd, et al., 2011)
- ✓ Receptor stimulation / inhibition and its function (Waldmeier et al., 1988)
- ✓ Reflex responses (Robertson et al., 1992)
- ✓ Release of Neurotransmitters mechanisms (Lee et al., 2016)
- ✓ Respiratory function (Edwards, Whitaker-Azmitia, & Harkins, 1990; King & Holtman, 1990)
- ✓ Reward pathway (Curley, Kydd, Kirk, & Russell, 2013)
- ✓ Sexual Behavior (Hayes & Adaikan, 2002)

- ✓ Sleep wake cycle (Pastel & Fernstrom, 1987)
- ✓ Synaptic Neurotransmission (Matsumoto, Hussong, & Truong, 1995)
- ✓ Synthesis of Neurotransmitters (da Silva et al. 2017)

1.6. Pharmacokinetic effects of TFMPP

TFMPP is typically obtained in the form of a powder, tablet or capsule and the primary route of administration is oral as reported by users. However, there are also reports of the drug being "snorted" or smoked, which have been noted for BZP and other piperazines and even injected. It is presumed that smoking and parenteral administration (injection) delivers substances of abuse to the CNS more rapidly, resulting in addiction as compared to other routes such as swallowing, which deliver the drugs more slowly. With regard to TFMPP, the plasma concentrations following a single 60mg oral dose in humans peaked at 24ng/mL (Tmax = 90 minutes). TFMPP had two disposition phases with calculated half-lives of 2 hours and 6 hours, with Cl/F of 384 L/hour. A single plasma metabolite, 4-OH TFMPP (C Max = 20 ng/mL; Tmax = 90 min), was detected in this study (Ushtana Antia, Tingle, & Russell, 2010). Urinary metabolites included 4-OH TFMPP and an N-glucuronide of TFMPP, with some evidence of conjugates of 4-OH TFMPP (Ushtana Antia et al., 2010). A more detailed analysis of TFMPP metabolism is presented below.

TFMPP, due to its structure, readily crosses the blood brain barrier. A positive relationship has been reported between plasma drug concentrations and subjective ratings indicating

that TFMPP have concentration-dependent subjective effects (Ushtana Antia, Tingle, & Russell, 2009). These findings suggest that elevated concentrations of these drugs (due to compromised clearance or larger doses) may result in elevated effects on mood (U Antia, Lee, Kydd, Tingle, & Russell, 2009).

A study of the tissue distribution of BZP and TFMPP has also noted a significant difference in the extent of distribution of these drugs in the rat (Chou, 2008). The organ with the highest concentration of BZP was the kidneys with a concentration ratio between the plasma and kidneys of approximately 1:20, while the TFMPP concentration ratio between the plasma and the lungs (organ with the highest TFMPP concentration) has a ten-fold difference at approximately 1:200, thirty minutes after the dose. This study reported that the ratios of BZP and TFMPP between plasma and all other analyzed tissue (brain, liver, kidneys, lungs, heart) were 1:40 and 1:385 respectively, thirty minutes after the dose. Therefore, the presence of a more obvious distribution phase in the human plasma profile of TFMPP when compared to BZP is in agreement with tissue distribution data from the rat (Chou, 2008). As TFMPP does not persist in plasma for longer than 24 hours, these results also suggest that subjective effects of these drugs should last no longer than 24 hours at the given dose. However, it is important to note that the drug effects are not the same for every individual, with a minority demonstrating the opposite relationship between concentration and effect. Conversely, reports from animal studies have indicated that the subjective effects of these drugs are synergized when they are co-administered (Baumann et al., 2005). This suggests that the interaction resulting in synergism between these drugs occurs at a pharmacodynamic level. This further suggests that by combining BZP and TFMPP, the doses of each can be reduced without compromising the effect of the drugs

which may explain why, when these drugs are sold in combined drug preparations, the doses of each drug are routinely far lower than in the single drug preparations. Interestingly, combining TFMPP with caffeine also resulted in lethal consequences due to pharmacokinetic interactions of elevated caffeine concentrations (Holmgren, Nordén-Pettersson, & Ahlner, 2004; Kerrigan & Lindsey, 2005; Walsh, Wasserman, Mestad, & Lanman, 1987).

Initially, the metabolism of TFMPP in rodents provided insight into probable routes for their metabolism in humans (Staack, Fritschi, & Maurer, 2003). By administering inhibitors quinidine, furafylline and troleandomycin it was found that CYP2D6, CYP1A2 and CYP3A4 metabolize TFMPP. CYP2D6 poor metabolizers have compromised metabolism of TFMPP both *in vitro* and *in vivo*. CYP2D1 (the rat orthologue of human CYP2D6) was claimed to be the principal enzyme responsible for the metabolism of TFMPP, accounting for 80.9 % of TFMPP metabolism in rats. CYP1A2 and CYP3A4 also contributed to the metabolism, but to a lesser extent, 11.5% and 7.6 % respectively (Staack, Paul, Springer, Kraemer, & Maurer, 2004). It was proposed that TFMPP metabolism occurs via hydroxylation of the phenyl group and to a lesser extent, dealkylation of the piperazine ring. Subsequent degradation, acetylation and conjugation (glucuronation and sulfonation) can result in a number of metabolites (Staack et al., 2003). Later it was found that TFMPP is metabolized by CYP2D6, CYP1A2 and CYP3A4 in the human liver (U Antia et al., 2009). However, it these enzymes have different affinities for TFMPP and its structural congeners like BZP. The metabolism of TFMPP has been shown to be diminished by the presence of the inhibitors of these enzymes and other substrates. An

important concern is that of compromised metabolism of TFMPP in 'poor metabolizers' can have a serious clinical interaction with antidepressants (paroxetine), atypical antipsychotics (olanzapine) and Antiepileptics (Carbamazepine). Previous studies with male Wistar rats (WI) had shown that TFMPP was metabolized mainly by aromatic hydroxylation. In another study, the role of CYP2D6 on TFMPP was examined. These investigators measured and compared TFMPP vs. hydroxy TFMPP ratios in urine from female Dark Agouti rats. Dark Agouti rats are a well-accepted animal model to study the CYP metabolism of the human. Male Dark Agouti rats are of the poor CYP2D6 metabolizer phenotype (PM) and WI is a model of the human CYP2D6 extensive metabolizer phenotype. Analysis of the plasma samples showed that female Dark Agouti rats exhibited significantly higher TFMPP plasma levels compared to those of male Dark Agouti rats and WI. Furthermore, pretreatment of WI with the CYP2D inhibitor quinine resulted in significantly higher TFMPP plasma levels (Peters, Schaefer, Staack, Kraemer, & Maurer, 2003; Staack et al., 2003, 2004; Staack & Maurer, 2005). The identified metabolites indicated that TFMPP was extensively metabolized, mainly by hydroxylation of the aromatic ring and by degradation of the piperazine moiety to N-(3-trifluoromethylphenyl) ethylenediamine, N-(hydroxy-3-trifluoromethylphenyl) ethylenediamine, 3-trifluoromethylaniline, and hydroxy-3-trifluoromethylaniline (Figure 1.6.). Phase II reactions included glucuronidation, sulfatation and acetylation of phase I metabolites (Staack & Maurer, 2005). Furthermore, the human hepatic CYPs involved in TFMPP hydroxylation were identified using cDNA-expressed CYPs and human liver microsomes. The urine studies suggested that TFMPP hydroxylation might be catalyzed by CYP2D6 in humans. Studies using human CYPs showed that CYP1A2, CYP2D6 and

CYP3A4 catalyzed TFMPP hydroxylation, with CYP2D6 being the most important enzyme accounting for about 81% of the net intrinsic clearance, calculated using the relative activity factor approach. The hydroxylation was significantly inhibited by quinidine (77%) and metabolite formation in poor metabolizer genotype human liver microsomes was significantly lower (63%) compared to pooled human liver microsomes.

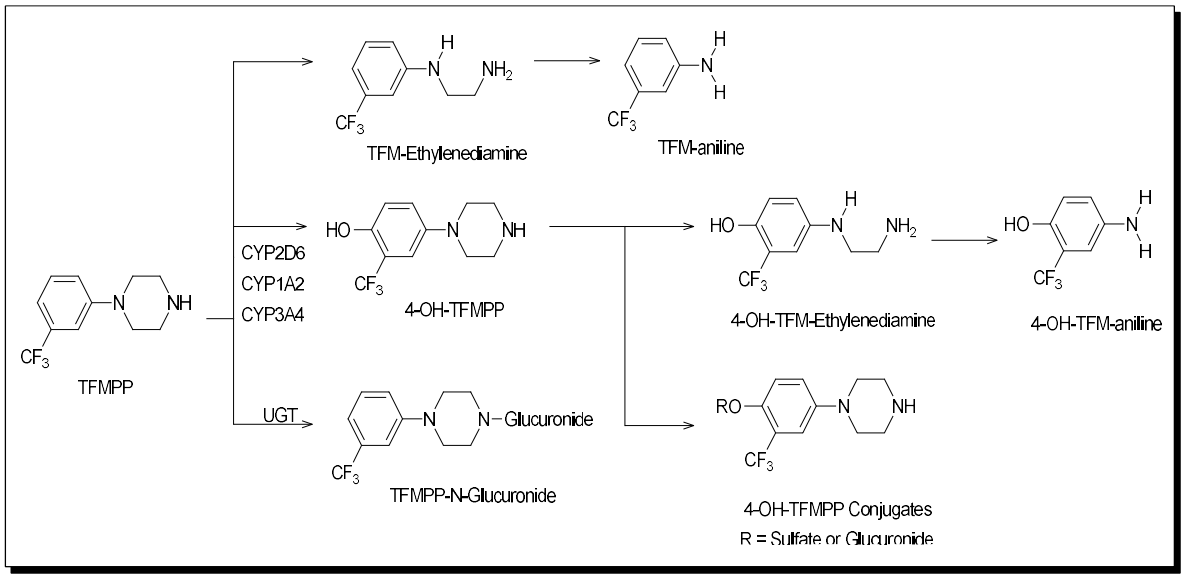


Figure 1.6. Metabolism of TFMPP

1.7. Pharmacodynamic effects of TFMPP

The pharmacodynamic effects of TFMPP result from its effects on monoaminergic neurotransmitters in particular serotonin (5-HT). TFMPP also affects other monoaminergic neurotransmitters dopamine (DA), and noradrenaline (NA). TFMPP has significant affinity towards 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, and 5-HT_{2C} receptors. TFMPP exhibits agonistic activity (binds with intrinsic effect) at all sites except the 5-HT_{2A} receptor, where it acts as a weak partial agonist or antagonist. Interestingly, TFMPP has insignificant affinity for the 5-HT_3 receptor (Baumann et al., 2005; Robertson et al., 1992).

