Assessing the toxic effects of ‘nyaope’ on the liver and brain, both *in vitro* (on HepG2 and SH-SY5Y cell lines) and in *vivo* (Wistar rats)

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Master of Science (Medicine)

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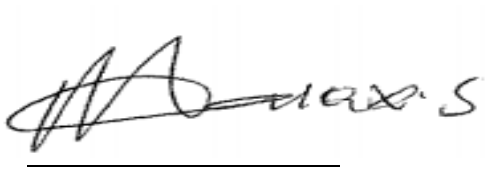
Declaration

I, Sharlot Malatswana, declare that this dissertation 'Assessing the toxic effects of 'nyaope' on the liver and brain, both *in vitro* (on HepG2 and SH-SY5Y cell lines) and *in vivo* (Wistar rats)' is my own unaided work. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University



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5 MAY 2021, Johannesburg

Abstract

The use and abuse of psychoactive drugs continue to escalate at an alarming rate worldwide, with South Africa identified as one of the leading drug regions in the world. The introduction of a new designer drug, ‘nyaope,’ has worsened the overwhelming increment in the drug use problem in South Africa. Nyaope, is a highly addictive illegal psychoactive cocktail drug presented as a fine white to brown powder, containing large amounts of heroin and other substances such as rat poison, caffeine, anti-retroviral drugs (ARVs) and detergent powder. Their contribution to the toxic effects of nyaope remains unknown despite literature reporting the detrimental effects of some of them.

Our study design included both *in vitro* and *in vivo* experiments. For in vitro experiments HepG2 cells were used as surrogate liver cells and SH-SY5Y cells represented neurons. These cells were treated with increasing concentrations of nyaope and the effects thereof on cell morphology, cell viability and cell death were subsequently investigated at various time points. For the *in vivo* experiments, Wistar rats received intraperitoneal injections of nyaope for 3 consecutive days. On day 3 liver tissue and the prefrontal cortex were dissected and subsequently analyzed by polymerase chain reaction (PCR) methodology for the presence of cell death markers.

Our data showed that nyaope caused a significant decrease in cell confluence and aberrant adherence with many cells floating in the cultured medium, suggesting that nyaope created an unfavourable growth environment for the cells. This observation was supported by a nyaope-induced, concentration dependent, decrease in cell viability in both HepG2 and SH-SY5Y cells. Measurement of lactase dehydrogenase (LDH) activity showed that nyaope exposure resulted in necrotic cell death in SH-SY5Y cells. The evidence for an apoptotic form of cell death caused by nyaope was less convincing with mixed findings observed for the markers (Bax/Bcl-2) studied. Our data further suggested that the autophagy process could have been partially initiated after nyaope exposure as increased p62 and reduced LC3, at the 24-hour time point, were recorded.

Results from the *in vivo* experiments reflect the *in vitro* findings with some evidence of the activation of apoptotic and autophagic pathways being observed, albeit differently in the two tissue types studied.

To our knowledge, this is the first study to determine the effects of nyaope on cell viability and death, with a focus on the liver and the brain. Nevertheless, our findings indicate that nyaope may have deleterious effects on these organs that may contribute to its malfunctioning. However, more studies are required to expand on these findings to create a more complete picture of nyaope toxicity.

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My siblings Dinah, Sipho and Boitumelo. Thanks for support and help in raising my kids.