Since the receptors for monoaminergic neurotransmitters are present in both the central and peripheral nervous system (PNS), TFMPP can have significant effects in both brain and periphery. The primary mechanisms associated with increased serotonergic neurotransmission may be due to its ability to bind with post-synaptic serotonergic receptors resulting in agonist activity (Fuller, Snoddy, Mason, Hemrick-Luecke, & Clemens, 1981; Toomey, Horng, Hemrick-Luecke, & Fuller, 1981) even though TFMPP exhibits both agonists and antagonistic effects on the serotonin receptors. In the CNS, it acts as a 5-HT agonist that results in neuroendocrine action, behavioral and serotonin turnover effects but in the periphery it exhibited potent antagonistic effect leading to serotonin-induced contraction of the jugular vein (Hashimoto et al., 1982). Similar to TFMPP, quipazine (a piperazine derivative) and Org 10155 also exhibited 5-HT agonistic activity and were sensitive to calcium entry blockade (Cohen et al., 1983). In addition to the effect on postsynaptic 5-HT receptors, TFMPP also can enhance the release of serotonin. When tested on rodent hypothalamic slices (*in vitro*), piperazines can induce a significant release of 5-HT, and this effect must be taken into account for their serotonergic pharmacological action in addition to its direct agonist activity when understanding the *in vivo* CNS effects of TFMPP (Brady & Barrett, 1985; Glennon, Titeler, & McKenney, 1984; Pettibone & Williams, 1984). With regard to the serotonergic release, the action may be attributed to the effect of TFMPP on 5-HT1 and 5-HT1b receptors (Cunningham & Appel, 1986; Dabire, Cherqui, Fournier, & Schmitt, 1987; Glennon, Pierson, & McKenney, 1988; Kennett et al., 1989; McKenney & Glennon, 1986; Murakami, Sano, Tsukimura, & Yamazaki, 1988; Pastel & Fernstrom, 1987; Sprouse & Aghajanian, 1987). The effect of TFMPP of serotonergic neurotransmission translates towards many actions in the body.

Due to the effect on 5-HT neurotransmission, it induces hallucination, psychotropic, anxiogenic, nociceptic effect, hypothermia, and affects rapid-eye-movement (REM) sleep, exploratory activity and release of other neurotransmitters.

Hallucinogenic effects of TFMPP may be due to its effect on serotonergic receptor (Titeler, Lyon, Davis, & Glennon, 1987). In general, the hallucinogenic effects are more prominent due to the stimulation of 5-HT2 receptor. With regard to the effect of TFMPP on rapid-eye-movement, it has shown that it causes suppression of rapid-eye-movement (REM) sleep. TFMPP (single injection) in rodents induced a substantial, dose dependent short-term (4-5h) suppression of rapid-eye-movement (REM) sleep. TFMPP augmented non-REM (NREM) sleep during the second hour. This study further confirms that suppression REM sleep is due to its effect on the central serotonergic neurotransmission (Pastel & Fernstrom, 1987). Hypothermia is action because it can result in a health emergency due to the fall in the body temperature below 95^{o}F (35^{o}C). In hypothermia, body the temperature drops faster than the body cannot than generate heat, leading to decreased body temperature. TFMPPP induces hypothermia in rats by binding at 5HT1b receptors (Maj, Chojnacka-Wójcik, Kłodzińska, Dereń, & Moryl, 1988). Interestingly TFMPP's ability to cause hypothermia was confirmed by another study where it they also showed that a lower dose of TFMPP evoked a hyperthermic and the higher a hypothermic response (Lecci et al., 1990). Due to its effect on 5HT1b receptors, TFMPP also possess psychotropic activity similar to imipramine and its derivatives (Frances, 1988). Nociception occurs due to the activation of nociceptors that leads to the processing of information about the internal or external environment in the peripheral and central nervous system. An injury can stimulate

nociceptors that are present in the periphery that triggers signals to the spinal cord dorsal horn or its trigeminal homologue, the nucleus caudalis. TFMPP due to its serotonergic stimulatory effect has anti-nociceptive effects (McKearney, 1989).

Exploratory behavior of laboratory rodents is of significance to understand the behavioral pharmacology of humans. A rodent introduced to an unfamiliar settings or entity displays behavioral changes that is referred to as exploration. The exploratory activity can be referred to the movement around an environment, positioning/adjusting towards a novel object, exploring (touching or sniffing) a new and /or novel objects (Berlyne, 1960; Glickman & Sroges, 1966; Welker, Benjamin, Miles, & Woolsey, 1957). The exploratory activity offers innovative evidence about various behavioral activities associated with feeding, accommodations and sexual activities. Introducing an animal to a new environment or exposing to a novel stimulus, escalates its risk of predation, aggression from conspecifics or other hazards (Greenberg, 2003; MONTGOMERY & MONKMAN, 1955). Neophilia and neophobia are behavioral concepts that explain the curiosity-based approach to, and fear-based avoidance of, a novel stimulus (Hughes, 2007). Neophilia is the attraction exhibited by an animal towards a novel object by an animal while neophobia is the act of displaying aversion (Greenberg, 2003). Neophilia is related to the neuronal functions associated with the rewarding effects of addiction / abuse (Bardo, Donohew, & Harrington, 1996). The most regularly used behavioral tests associated with exploratory behavior are the open field (Crawley, 1985; Gharbawie & Whishaw, 2006; Hall, 1934). The pharmacological mechanism associated with the exploratory activity in this model is due to the activation of 5-HT1C, or 5-HT1B, receptors (Lucki, 1998). TFMPP and m-

CPP-induce a decrease in the exploratory activity (Kłodzińska, Jaros, Chojnacka-Wójcik, & Maj, 1989). TFMPP also decreased the total interaction time in a rat social interaction test. The total social interactions test takes into the consideration the following behavior: grooming, following, crawling over, fighting and sniffing. The results from the study reveal that TFMPP has an anxiogenic effect without the sedative action (Kennett et al., 1989). There was another study that confirmed the above behavioral effect where TFMPP-increased conditioned avoidance response and this action was also attributed to the action on the 5HT1C/5-HT2 receptors (Alhaider, Ageel, & Ginawi, 1993).

Serotonin has shown to affect the release of other neurotransmitters including norepinephrine, dopamine and acetylcholine. Serotonin is mainly synthesized in the rostral, median and caudal raphe complex (perikarya, cell body, in the CNS) and the enterochromaffin cells in the PNS. The serotoninergic neuronal tracts from the rostral raphe complex neurons project to the forebrain, while those from the caudal raphe complex neurons project to the brainstem and spinal cord. The serotoninergic neuronal tracts from the median raphe and dorsal raphe nuclei neuros provide parallel and overlapping projections to many forebrain regions. These neuronal tracts then regulate the release of other neurotransmitters in the regions to which they project. Therefore, since TFMPP affects serotoninergic neurotransmission it can have a significant influence on the release of other neurotransmitters. The effect on the release of other neurotransmitters may be due to inhibiting synaptic potentials in the serotonergic perikarya-locus ceruleus and its prominent effect on the terminal axons of serotonergic neurons (Bobker & Williams, 1989; Dolzhenko, Komissarov, & Kharin, 1989). TFMPP also has been shown to inhibit the K+-evoked release of acetylcholine from rat hippocampal synaptosomes (Bolanos & Fillion,

1989) and induce in vitro and in vivo dose-dependent extracellular dopamine release (Benloucif & Galloway, 1991). TFMPP increased dopamine release in the substantia nigra, striatum and limbic forebrain and this was confirmed by the accumulation of dopamine metabolite 3-MT (Elverfors & Nissbrandt, 1992). In the ventral tegmental area, TFMPP showed maximal inhibition of the basal activity of dopamine neurons (Prisco & Esposito, 1995). TFMPP also decrease epinephrine content in rat hypothalamus (Hemrick-Luecke & Fuller, 1995). These findings exhibit TFMPP and MDMA share the ability to evoke monoamine release, and dangerous drug-drug synergism may occur when piperazines are co-administered at high doses (Baumann et al., 2005).

With regard to other behavioral activity, TFMPP has shown been shown to suppress aggression in rats (Olivier & Mos, 1992), and to facilitates lordosis in 5,7-DHT-treated and non-treated rats (Aiello-Zaldivar et al., 1992). Lordosis is the normal inward lordotic curvature of the lumbar and cervical regions of the human spine. TFMPP amplified vacuous chewing movements (Liminga et al., 1993) and induced inhibition of saccharin taste preference (Cooper & Barber, 1994). TFMPP attenuated posthypoxic myoclonus (Matsumoto et al., 1995). Serotonin controls the phase adjusting effects of light on the mammalian circadian clock through the activation of presynaptic 5-HT1b receptors located on retinal terminals in the suprachiasmatic nucleus (SCN). TFMPP also attenuated the inhibitory effect of light on pineal melatonin synthesis in a dose-related manner (Rea & Pickard, 2000). Finally TFMPP also reduces the frequency of pilocarpine-induced epilepsy in rats (Hernandez et al., 2002).

1.8. TFMPP on the Peripheral Nervous System

With regard to its actions in the periphery, TFMPP can modify the function of a host of tissues including those of the ophthalmic, cardiovascular, respiratory, gastrointestinal, urinary, reproductive and endocrine systems. In the eye, TFMPP acts on the presynaptic 5HT1B receptors, of the retinal terminals in the suprachiasmatic nucleus and this activation of these receptors by TFMPP inhibits retinohypothalamic input (Pickard et al., 1996). TFMPP displays a pharmacological profile comparable to a serotonergic agonist on the cardiovascular system. Multiple studies have demonstrated that TFMPP administration produces a dose-dependent hypotension and bradycardia (Dabire et al., 1987; King & Holtman, 1990). TFMPP causes contractions of uterine arteries and also umbilical veins and arteries from fetal lambs (Zhang & Dyer, 1990). In the respiratory tract, the effect of TFMPP was similar to the activation of 5-HT1A, 5-HT1B and 5-HT2 receptor subtypes at the intermediate area of the ventral surface of the medulla. TFMPP affects the laryngeal and phrenic nerve. The phrenic nerve originates in the neck and descends through the thorax to reach the diaphragm. It is associated with the motor innervation of the diaphragm and helps regulate breathing. The larynx, under control of the laryngeal nerve, regulates respiration, and aids in airway protection, coordination of swallowing, and phonation. TFMPP has been shown to reduce the d amplitude of the recurrent laryngeal and phrenic nerve signals (King & Holtman, 1990). Furthermore, TFMPP by acting on the 5HT1B receptors decreases the respiratory activity, increase pulmonary resistance and decrease in dynamic lung compliance (Edwards et al., 1990). TFMPP also affects the pharynx by increasing the basal tone and affects phasic contractions (O'Gara et al., 1999).

In the gastrointestinal tract, TFMPP causes hypohagia by interaction with 5HT1b receptors (Hutson, Donohoe, & Curzon, 1988). Hypohagia refers to the suppression of caloric intake due to the reduction in feeding due to administration of drugs surgery or environmental interventions (such as change in diet). The hypophagic effect may be due to the effect of TFMPP on the paraventricular nucleus of the hypothalamus. TFMPP induces anorexia by interacting with 5-HT2 and 5-HT1C receptors (Kennett et al., 1989). TFMPP causes relaxation of smooth muscle-anterior byssus retractor muscle of Mytilus (Murakami et al., 1988). With regard to the effect on sexual behavior, TFMPP exerts mixed actions. TFMPP reduces the rodent's sexual masculine behavior as it reduces the copulation of animals (Fernández-Guasti, Escalante, & Agmo, 1989). However, (Berendsen, Jenck, & Broekkamp, 1990) showed that TFMPP induced penile erection at 5HT1C receptors. TFMPP also affects the hormonal secretion and affects the endocrine functions which may impact on sexual behavior. The actions of TFMPP on the endocrine system are complex. There various studies that demonstrate an effect of TFMPP on the glucose level. Pretreatment with 1-(3-chlorophenyl)-piperazine (mCPP) or TFMPP decreased 2,5-Dimethoxy-4-iodoamphetamine-induced hyperglycemia in a dose-dependent manner (Chaouloff, Laude, & Baudrie, 1990). TFMPP also has been shown to affect insulin level without disrupting glucose homeostasis (Rouru et al., 1993). TFMPP can promote the release of adrenocorticotropin (ACTH) and increase serum corticosterone levels. It also can increase prolactin levels and promote the release of arginine vasopressin (AVP) into the portal vessels from the anterior pituitary via the central serotonergic mechanism (Poland & Frazer, 1991). Finally there were studies showing that TFMPP acts additively with BZP to produce significant hepatotoxicity. In vitro hepatotoxicity of 'Legal X': the

combination of BZP and TFMPP triggers oxidative stress, mitochondrial impairment and apoptosis (da Silva et al. 2017). Piperazine designer drugs have also been shown to affect cholesterol biosynthesis and escalates the risk of phospholipidosis and steatosis (M. D. Arbo et al., 2012).

1.9. Toxicological effects & Identifications of TFMPP

TFMPP may be an ingredient in clandestine drug products marketed as ecstasy and BZP, or and abusers hoping for an extended or intensified "high" from ecstasy sometimes deliberately combine these drugs. The median consumption of TFMPP is 400 mg but can range from 43-2500 mg. In humans, combined BZP and TFMPP (mean quantity) consumed on an incident of utmost use has been reported to be 533 mg. TFMPP has shown to induce bradycardia and reduce the rate of breathing, impair the ability to move and to regulate of body temperature. This results in high fevers that cannot be reversed, leading to heart, liver and kidney failure. The other general adverse effects are insomnia, anxiety, nausea, vomiting, headache, migraine, seizures, impotence, psychosis interference with circadian system and hypophagia. In New Zealand toxic seizures and respiratory acidosis has been reported in several patients. As of 2002, there had been two reported deaths from BZP/TFMPP. The mechanisms of toxicity may be due to its effect on the serotonergic neurotransmission and endocrine function. In the cellular level, TFMPP decreases in intracellular ATP, accompanied by increased intracellular calcium levels, reactive oxygens species, depletion of antioxidants and a decrease in mitochondrial membrane potential that seems to involve the mitochondrial permeability transition pore. The cell death mode revealed early apoptotic cells and high number of cells undergoing secondary necrosis

(Arbo et al. 2014; Dias-da-Silva et al. 2015; da Silva et al. 2017). TFMPP also increases the the biosynthesis of cholesterol acting on the synthetic enzymes and potentiate increase the risk of phospholipidosis and steatosis (M. D. Arbo et al., 2016). Like any other stimulants, TFMPP also increases the monoaminergic neurotransmission and inhibits the GABAergic inhibitory neurotransmission. TFMPP exhibits antagonistic effect on the GABA-A receptor which leads to increased monoaminergic neurotransmission resulting overdoses (Hondebrink et al., 2015). Among the tested drugs, TFMPP seems to be the most potent cytotoxic compound. Overall, piperazine designer drugs are potentially cardiotoxic, supporting concerns about the risks associated with abuse of this drug class (B. D. Arbo et al., 2014). Finally the toxic effects of TFMPP and TFMPP-containing drugs of abuse are more prominent in females as compared to the males. Females may be at greater risk of experiencing toxicity from BZP/TFMPP party pills due to their smaller physical size and therefore greater exposure. Furthermore, consuming enormous amounts of TFMPP-containing drug products in a single party setting and concurrently with cannabis, BZP and 5-hydroxytryptophan (5-HTP) recovery pills definitely has shown to increase detrimental toxic effects in both males and females, presumably due to the ability of all of these substances to potentiate serotonin release (Wilkins et al., 2008).

A number of methods for the identification and quantification of 3-TFMPP in body fluids have been published (Peters et al. 2003; Bishop et al. 2005; Tsutsumi et al. 2005; Vorce et al. 2008; Maher et al. 2009; Wohlfarth et al. 2010; Dickson et al. 2010; Wada et al. 2011; Maskell et al. 2011; Bell et al. 2011; Elie et al. 2012; Wada et al. 2012; Moreno et al. 2012b; Moreno et al. 2012a; Rust et al. 2012; Johnson and Botch-Jones 2013; Zuba and Byrska 2013; Curley et al. 2013; Siroká et al. 2013; Stojanovska et al. 2014; Beckett et al.

2015). Most of these rely either on gas chromatography with mass spectrometry (GC/MS) or liquid chromatography coupled with mass spectrometry (LC/MS). In most MS analyses TFMPP shows the expected molecular ion at 230 mass units and gives other characteristic ions at m/z of 188, 174, 173,172 and 145 arising from fragmentation of the piperazine ring. More recently (Maher, Awad, DeRuiter, & Clark, 2010) has published a method to differentiate 3-TFMPP from its 2- and 4-TFMPP regioisomers using GC-MS and GC-IRD.

Table 1.1. Effect of TFMPP attributed to the effect on serotonergic neurotransmission.

Organs	Action of TFMPP
CNS	Increases serotonergic neurotransmission Release ACTH from the anterior pituitary Release arginine vasopressin (AVP) from posterior pituitary Thermoregulation:lower dose of TFMPP evoked hyperthermic response and high dose hypothermic response Affects Mood and Behavior: Hallucinogenic, psychotropic, anxiogenic, Anti-nociceptive properties Modulates release of other neurotransmission serotonin (5-HT), dopamine (DA), and noradrenaline (NA) Involved in sleep regulation: suppression of REM sleep, augmentation of NREM sleep Facilitate lordosis Regulation of nocturnal pineal melatonin production: attenuated the inhibitory effect of light on pineal melatonin synthesis
Eye	inhibits retinohypothalamic input

CVS	Decrease in blood pressure and heart rate
Respiratory Tract	Decreases respiratory activity Increase in pulmonary resistance and decrease in dynamic lung compliance
Gastrointestinal Tract	Hypohagia Anorexia
Urinary Tract	Hyponatremia Acute urinary retention Acute tubular necrosis
Reproductive system:	Reduces sexual masculine behavior Contraction of uterine arteries
Endocrine system:	Induces hyperglycemia Increases both serum corticosterone and prolactin concentrations
Immune system	Suppresses Delayed Hypersensitivity response

2. Materials and Methods

2.1. Chemicals and Reagents

Thiazolyl Blue Tetrazolium Bromide (MTT) was purchased from Tokyo Chemical Industry America. Trypsin-EDTA solution and Penicillin-Streptomycin solution were purchased from Thermofisher. RPMI 1640 Medium, ES Cell Qualified Fetal Bovine Serum (FBS) and L-Glutamine Solution were purchased from Emdmillipore. 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 was 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.

2.2. Rat dopaminergic neuron cells (N27)

N27 rat dopaminergic neuron cells were cultured in RPMI 1640 Medium supplemented with Fetal Bovine Serum (10%), Penicillin-Streptomycin Solution (1%) and L-Glutamine Solution (100x) (1%). For the MTT assay, cells were grown into 75 cm^2 flasks, harvested

by trypsinization after achieving 80% confluency (4-5 days) and seeded 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 6-12 passages after the cells were received (Holmes, Abbassi, Su, Singh, & Cunningham, 2013).

2.3. Treatment Design

Prior to each experiment 2-TFMPP, 3-TFMPP and 4-TFMPP were diluted in Phosphate Buffered Saline (PBS) to a 10mM stock solution. For cytotoxicity testing, eight different concentrations of 2-TFMPP, 3-TFMPP and 4-TFMPP (1, 2, 10, 50, 100, 250, 500, 1000 uM) were attained by serial dilution with PBS followed by additional dilution in serum-enriched fresh culture medium. Test concentrations were exposed to the cell line for 48 hours demonstrating relatively long exposure of drug in vitro based toxicity testing. For the collection of cell homogenate, drug concentrations (100 µM and 1mM) 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.

2.4. Cytotoxicity Assay

For the evaluation of cytotoxicity, MTT cell viability assay was performed. The notion of MTT assay is that the mitochondria of viable cells through succinate dehydrogenases reduce the yellow colored water soluble tetrazole reagent MTT (3-(4,5-dimethylthiazol-2-

yl)-2,5-diphenyltetrazolium bromide) to an insoluble blue crystal formazan that can be measured colorimetrically (Berridge, Herst, & Tan, 2005; Mosmann, 1983).

After 24 hours and 48 hours incubation with 3-TFMPP in serum-fed and serum-free medium, 12 mM MTT stock solutions was prepared and then added on each well along with fresh culture medium. Following a 2 hours incubation at 37° C the medium was aspirated and 200 µl of DMSO was added to solubilize the formazan crystal. Afterward 10 minutes incubation at 37° C the absorbance was measured using a microtiter plate reader (Synergy HT, Bio-Tek Instruments Inc., Winooski, VT, USA) at 540 nm.

Results showed time dependent and dose dependent cell death with the three drugs along with Hydrogen peroxide which served as positive control. Furthermore, results were expressed graphically as % viability vs. concentration (uM). Cells were imaged using an Axiovert 25 inverted microscope equipped with a Nikon Coolpix 4500 camera (M. Zheng et al., 2014).

2.5. Protein quantification

Protein was quantified using Thermo Scientific Pierce 660 nm Protein Assay reagent kit (Pierce, Rockford, IL). Bovine serum albumin (BSA) was used as a standard for protein measurement.

2.6. Quantifying Reactive Oxygen Species

The generation of reactive oxygen species in the N27 rat dopaminergic cells treated with 3-TFMMP, 2-TFMPP and 4-TFMPP was estimated spectrofluorometrically by measuring the conversion of non-fluorescent chloromethyl-DCF-DA (2′, 7-

dichlorofluorescindiacetate, DCF-DA) to fluorescent DCF using excitation wavelength of 492 nm and emission wavelength of 527 nm. A mixture of 0.05% w/v solution of DCF-DA in ethanol (10 µl), phosphate buffer (150 µl) and cell homogenate (40 µl) were incubated for 1 h at 37 °C. DCFH reacted with ROS to form the fluorescent product DCF. Readings were measured by BioTek Synergy HT plate reader (BioTek, VT, USA). Results were expressed as percentage change from the control (Dhanasekaran, Tharakan, & Manyam, 2008).