List of abbreviations

µg	Microgram
µL	Microliter
µg/µl	microgram/microliter
AIDS	Acquired Immune Deficiency Syndrome
ANOVA	One-way analysis of variance
ARVs	Anti-retroviral drugs
BLA	Basolateral amygdala
CGC	Cerebellar granule cells
CNS	Central Nervous System
CT	Threshold Cycle
DSM-V	Diagnostic and Statistical Manual of Mental Disorders
DMSO	Dimethyl-sulfoxide
D1	Dopamine type 1 receptor
D2	Dopamine type 2 receptor
dHPC	Dorsal hippocampus
DMEM	Dulbecco's Modified Eagle's Medium
JKN	c-Jun-N-terminal kinase
FCS	Foetal Calf Serum
FC	Frontal Cortex
Hipp	Hippocampus
HIV	Human Immunodeficiency Virus
IL	Infralimbic
GC-MS	Gas Chromatography Mass Spectrometry
LDH	Lactate dehydrogenase
LHb	Lateral habenula
LTD	Laterodorsal tegmentum
Mpfc	Medial pre-frontal cortex
MDT	Mediodorsal thalamus
NAcc	Nucleus accumbens
MTT	3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide
OFC	Orbitofrontal cortex
PVT	Paraventricular nucleus
PE	Phosphatidylethanolamine
THC	D9-tetrahydrocannabinol
PFC	Pre-frontal cortex
ROS	Reactive Oxygen Species
RMTg	Rostromedial tegmentum
SUD	Substance Use Disorder
STN	Subthalamic nucleus
SOD	Superoxide dismutase
Thal	Thalamus
TOF-DSA	Time-of-Flight Mass Spectrometry
vHPC	Ventral hippocampus
VTA	Ventral tegmental area
VP	Ventral Pallidum

WDR	World Drug Report
WST1	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt
XXT	2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-carboxanilide-2H- tetrazolium, monosodium salt

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Chapter 1: Introduction

1.1 Introduction

The recreational use of psychoactive substances has become the major risk factor in the development of substance-related addictive behaviours globally and in South Africa (World Drug Report, 2017; Mokwena, 2016; EMCDDA, 2015). The trafficking and use of psychoactive substances has seen a steady increase, specifically within the last 25 years (Mokwena, 2016). Drug use and dependence presents itself with complicated challenges that threatens the social fabric of society and has severe complications for both users and non-users (Khine *et al.*, 2015; Mokwena and Huma, 2014). The patterns of drug use have been linked to regional and country variations, socio-economic status, racial and geographical difference.

In South Africa, heroin is the most commonly abused drug (Morgan *et al*., 2019). Currently the abuse of heroin-based powder known as nyaope has gained popularity mostly in disadvantaged communities across South African provinces (Mokwena and Huma, 2014; Khine *et al*., 2015; Morgan *et al*., 2019). Previous studies conducted across twelve different regions in South Africa indicate that nyaope mainly consists of a high quantity of heroin together with minor quantities of other substances such as caffeine, antiretroviral drugs, and antidepressants (Khine *et al*., 2015: Mthembi *et al*., 2018). Nyaope compound is a highly addictive mixture, which is mostly smoked in combination with cannabis (Mokwena and Huma, 2014 and Khine *et al*., 2015).

Nyaope use have been identified as the main contributors to the social challenges such as crime, gangsterism, domestic violence, and unemployment as the user resorts to criminal behaviour to quench the drug thirst (Henkel, 2011; Gauteng Department of Community Safety, 2014). Despite this growing concern, treatment and management of nyaope use disorder remains a challenge. The neurotoxic effects of nyaope are largely unknown or, in many instances, not well understood. Recently, nyaope use was associated with neuroanatomical changes such as extensive grey matter atrophy in the right hemisphere (medial orbitofrontal, rostral middle frontal, superior temporal, superior frontal and supra-marginal gyri). These cortical changes were therefore concentrated in areas involved in decision making, social and self-perception, impulse control and executive functions (Ndlovu *et al*., 2021).

1.2 Aim and objectives of the present study

The overall aim of this study therefore was to investigate the toxic effects of nyaope on liver and brain tissue. This was done in order to demonstrate peripheral and central deleterious effects following nyaope consumption. Hepatocytes (HepG2 cells) and neuroblastoma (SH-SY5Y) cells were used for *in vitro* characterization of nyaope-induced harmful effects. *In vivo* experiments were subsequently performed to see whether some of the effects could be repeated in a whole body system.

Our specific objectives for the study were

a. To assess whether nyaope was toxic to hepatocytes. Here HepG2 cells were exposed to nyaope and evaluated microscopically together with the trypan blue exclusion assay.
b. To assess whether nyaope was toxic to neurons. Here neuroblastoma cells were exposed to various concentrations of nyaope for different time periods to determine the optimal conditions at which nyaope toxicity occurs. MTT assays were done to assess cell viability after nyaope exposure.
c. To determine the mode of cell death induced by nyaope. Here we determined whether the drug activated necrotic, apoptotic, and/or autophagic modes of cell death. For necrosis the levels of lactate dehydrogenase in the culture medium was measured. For apoptosis we measured the expression of Bcl-2 and Bax. For autophagy the expression levels of LC3 and p62 were determined.
d. To establish whether some of these effects also occurred *in vivo*, we measured Bcl-2, Bax, LC3 and p62 in the liver and frontal cortex of nyaope-treated rats.

1.3 Organisation of thesis

This thesis is arranged into six chapters, the first being a brief introduction about drug use and abuse, use of nyaope and aim and objectives. Chapter two addresses the relevant literature surrounding the trend of drug abuse worldwide, Africa and South Africa, nyaope problems, its use and composition. It further focuses on substance use disorder, mechanism of action for drugs of abuse, brains areas affected by drug of abuse, linking into literature discussing endogenous opiates and opiates receptors. The chapter finally moves onto the concept of cellular stress and *in vitro* methods used to assess neurotoxicity. The third chapter describes the methods used to attain results and is divided into two parts: the 1) the *in vitro* experiments and *in vivo* experiments. The fourth chapter presents the results of the morphological, biochemical and statistical analyses, which will be used to investigate the effect of nyaope on cells and rats. The fifth chapter presents a discussion of these results. Chapter six provide conclusions and suggest further research avenues.

Chapter 2: Literature review

2.1 World drug situation

According to the World Drug Report (WDR) of 2017 about 29.5 million people globally suffer from drug use disorder. The report further states that in 2016 an estimated 5% of the global population used psychoactive drugs at least once in a year. Opioids were the most harmful drug type reported and it accounted for more than 70% of the disorders and negative health consequences associated with drugs worldwide (World Drug Report, 2017). A review conducted by Peacock and colleagues (2017), found that in 2015 the prevalence of heavy episodic alcohol use was estimated to be 18.4% among adults, 15.2% of adults consumed tobacco daily, while the past-year use of cannabis, amphetamine, opioid and cocaine was 3.8, 0.77, 0.37 and 0.35% respectively (Peacock *et al*., 2017).

The age-standardized prevalence of alcohol dependence was 843.2 per 100 000 people; for cannabis, opioids, amphetamines and cocaine dependence it was 259.3, 220.4, 86.0 and 52.5 per 100 000 people, respectively. The North America region has been indicated to have amongst the highest rates of cannabis, opioid and cocaine dependence (Peacock *et al.,* 2017). In 2015, 307 400 substance use disorder (SUD) mortalities were reported, of these the highest numbers were from alcohol use disorder with 137 500 mortalities, followed by opioid use disorder, amphetamines and cocaine with 122 100, 12 200 and 11 100 respectively (Lancet, 2016).

2.2 Drug use in Africa

African countries are experiencing similar increases in drug abuse compared to the rest of the world, adding to the existing double burden of communicable and non-communicable diseases. In Africa this unexpected escalation in drug users pose a big problem to the healthcare system which is already struggling to provide basic health service. Rapid changes in the economy, political instability, social inequality and poverty play an important role in driving young adults to engage in risky behaviour. In general, African countries also experience a huge surge in urbanisation. Despite signs of a growth in disposable income and improved gender inequality, unemployment rates amongst the youth remains high. These factors strongly contribute to the substance disorder conundrum (Odejide, 2006).

Focusing on sub-Saharan Africa a review of 27 studies showed that overall prevalence of substance use among adolescents were 41,6%, with the highest rate of 55.5% recorded for central Africa. Caffeine containing products were among the mostly abused substances (41.2%), followed by alcohol (32.8%), tobacco products (23.5%), cannabis (15.9%), depressants (11.3%), amphetamine (9.4%), heroin (4.0%) and lastly cocaine (3.9%) (Olawole-Isaac et al., 2018).

2.3 Drug use in South Africa

The past few years have seen an increase in the manufacturing, use and distribution of drugs, either for medical or non-medical use, in South Africa (Khine *et al*., 2015; Mokwena and Huma, 2014). The problem has grown to such an extent that the country was identified as one of the leading drug capitals in the world. Substance dependency statistics show that drug use in South Africa is approximately twice the global average (UNODC, 2014). In 2016 about 60% of substance use was reported with cannabis been the most abused drug, followed by cocaine, benzodiazepines, opiates, hallucinogens and meth cathinone (UNODC, 2016). Recently researchers have reported a shift in the drug consumption pattern of youth in South Africa (especially the Northern provinces) with a great concern being expressed for the use of nyaope (Fernandesa and Mokwena, 2016).