2.7. Lipid Peroxide Content

Lipid peroxidation is a sequence reaction process in which ROS attack polyunsaturated fatty acids causing the oxidative breakdown of lipids. Lipid peroxidation content was measured by calculating the quantity of malondialdehyde (MDA) content in the form of Thiobarbituric acid-reactive substances (TBARS) (Ohkawa, Ohishi, & Yagi, 1979). 100 µl ice cold Trichloroacetic acid (TCA) (20 % w/v) was added to Cell homogenate (100 µl) then it was mixed with 400 µl Thiobarbuturic acid (TBA) (0.5 % w/v) and 500 µl deionized water. Additionally, the mixture was incubated in water bath for 15 minutes (80º C) then cooled at ice for 5 minutes. Afterwards the mixture was centrifuged at 4°C for 5 minutes at 10,000 RPM. Following the centrifuging, samples supernatant was placed at 96-well plate and the absorbance was measured at 532 with a plate reader (Synergy HT, Bio-Tek Instruments Inc., Winooski, VT, USA) using duplicate reading 200 µl in each well and MDA levels were calculated as TBARS reactive substances per mg protein. Results were expressed as percentage change from the control (Dhanasekaran et al., 2007; M. Zheng et al., 2014).

2.8. Superoxide Dismutase Activity

The autoxidation of pyrogallol in an alkaline environment results in the generation of superoxide anion radicals. Superoxide dismutase (SOD) is an antioxidant enzyme that rapidly dismutase superoxide anion radicals into hydrogen peroxide and water. Spectrophotometric measurement of the inhibition of pyrogallol autoxidation induced by SOD can be performed rapidly and conveniently by reading the absorbance of a mixture of 2 mM pyrogallol solution, 50 mM Tris buffer pH 8.2 and cell homogenate using visible light at 420 nm for 3 minutes (Marklund & Marklund, 1974). Superoxide dismutase activity was measured as the change in absorbance at 420 nm and expressed as percentage change from the control.

2.10. Catalase Activity

Catalase is an antioxidant enzyme that stimulate the transformation of hydrogen peroxide into water and oxygen. An assay mixture of 50 mM PBS at pH 7.0 and cell homogenate was prepared. Following the addition of 30 mM hydrogen peroxide, which yielded approximately 0.5 absorbance, the decomposition of hydrogen peroxide was monitored spectrophotometrically using ultraviolet light at 240 nm for 1 minute (Aebi, 1984). A standard curve was created from commercially procured hydrogen peroxide. The change in absorbance was observed and the enzyme activity was calculated as percentage change from control (Muralikrishnan & Mohanakumar, 1998).

2.11. Glutathione Content

In the presence of glutathione (GSH), Glutathione peroxidase (GSH-Px) stimulate the conversion of hydrogen peroxide to water. The condensation reaction between GSH and

o-phthalaldehyde (OPT) produce a fluorescence at pH 8.0 that can be measured spectrofluorometrically (Cohn & Lyle, 1966). The assay mixture was made of cell homogenate, 0.1 M phosphoric acid, 0.1% OPT solution in methanol and 0.01 M phosphate buffer. In the beginning of the experiment, cell homogenate was mixed with the 0.1 M phosphoric acid in order to precipitate the protein. Then, the mixture was centrifuged at 12000 RPM for 10 minutes. Following the addition of OPT to the supernatant, the mixture was incubated in dark for 20 minutes 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 acquired GSH. The GSH content was calculated as mmol of GSH/µg protein and expressed as percentage control (Muralikrishnan & Mohanakumar, 1998; Y. Zheng et al., 2014).

2.12. Mitochondrial Complex-I Activity

NADH oxidation to NAD^+ is catalyzed by Mitochondrial Complex-I (NADH dehydrogenase). Cell homogenate was added to phosphate buffered saline and NADH in order to measure NADH dehydrogenase activity spectrophotometrically at 340 nm using visible light. A standard curve was composed from commercially obtained NADH. The extent of NADH oxidation was quantified by determining the decrease in absorbance at 340 nm for 3 minutes. Results were reported as percentage change from the control (Ramsay, Dadgar, Trevor, & Singer, 1986).

2.13. Mitochondrial complex IV activity

Cytochrome C oxidation is catalyzed by Mitochondrial complex IV (Cytochrome C oxidase). Cell homogenate was added to phosphate buffered saline and Cytochrome C in order to determine the activity of the Cytochrome C oxidase activity spectrophotometrically at 550 nm using visible light. A standard curve was created from commercially obtained Cytochrome C. The magnitude of Cytochrome C oxidation was measured by following the oxidation of reduced Cytochrome C as an absorbance decrease at 550 nm for 3 minutes. Results were reported as percentage change from the control (Ramsay et al., 1986; Wharton & Tzagoloff, 1967).

2.14. Mitochondrial monoamine oxidase (MAO) activity

Total monoamine oxidase activity was measured fluorometrically by determining the amount of 4-hydroxyquinoline formed as a result of kynuramine oxidation (Morinan & Garratt, 1985). MAO activity was reported as 4-hyroxyquinoline formed/hour/mg protein (Albano, Muralikrishnan, & Ebadi, 2002; Muralikrishnan & Mohanakumar, 1998).

2.15. Nitrite assay

Nitric oxide (NO) oxidation pathways produce nitrite and nitrate as final products which allow to use their concentrations as an expression of NO production. Nitrite was measured using Griess reagent which was developed by Griess in 1879. This method relies on Reaction of NO2 with sulfanilamide under acidic condition resulting in the production of diazonium ion which then combine with N-(1-naphthyl) ethylenediamine to form

chromophoric azo product which can be measured spectrophotometrically at 545 nm (Giustarini, Dalle-Donne, Colombo, Milzani, & Rossi, 2008).

2.16. Statistical Analysis

Data was reported as mean ± SEM. Statistical analysis were accomplished 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).

3.Results

3.1. TFMPP derivatives induce Dose-Dependent and Time-Dependent reduction N27 Cell viability:

Different doses (50µM, 100 µM, 250 µM, 500µM, 1mM, 2.5mM, 5mM, 10mM) of TFMPP derivatives (3-TFMPP, 2-TFMPP, and 4-TFMPP) were treated with N27 cells for two different time points (24 and 48 hours). Controls cells were cultured under the same conditions without exposure to TFMPP derivatives. Hydrogen peroxide, an endogenous neurotoxin, served as a positive control.

TFMPP derivatives significantly reduced cell viability in a dose-dependent and time-dependent approach when compared to the control ($n=12$, $p<0.0001$; Figure 3.2.a. and Figure 3.3.a. and Figure 3.4.a.).

After 24 hours incubation with TFMPP derivatives, all the derivatives caused dose-dependent decrease in N27 cell viability. TFMPP derivatives reduced the cell viability approximately by 40% and 60% at the dose of 100µM and 1mM respectively. There was no significant effect on the cell viability at the dose of 10 µM. However, after 48 hours incubation, there was significant increased reduction in cell viability by with TFMPP derivatives as compared to 24hours. TFMPP derivatives reduced the cell viability approximately by 75% and 100% at the dose of 100µM and 1mM respectively. Interestingly, there was significant effect on the cell viability at the dose of 10 µM (60% decrease in cell viability).

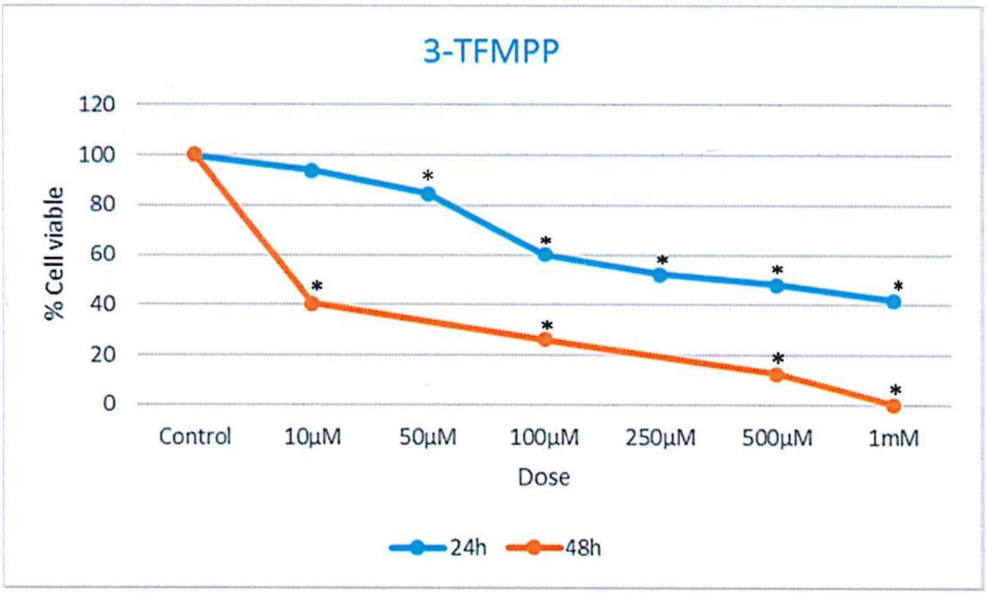
Hydrogen peroxide (positive control) also caused dose-dependent decrease in cell viability ($n=12$, $p<0.05$; Figure 4.1.b). Hydrogen peroxide (50 µM) treatment approximately reduced the cell viability by 50% at 24 hours.

With regard to the morphological changes in N27 cells, TFMPP derivatives induced well defined cell structural deformation. There was significant neuronal shrinkage, decreased synaptic connections and cells getting rounded in shape which led to decreased viability.

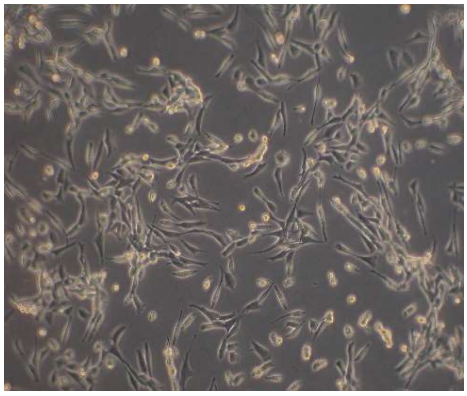
a) b)

Figure 3.1. Morphological characterization of N27 rat dopaminergic cells a) Control at 24 hours b) Control at 48 hours

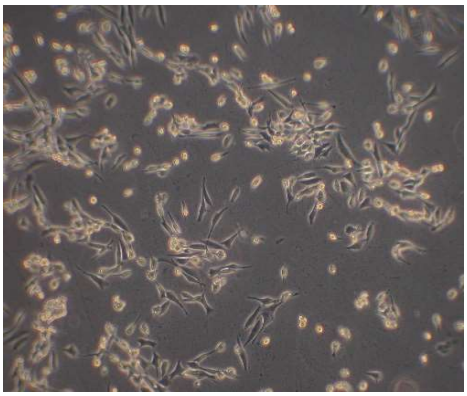
a)



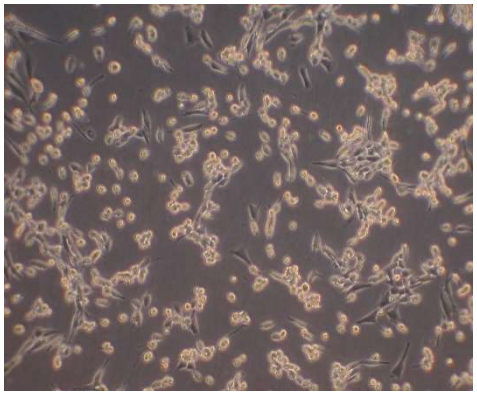
b)



c)



d)