2.4 Nyaope problem in South Africa

The introduction of a new designer drug ‘nyaope’ has worsened the overwhelming increment in the drug use problem in South Africa (Mokwena 2015; Grelotti *et al*., 2014; Skeen *et al.,* 2010). Nyaope, is an illegal psychoactive cocktail drug presented as a fine white to brown powder. It is a highly addictive mixture which is smoked, snorted or injected by users (Morgan *et al*., 2019; Mokwena, 2015). Its psycho-stimulatory effect is mainly attributed to heroin, the major constituent of nyaope (Khine *et al*., 2015). Apparently nyaope first appeared in a South African township (Durban) around 2010 and gradually spread to other impoverished areas across the country (Khine *et al*., 2015). It is prepared and distributed primarily among young, unemployed adults, mostly in townships and disadvantaged areas in the northern provinces of

South Africa. The increase in the use and abuse of nyaope has been largely observed among males of non-Caucasian origin (Mokwena and Huma, 2014).

The increase in the distribution and use of nyaope across South African communities is further associated with high levels of criminality as nyaope users resort to any means to quench their drug habits. This includes the stealing of valuable goods to less valuable items such as kitchen utensils (Mthembi *et al*., 2018). Nyaope is readily available and easily accessible due to its low cost. One joint can be readily sourced from as little as R30.00 per “brown bag” or “joint”, which makes nyaope one of the cheapest illicit drugs in South Africa (Fernandes and Mokwena, 2016).

2.5 Chemical composition of nyaope

A few studies have focused on the chemical analysis and profiling of nyaope (Mthembi *et al*., 2018, Khine *et al*., 2015). It is believed that a thorough understanding of the relative proportions of the various components, as well as chemical profiling of nyaope, will assist relevant stakeholders in prosecuting those involved in the manufacture, trafficking and distribution of the drug, thereby hopefully minimizing the availability and eventual use of the drug.

Time-of-Flight Mass Spectrometry with direct sample analysis (TOF-DSA MS) and Gas Chromatography Mass Spectrometry (GC-MS) are methods that have been used for the chemical analysis of nyaope (Mthembi *et al*., 2018; Khine *et al*., 2015). Data obtained from our collaborators (Department of Chemical Pathology, School of Pathology, University of the Witwatersrand), and others (Mokwena, 2015; Khine *et al*., 2015), show that nyaope is composed mainly of heroin mixed with other substances such as rat poison, caffeine, anti-retroviral drugs (ARVs) and detergent powder. These latter substances occur in lesser amounts than heroin and are generally used as cutting / bulking agents (Khine *et al*., 2018; Mokwena and Huma, 2014). Their contribution to the toxic effects of nyaope remains unknown despite literature reporting the detrimental effects of some of them.

Data from a recent study (Morgan *et al*., 2019) in our laboratory showed that nyaope is mostly used in combination with cannabis. The combination of heroin and cannabis may be prepared by the drug dealers or mixed together by the users of the drugs themselves, resulting in a mixture of powder and plant material. In comparison to other

street drugs, such as crack cocaine and tik (methamphetamine), nyaope is considered more problematic in that it appears to have greater toxic effects on the central nervous system because of its high content of heroin (Monyaka, 2018). A short overview of the ingredients used in the manufacturing of nyaope follows below.

2.5.1 Heroin

Heroin is being used as the main ingredient for nyaope. Heroin is a white powder that is readily soluble in water. Heroin is commonly used in conditions where patients experience severe pain, including cancer (Kaye *et al*., 2014). However, it is highly addictive and carry a high risk of being abused (Scholar, 2015), as the drug does not only reduce pain perception, but also produce pleasurable, euphoric effects (Dickson *et al*., 2010).