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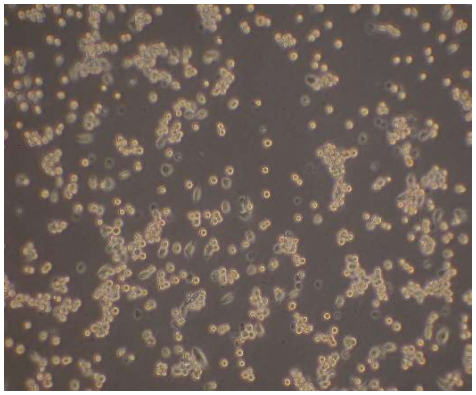
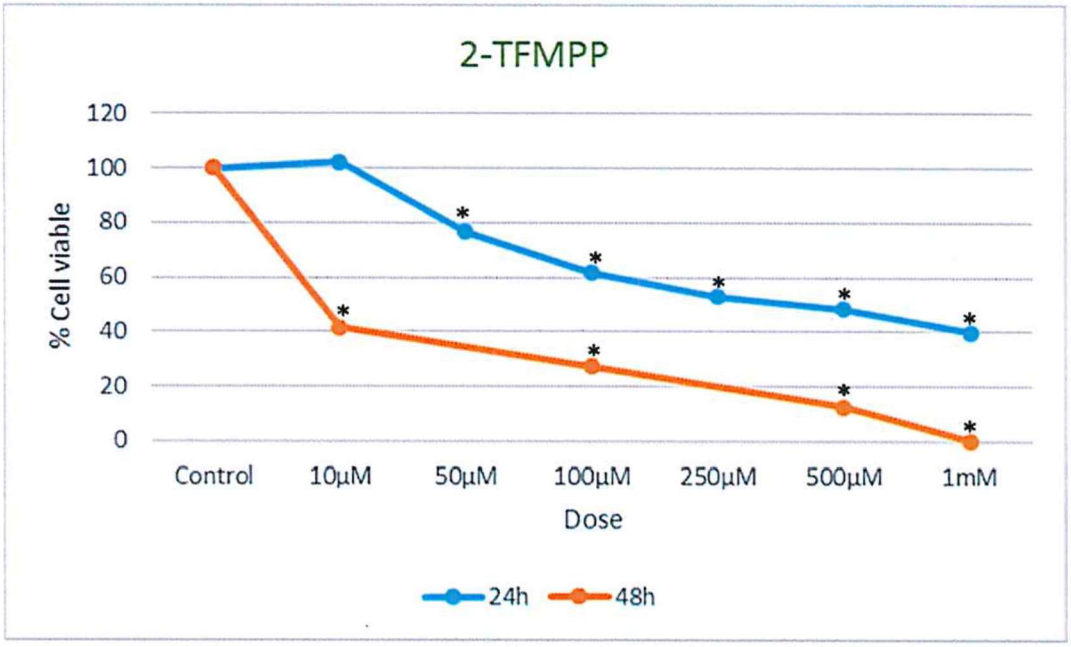


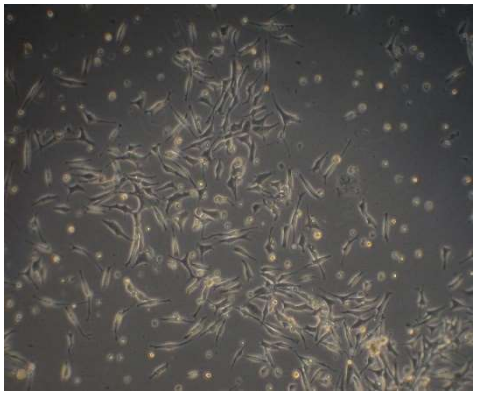
Figure 3.2. Concentration-response (cell viability) curve and Morphological characterization in N27 cells treated with 3-TFMPP

a) Cells were treated with different doses of 3-TFMPP for 24 hours and 48 hours as well at 37°C. Cell viability was evaluated through the MTT reduction assay (n=12). After incubation, the cells were washed with warm PBS and visualized under microscope (magnification 10x). Morphological characterization of N27 rat dopaminergic cells treated with b) 3-TFMPP 100 µM after 24 hours c) 3-TFMPP 100 µM after 48 hours d) 3-TFMPP 1mM after 24 hours e) 3-TFMPP 1mM after 48 hours.

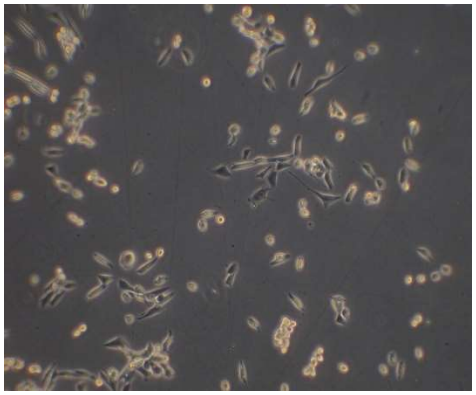
a)


2-TFMPP
120
100
80
60
40
20
0
% Cell viable
Control
10µM
50µM
100µM
250µM
500µM
1mM
Dose
24h
48h

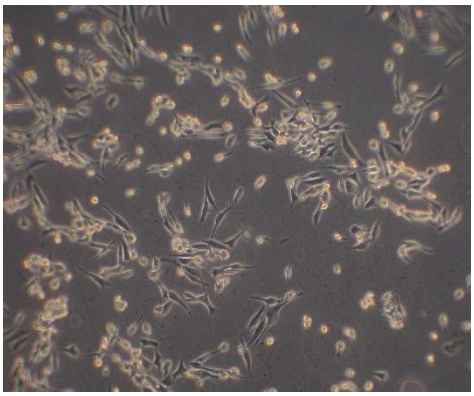
b)



c)



d) e)



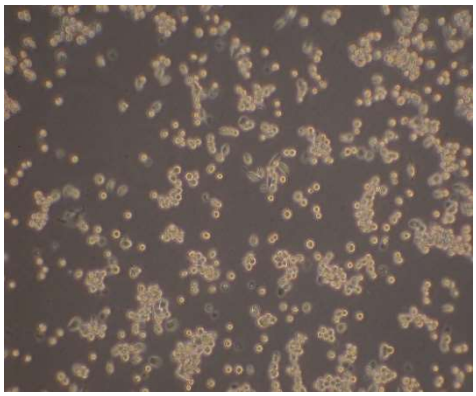
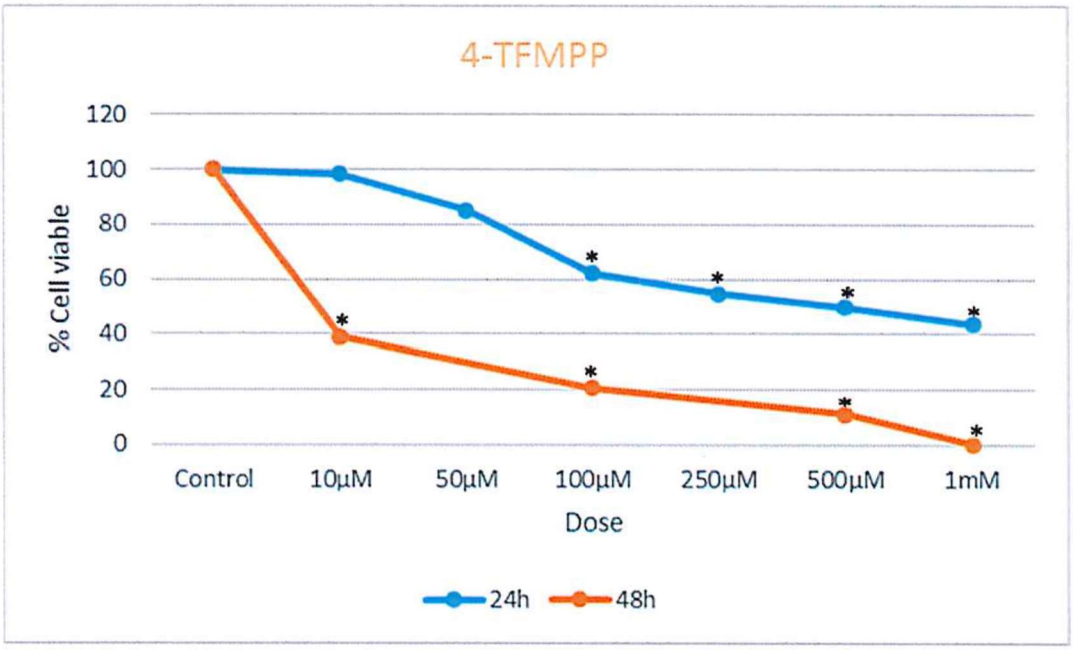


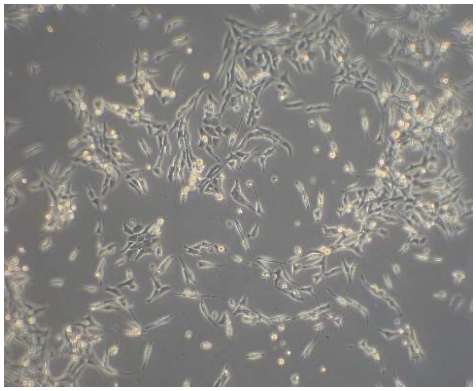
Figure 3.3. Concentration-response (cell viability) curve and Morphological characterization in N27 cells treated with 2-TFMPP

a) Cells were treated with different doses of 2-TFMPP for 24 hours and 48 hours as well at 37°C. Cell viability was evaluated through the MTT reduction assay (n=12). After incubation, the cells were washed with warm PBS and visualized under microscope (magnification 10x). Morphological characterization of N27 rat dopaminergic cells treated with b) 2-TFMPP 100 µM after 24 hours c) 2-TFMPP 100 µM after 48 hours d) 2-TFMPP 1mM after 24 hours e) -TFMPP 1mM after 48 hours.

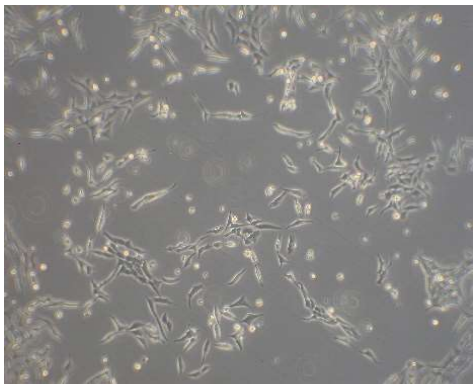
a)



b)



c)



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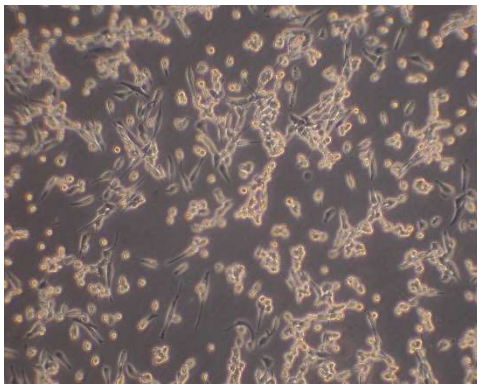

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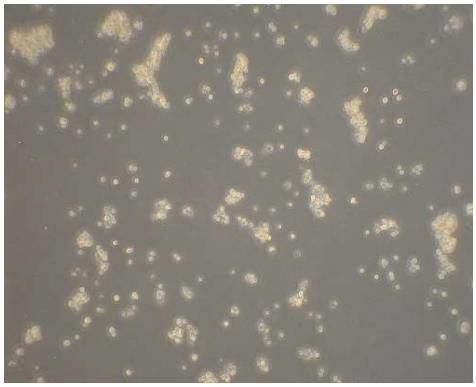


Figure 3.4. Concentration-response (cell viability) curve and Morphological characterization in N27 cells treated with 4-TFMPP

a) Cells were treated with different doses of 4-TFMPP for 24 hours and 48 hours as well at 37°C. Cell viability was evaluated through the MTT reduction assay (n=12). After incubation, the cells were washed with warm PBS and visualized under microscope (magnification 10x). Morphological characterization of N27 rat dopaminergic cells treated with b) 4-TFMPP 100 µM after 24 hours c) 4-TFMPP 100 µM after 48 hours d) 4-TFMPP 1mM after 24 hours e) 4-TFMPP 1mM after 48 hours.