The major pharmacological effects of heroin can be attributed to some structural properties of the morphine molecule. The introduction of just two esters onto the morphine molecule changes the physical properties of the substance such that there is a significant increase in solubility, permitting solutions with increased drug concentrations (Hosztafi, 2003). Hence heroin is more potent and faster acting than morphine as an analgesic drug (Robert *et al*., 2019). Heroin active metabolites, 6-O-acetylmorphine and morphine, bind specifically to the mu-opioid receptors of the central nervous system. Furthermore, chronic administration of heroin was shown to be characterized by a shortened duration and decreased intensity of the analgesic, euphoric, sedative respiratory depression and physical dependence. These pharmacological actions are all mediated by mu receptors (Hosztafi, 2003).

2.5.2 Antiretroviral drugs (ARVs)

Efavirenz, belongs to a class of medications known as non-nucleoside reverse transcriptase enzyme inhibitors, which is used as part of the human immunodeficiency virus/ acquired immunodeficiency syndrome (HIV/AIDS) treatment regime due to its highly effective inhibitory activity against the transcription of the human immunodeficiency virus-1 (HIV-1) (Best and Goicoechea, 2008). Interestingly efavirenz was reported to be a hallucinogen (Turjanski and Lloyd, 2005). A combination of efavirenz and D9-

tetrahydrocannabinol (THC found in cannabis) has been proposed to result in stronger hallucinating power and increased psychoactive potency (Larkan *et al*., 2010; Turjanski and Lloyd 2005). This apparent “benefit” of ARVs has led to a number of other social challenges. The use of ARVs during the manufacturing process of nyaope has reportedly led to health facilities being robbed and/or corrupt officials selling the ARVs to nyaope producers. More sadly, HIV positive patients are reported to either being robbed or sell the ARVs themselves, thereby defaulting on their treatment (Larkan *et al*., 2010; Inciardi *et al*., 2007). Some of the common psychiatric side-effects of ARVs include agitation, anxiety, hallucinations, insomnia, lethargy, nervousness, mood disorders, depression, suicidality, antisocial behaviour, psychosis, catatonia, delirium and vivid dreams (Turjanski and Lloyd, 2005). One can therefore postulate that the combined consumption of heroin and ARVs will have a significant negative impact on the central nervous system.

2.5.3 Caffeine

Caffeine is another prominent substance that is included in nyaope as a cutting agent. Caffeine is the most widely used psychoactive substance and has sometimes been considered a drug of abuse. Classic drugs of abuse lead to specific increases in cerebral functional activity and dopamine release in the shell of the nucleus accumbens (the key neural structure for reward, motivation, and addiction). In contrast, caffeine, at doses reflecting daily human consumption, does not induce a release of dopamine in the shell of the nucleus accumbens, but leads to a release of dopamine in the prefrontal cortex, which is consistent with its reinforcing properties (Górska *et al*., 2018).

2.6 Substance-related disorders

According to the Diagnostic and Statistical Manual of Mental Disorders (DSM–V) the repeated consumption of illicit substances can lead to two broad classes of pathological conditions namely, substance-induced and substance use disorders. Substance-induced disorders refer to conditions such as intoxication, withdrawal, or substance-induced mental abnormalities (for example psychosis, depression, anxiety,

sleep abnormalities, delirium). The focus of the present dissertation however will mainly be on substance use disorder (SUD).

Substance use disorder (SUD) is a chronically relapsing disorder that has been characterized by a compulsive desire for the drug, loss of control in limiting intake, and the emergence of a negative emotional state (e.g, dysphoria, anxiety, irritability) reflecting a motivational withdrawal syndrome when access to the drug is prevented (Koob and Le Moal, 1997). It has been claimed that repeated exposure to an addictive stimulus is the core pathology that drives the development and maintenance of an addictive behaviour (Nestler, 2013). There are 10 classes of substances that fall within the category of having addictive potential. These are alcohol, caffeine, inhalants, stimulants, opioids, tobacco, sedatives, hypnotics, hallucinogens and other unknown substances (DSM-V). A common feature of these substances is that they all activate the brain's reward system, albeit by different mechanisms, that drive the development of additive behaviours and drug-associated memories (Goodman and Packard, 2016).