3.2. TFMPP derivatives generates ROS

ROS generation stimulate oxidative stress which result in destruction of biological molecules such as proteins, DNA and lipids (Freeman & Crapo, 1982). Various human diseases including neurodegenerative diseases, aging, atherosclerosis, cancer and

pulmonary fibrosis are linked to the damage caused by ROS generation (Cross et al., 1987; Halliwell, Gutteridge, & Cross, 1992). Antioxidants such as catalase, superoxide dismutase and glutathione neutralize the harmful effects of ROS.

3-TFMPP, 2-TFMPP and 4-TFMPP dose-dependently stimulated ROS generation in N27 cells as compared to the control (n=5, $p< 0.0001$; Figure 4.A). At the lower dose (100 µM) TFMPP derivatives increased the ROS production by 2-3 times approximately. However, at the higher dose 30-50 times approximately as compared to control. Interestingly, 3-TFMPP significantly increased ROS production as compared to 2-TFMPP and 4-TFMPP at both the doses. The positive control (Hydrogen peroxide) also generated comparatively significant ROS.

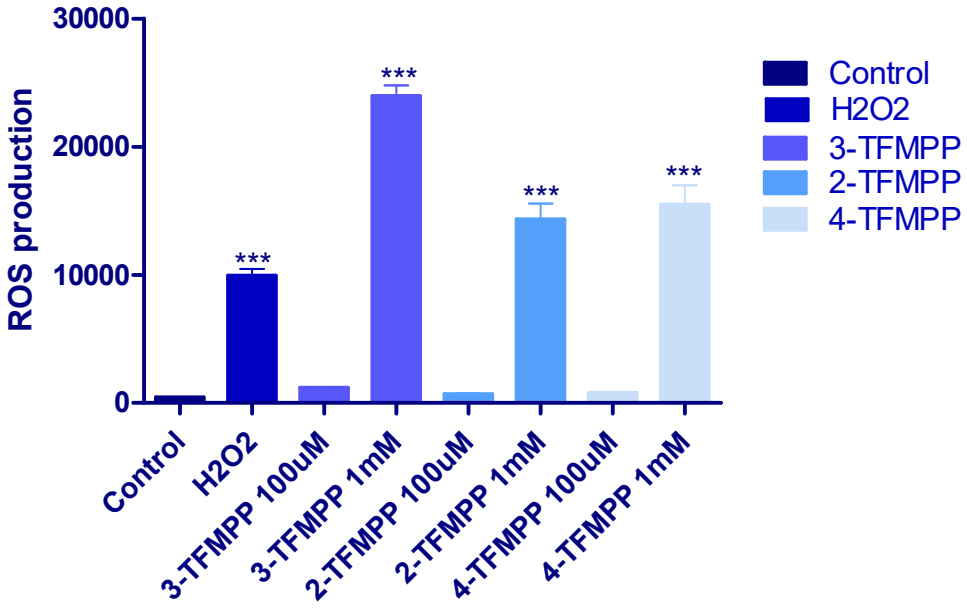


Figure 3.5. Effect of TFMPP derivatives on ROS generation in N27 cells

3-TFMPP, 2-TFMPP and 4-TFMPP generate oxidative stress by aggregating reactive oxygen species generation in N27 cells after 24 hours. The fluorescent product DCF was measured spectrofluorometrically. 3-TFMPP, 2-TFMPP and 4-TFMPP (1mM) showed a significant increase in ROS generation ($p < 0.05$, n=5). 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

3.3. TFMPP derivatives increases nitrite production

Different studies indicated that the production of nitric oxide is increased in the brain of Parkinson's disease patient resulting in dopaminergic neuron damage through oxidative stress (Qureshi et al., 1995). TFMPP caused a significant increase in nitrite formation in a dose-dependent manner. This increase was significant at the higher dose (1mM) where 3-TFMPP, 2-TFMPP and 4-TFMPP increased the nitrite production by 219%, 210% and 198% respectively (n=5, $p<0.05$; Figure). At the same time, 3-TFMPP, 2-TFMPP and 4-TFMPP also increased nitrite formation by 192%, 114% and 137% respectively at the lower dose (100µM). Furthermore, 3-TFMPP had formed higher nitrite content as compared to 2-TFMMP and 4-TFMPP.

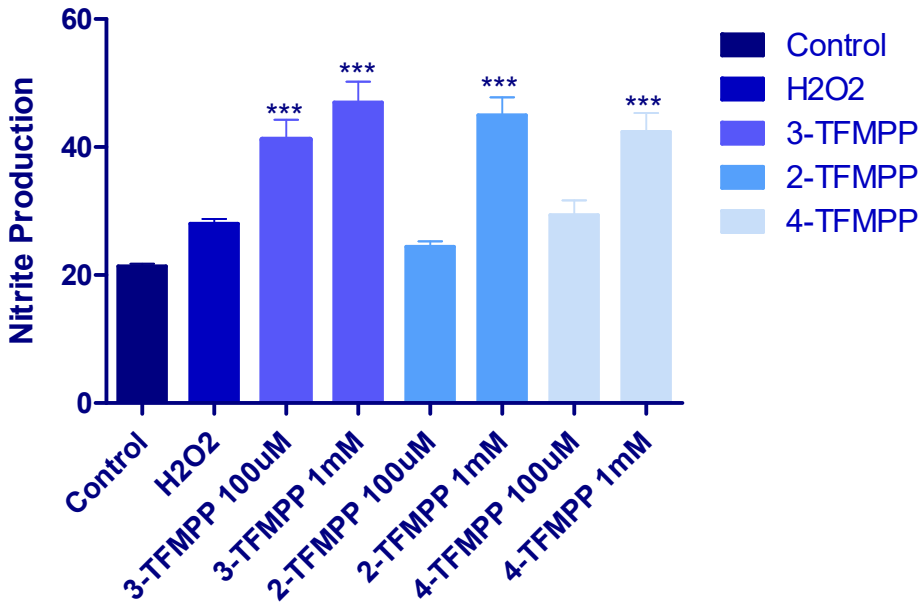


Figure 3.6 Effect of TFMPP derivatives on Nitrite production in N27 cells

TFMPP caused an increase in nitrite production in a dose-dependent manner. This increase was significant at the higher dose (1mM) (n=5, $p<0.05$; Figure) in N27 cells after 24 hours incubation. However, the increase in nitrite production was not statistically significant at the lower dose (100µM). Nitrite production was determined spectrophotometrically at 540 nm. 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.

3.4. TFMPP derivatives induces lipid peroxidation

Lipid peroxide production is known to be increased by free radicals/ROS interaction with lipids. When compared to control, 3-TFMPP, 2-TFMPP and 4-TFMPP at dose of 100µM significantly increased lipid peroxidation by 157%, 139% and 149% respectively. While at dose of 1mM 3-TFMPP, 2-TFMPP and 4-TFMPP increased lipid peroxide level by 331%, 284% and 300% respectively (n=5, p< 0.0001; Figure 4.B.).

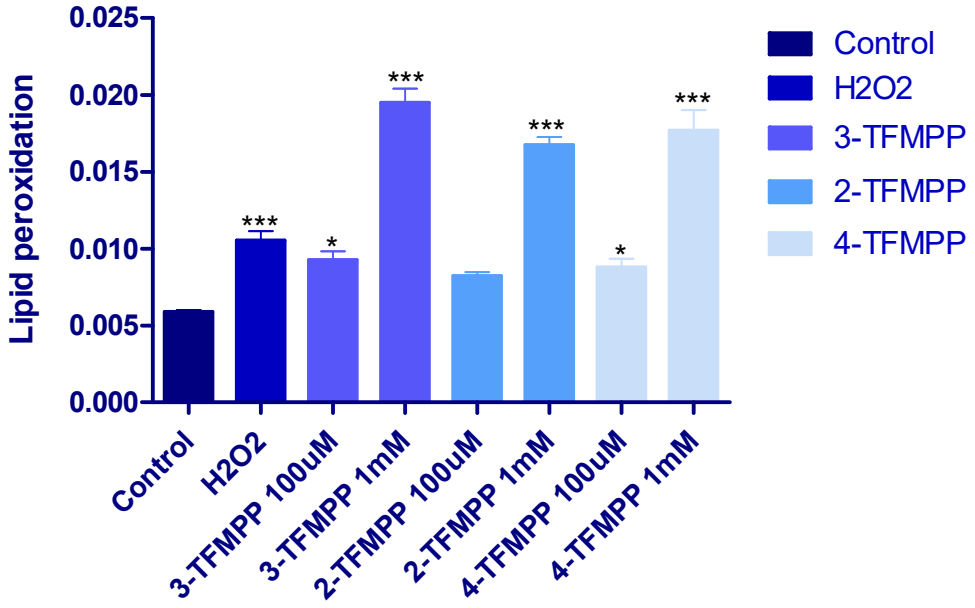


Figure 3.7. Effect of TFMPP derivatives on lipid peroxidation in N27 cells

3-TFMPP, 2-TFMPP and 4-TFMPP (1mM) significantly increased lipid peroxidation in dose dependent manner (n=5, $p < 0.05$) in N27 cells after 24 hours incubation. Lipid peroxidation was measured colorimetrically as TBARS, a marker 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.

3.5. TFMPP derivatives depletes GSH content and increases GSH-Px activity

TFMPP derivatives dose-dependently depleted GSH in N27 cells as compared to the control. 3-TFMPP, 2-TFMPP and 4-TFMPP caused significant depletion of GSH by 80% approximately at 1mM. While at lower dose (100uM), 3-TFMPP, 2-TFMPP and 4-TFMPP demonstrated less effect on GSH depletion approximately by 10% (n=5, $p< 0.0001$; Figure 5.A). In a dose-dependent manner, TFMPP derivatives increased the activity of glutathione peroxidase. While at the lower dose (100uM), 3-TFMPP increased the activity as compared to 2-TFMPP (non-significantly) and 4-TFMPP (significantly) (n=5, $p<0.05$; Figure 5.B). interestingly, there was no significant difference observed the higher concentration between TFMPP derivatives.

A. Glutathione content

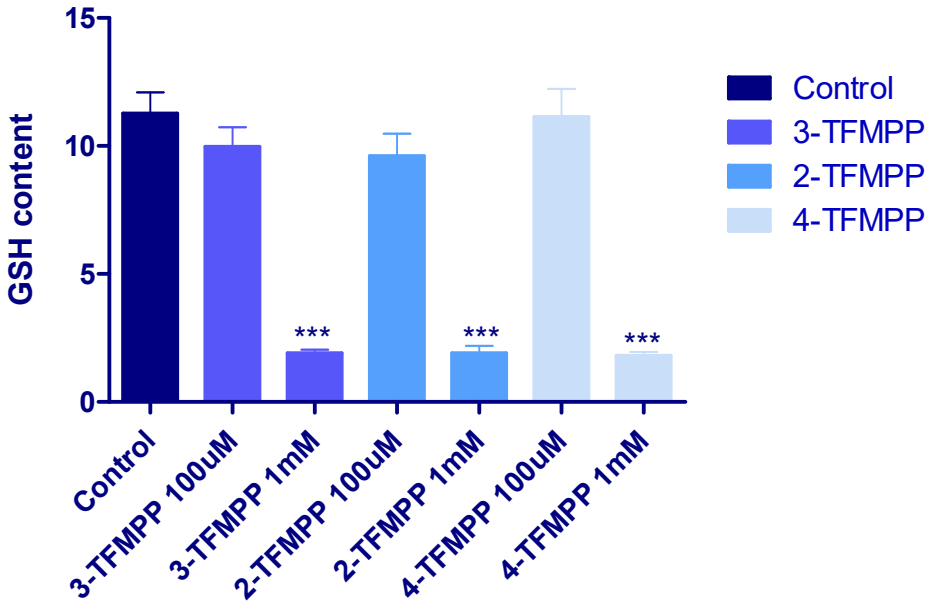


Figure 3.8.A. Effect of TFMPP derivatives on GSH content in N27 cells

In dose-dependent manner 3-TFMPP, 2-TFMPP and 4-TFMPP depleted GSH content in N27 cells after 24 hours. They all reduced GSH content significaly at dose of 1mM (n=5, $p<0.05$; Figure 4.A) while they showed less effect with lower dose (100uM). The condensation reaction between GSH and o-phthalaldehyde (OPT) produce a fluorescence at pH 8.0 that was measured spectrofluorometrically. 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.

B. Glutathione peroxidase

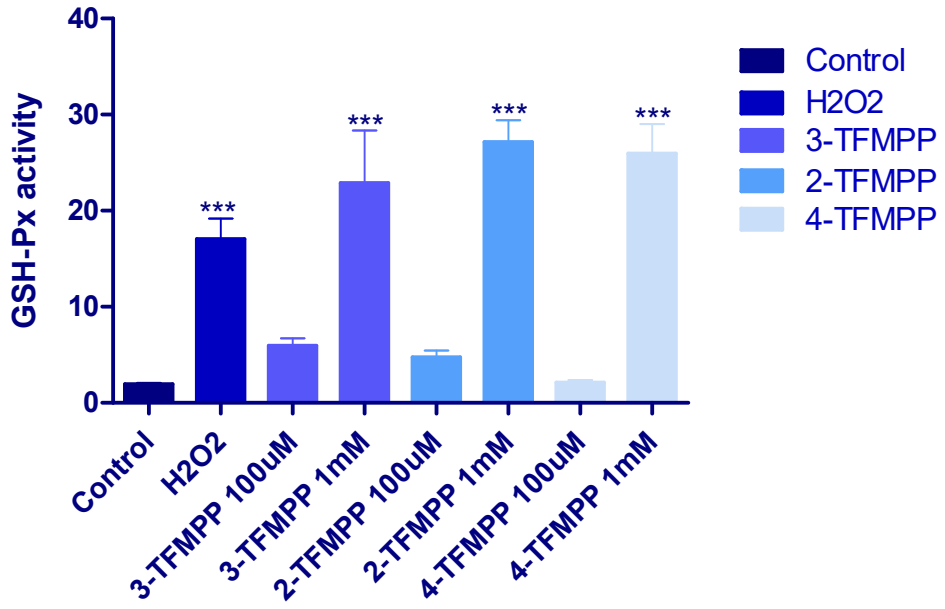


Figure 3.8.B. Effect of TFMPP derivatives on glutathione peroxidase activity in N27 cells

In dose-dependent manner 3-TFMPP, 2-TFMPP and 4-TFMPP increased the activity of Glutathione peroxidase in N27 cells after 24 hours. They all increased Glutathione peroxidase significantly at dose of 1mM ($n=5$, $p<0.05$; Figure 4.B). The activity of GSH-Px were increased significantly when the cells were treated with hydrogen peroxide which served as positive control. While at the lower dose (100uM), they showed less effect on Glutathione peroxidase activity. 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.

3.6. TFMPP derivatives alters antioxidant enzymes (SOD and CAT) activities

Superoxide dismutase provides protection to the cell by stimulating the breakdown of superoxide anion to hydrogen peroxide and water. When compared to control, higher doses of 3-TFMPP, 2-TFMPP and 4-TFMPP (1mM) increased SOD activity (n=5, $p < 0.0001$; Figure 6.A.). To counteract the oxidative stress caused by higher level of hydrogen peroxide, the neurons boosted their catalase activity which break hydrogen peroxide resulting in the byproducts water and molecular oxygen. TFMPP derivatives did not affect the catalase activity as compared to control (n=5; Figure.).

A. Superoxide Dismutase

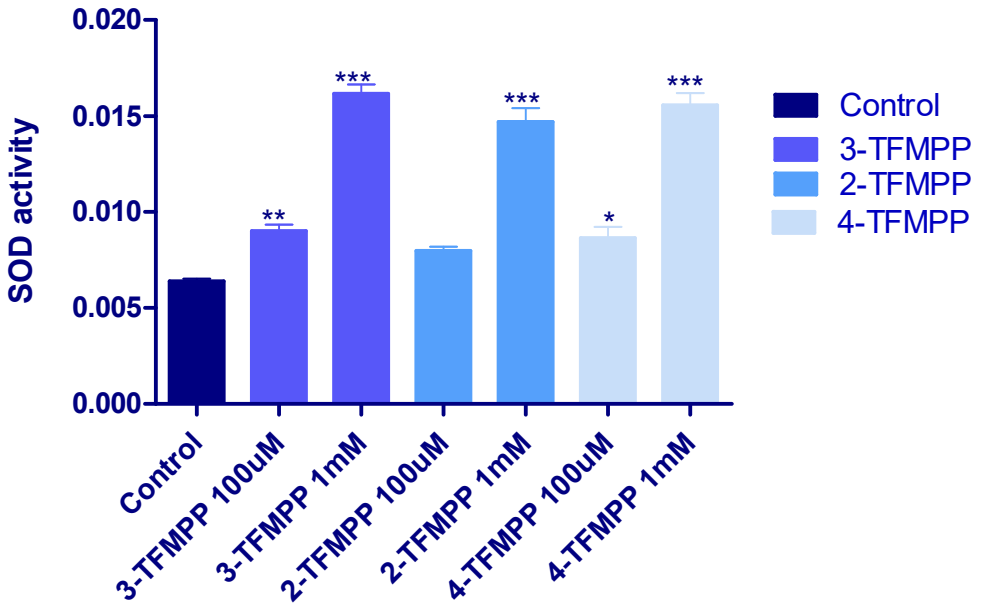


Figure 3.9.A. Effect of TFMPP derivatives on SOD activity in N27 cells

3-TFMPP, 2-TFMPP and 4-TFMPP significantly increased Superoxide Dismutase Activity at the higher dose (1mM) (n=5, $p < 0.05$) in N27 cells after 24 hours incubation. The inhibition of pyrogallol autoxidation induced by Superoxide Dismutase 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.

B. Catalase activity

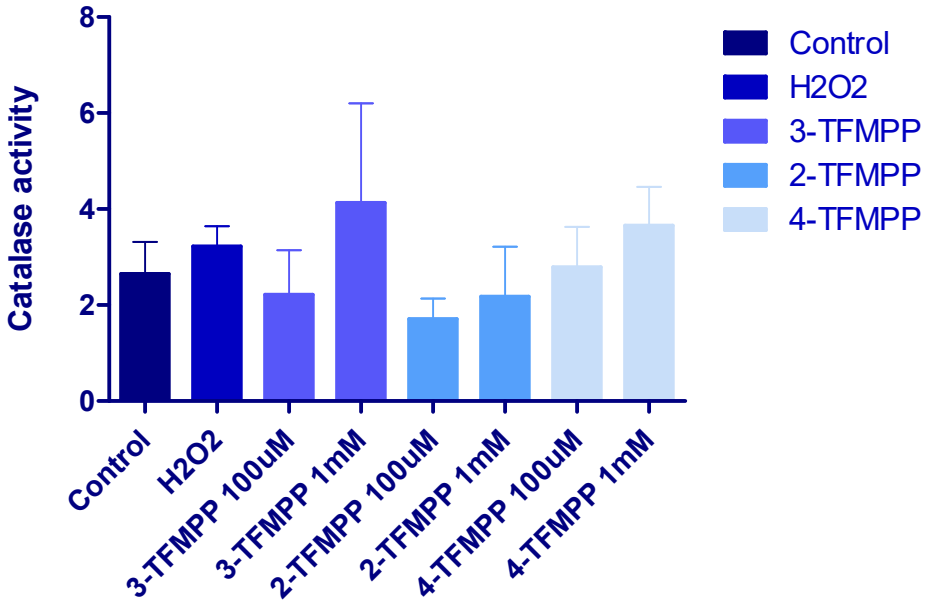


Figure 3.9.B Effect of TFMPP derivatives on Catalase activity in N27 cells

3-TFMPP, 2-TFMPP and 4-TFMPP showed fluctuated effect on catalase activity where the highest increase was produced by 3-TFMPP 1mM (156%) and 4-TFMPP 1Mm (138%). However, the difference in catalase activity was not significant when compared to control. 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.

3.7. TFMPP derivatives increases Monoamine oxidase activity (MAO) in N27 cells

Multiple studies has shown that MAO activity is linked to neurodegenerative diseases such as Parkinson's disease (Youdim & Lavie, 1994). MAO plays a role in neurodegeneration through oxidative stress (Siddiqui 2011), neuroinflammation (Bielecka, Paul-Samojedny, & Obuchowicz, 2010), apoptosis (Merad-Boudia, Nicole, Santiard-Baron, Saillé, & Ceballos-Picot, 1998; Naoi, Maruyama, Akao, Yi, & Yamaoka, 2006), glial activation (Weinstock, Luques, Poltyrev, Bejar, & Shoham, 2011) and decreasing aggregated-protein clearance (Konradi, Riederer, Jellinger, & Denney, 1987). By stimulation of oxidation of monoamine which produces hydrogen peroxide, MAO induces oxidative stress resulting in neuronal degeneration (J. J. Chen & Wilkinson, 2012; Naoi et al., 2006).

TFMPP derivatives dose-dependently increased MAO activity in N27 cells as compared to the control (n=5, p< 0.0001; Figure 5.A).

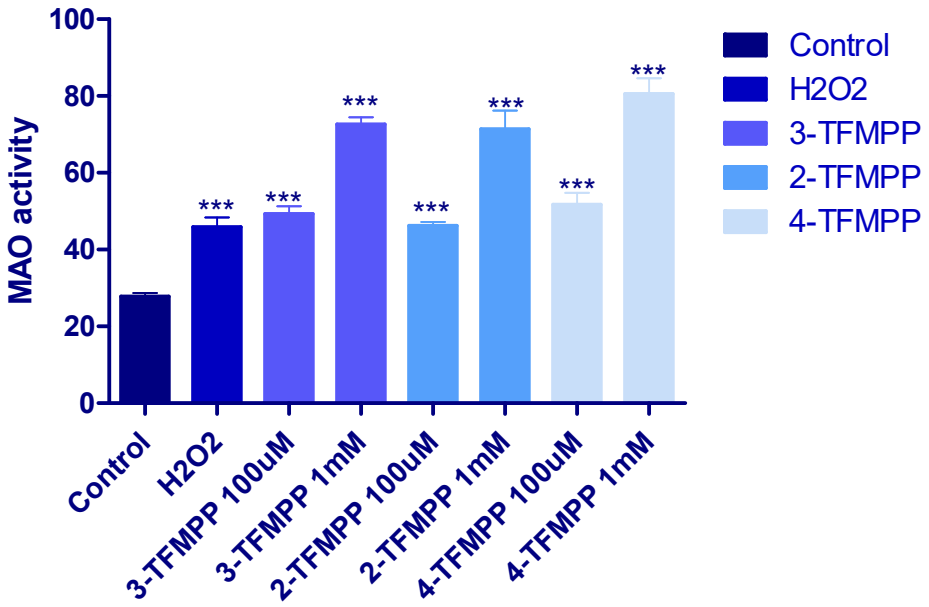


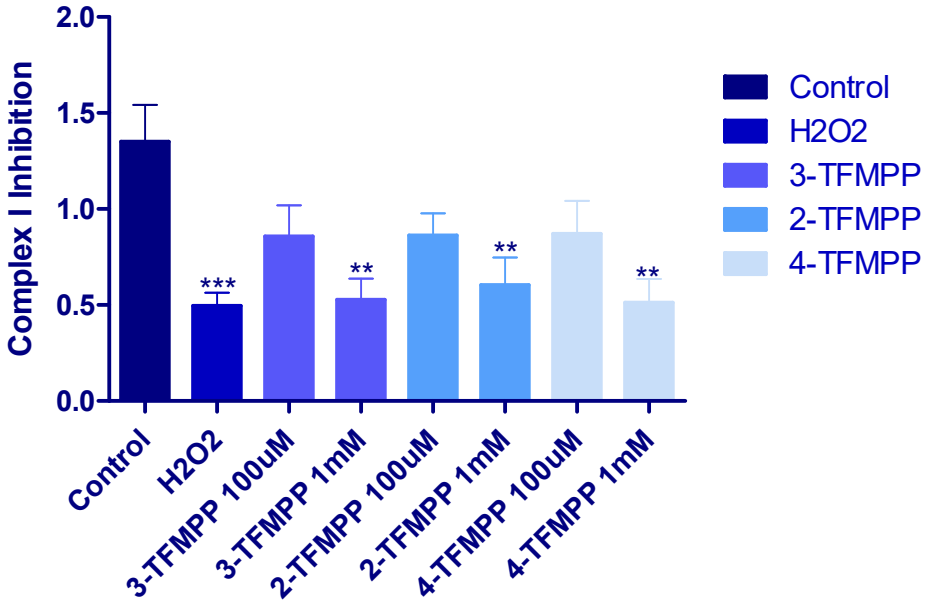
Figure 3.10. Effect of TFMPP derivatives on mitochondrial monoamine oxidase (MAO) activity in N27 cells

TFMPP caused significant increase in MAO activity in a dose-dependent manner (n=5, p<0.05; Figure) in N27 cells after 24 hours incubation. Total MAO activity was determined fluorometrically at 315 nm excitation / 380 nm emission. 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.

3.8. TFMPP derivatives Inhibits Mitochondrial Complex-I activity without affecting Complex IV activity

The main role of mitochondria in the cell is energy production (ATP) through respiration. Thus, the mitochondria play vital role in regulating cell survival and death. Mitochondrial complex-I and complex- IV deficits are involved in the aging process and various neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, Huntington's disease and Amyotrophic lateral sclerosis (M. T. Lin & Beal, 2006). TFMPP derivatives exhibited inhibition of Complex-I activity in a dose-dependent manner. However, TFMPP derivatives at high dose (1mM) exhibited significant inhibition of Complex-I activity as compared to the control (n=5, $p < 0.0020$; Figure 3). However, TFMPP did not demonstrate similar inhibitory effects on Complex-IV activity.

a) Complex I



b) Complex IV

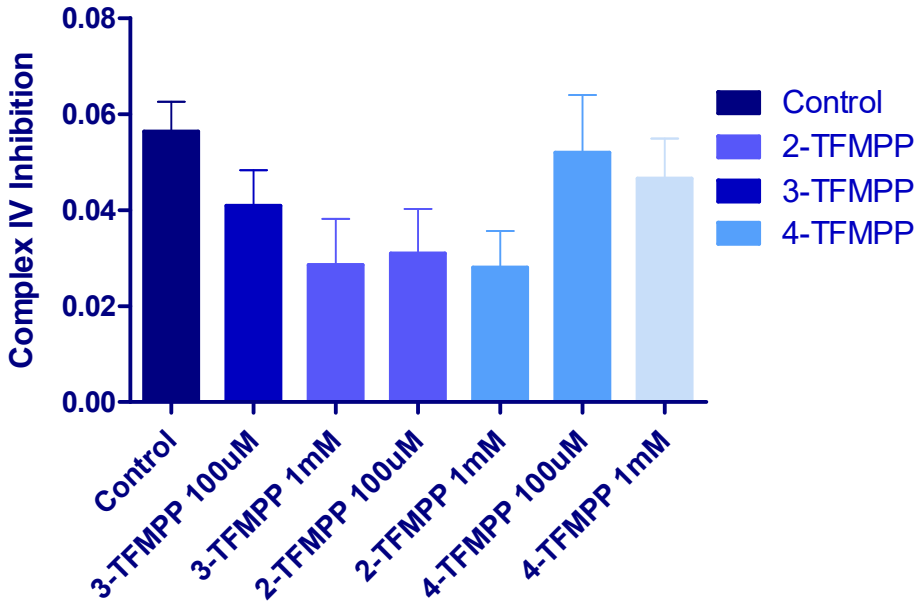


Figure 3.11. Effect of TFMPP derivatives on Mitochondrial complex activity in N27 cells

TFMPP showed remarkable inhibition of Complex-I activity in a dose-dependent manner (n=5, $p<0.05$; Figure 3. A) in N27 cells after 24 hours incubation. Nonetheless, TFMPP did not show comparable inhibitory effect on Complex-IV activity (n=5; Figure 3. B). 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.

4. Discussion

The usage of illegal drugs in the United States has increased significantly in the past few years (National Survey on Drug Use and Health (NSDUH) conducted by the Substance Abuse and Mental Health Services). Substance abuse (drug abuse) denotes to a pattern of harmful or hazardous use of psychoactive substances, including alcohol and illicit drugs. These substances of abuse results in physical and psychological dependence. Dependence indicates a cluster of behavioral, mental, and physical symptoms resulting from chronic drug use. The dependence comprises of a strong craving to take the drug, difficulties in controlling drug use, and persisting in drug abuse despite the harmful consequences. Thus, dependence for substances of abuse leads to higher priority to drug use than to other social, occupational, interpersonal, and scholastic activities and obligations. Furthermore, repeated drug abuse leads to increased tolerance of the drug (requiring more of the drug to achieve the same effects), drug addiction, and a withdrawal state if the drug use is abruptly discontinued. Many individuals who develop substance abuse and addiction are also struggling with an undiagnosed and untreated mental illness. The most frequent co-occurring disorders include:

- ✓ Alcoholism
- ✓ Anxiety disorders
- ✓ Bipolar disorder

- ✓ Conduct disorders
- ✓ Depressive disorders
- ✓ Eating disorders
- ✓ Panic disorder
- ✓ Post-traumatic stress disorder
- ✓ Schizophrenia

The effects of addiction and abuse of drugs can be all-encompassing, leaving virtually no part of an addict's life untouched. While the effects of chronic drug abuse will vary among individuals, the most common effects of drug abuse may include:

- ✓ Accidents
- ✓ Addiction
- ✓ Cardiovascular complications
- ✓ Changes in the structure or functioning of the brain
- ✓ Child abuse
- ✓ Crumbling interpersonal relationships
- ✓ Damage to all organ systems in the body
- ✓ Divorce
- ✓ Domestic abuse
- ✓ Financial ruin
- ✓ Heart attacks
- ✓ Impaired decision-making
- ✓ Incarceration
- ✓ Increased infections

- ✓ Increasing medical problems
- ✓ Legal problems
- ✓ Liver damage and/or failure
- ✓ Nausea, vomiting, and abdominal pain
- ✓ Permanent brain damage
- ✓ Seizures
- ✓ Strokes
- ✓ Tolerance
- ✓ Unintentional injuries
- ✓ Weakening of immune system
- ✓ Worsening of emotional wellbeing

The most common drugs of abuse are cocaine, heroin, inhalants, marijuana, methamphetamine and prescription drugs. However, designer drugs are currently being abused more throughout the world. Our studies show generation of reactive oxygen species leading to the depletion of antioxidant glutathione, which resulted in lipid peroxidation. Increased lipid peroxide formation due to ROS affects the cell membrane integrity and permeability; and affects mitochondrial function. In our study, TFMPP derivatives induced oxidative stress by affecting the superoxide dismutase activity but had no effect on the catalase activity. Furthermore, TFMPP also significantly increased the monoamine oxidase activity which further can increase the formation of hydrogen peroxide which can increase the generation of free radicals. With regard to the Complex-I activity, TFMPP derivatives had similar effect as compared to other dopaminergic neurotoxins. TFMPP derivatives inhibited the Complex-I activity. Interestingly, they had less effect on Complex-IV

activity. Thus oxidative stress and mitochondrial dysfunction can lead to cell death. Based on our results, within the three TFMPP derivatives, 3-TFMPP looks to be more toxic as compared to the 2 and 4 TFMPP.

5. Conclusion

TFMPP is an incipient psychoactive designer drug, whose abuse is engaged for stimulating and recreational activities worldwide. As with other drugs of abuse, TFMPP acts predominantly on monoaminergic neurotransmission, thus causing psychostimulatory effects. Abuse of TFMPP has led to increased mortality and morbidity. Few studies have investigated the potential risks and mechanisms of toxicity associated with TFMPP. Our *in vitro* study, clearly indicates that TFMPP derivatives are neurotoxic to the dopaminergic cells. TFMPP derivatives exert their neurotoxic effects by inducing oxidative stress and mitochondrial dysfunction. *In vivo* and extensive clinical investigations into the toxic effects / abuse of TFMPP are much required because these drugs are available easily in many countries and online. Consequently, there is an urgent need for detailed research to identify appropriate drug therapy to treat TFMPP-induced pathologies.

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