Dopamine plays a prominent role in the regulation of movement, cognition, motivation, emotion, and pleasure. As such dopamine is said to be the primary neurotransmitter responsible for the reinforcing properties of substances and therefore almost all addictive substances act either directly or indirectly upon the brains reward system by increasing dopaminergic activity (Volkow *et al*., 2007). Thus excessive use of addictive drugs results in prolonged release of dopamine, which in turn affects the reward pathways directly through heightened dopamine receptor activation. Prolonged and abnormally high levels of dopamine within the synaptic cleft can induce dopamine receptor downregulation, resulting in decreased receptor sensitivity. The intake of larger amounts of the substance is subsequently required to produce the desired pleasurable effects leading to tolerance and substance dependence (Volkow *et al*., 2007).

An alternative view of SUD is that it is a disorder that arises through the interplay of a number of neurophysiological processes such as neurotransmission, intraneuronal signalling, neuroplasticity, and transcriptional and epigenetic mechanisms (Racs, 2014; Chen *et al*., 2010; Nestler, 2008). These processes then manifest as alterations in (i) dopamine activity and release (Nutt *et al*., 2015; Volkow, 2007), (ii) gene expression in mesocorticolimbic projections (Rawas *et al*., 2012) and (iii) epigenetic

regulation of gene expression (Ajonijebu *et al*., 2019). The greater comprehensivity of this view has enjoyed substantial acceptability by researchers in the field of substance abuse.

2.7 Brain areas important in SUD

Central to the brain reward system is the meso-cortico-limbic dopaminergic projection that arises in the ventral tegmental area and projects to forebrain structures such as the ventral striatum (nucleus accumbens), prefrontal cortex and the limbic region (Roberts and Koob, 1997). However other brain structures such as the thalamus (Zhu *et al*., 2016), hypothalamus (Aston-Jones *et al*., 2010) and habenula (Velasquez et al., 2014) have also been associated with addictive behaviours.

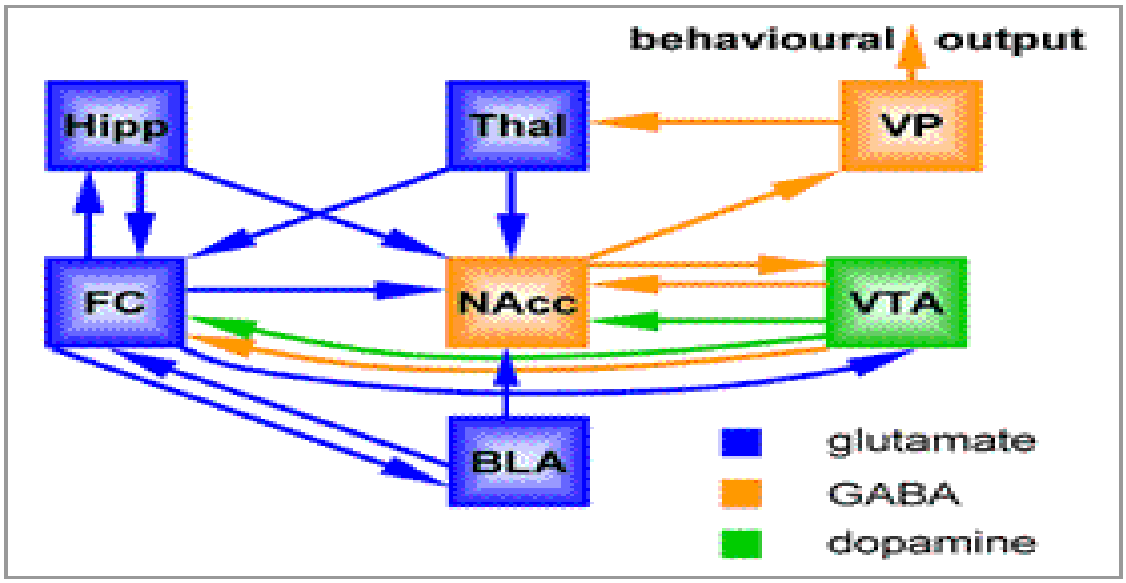

Figure 1: Neuronal pathways involved in substance use disorder (SUD). GABAergic projections are shown in orange, Glutamatergic projections in blue and dopaminergic neurons in green. Abbreviations: VTA, ventral tegmental area; NAcc, nucleus accumbens; FC, frontal cortex; Hipp, hippocampus; Thal, thalamus; VP, ventral pallidum; BLA, basolateral amygdala (NIDA, 2007)

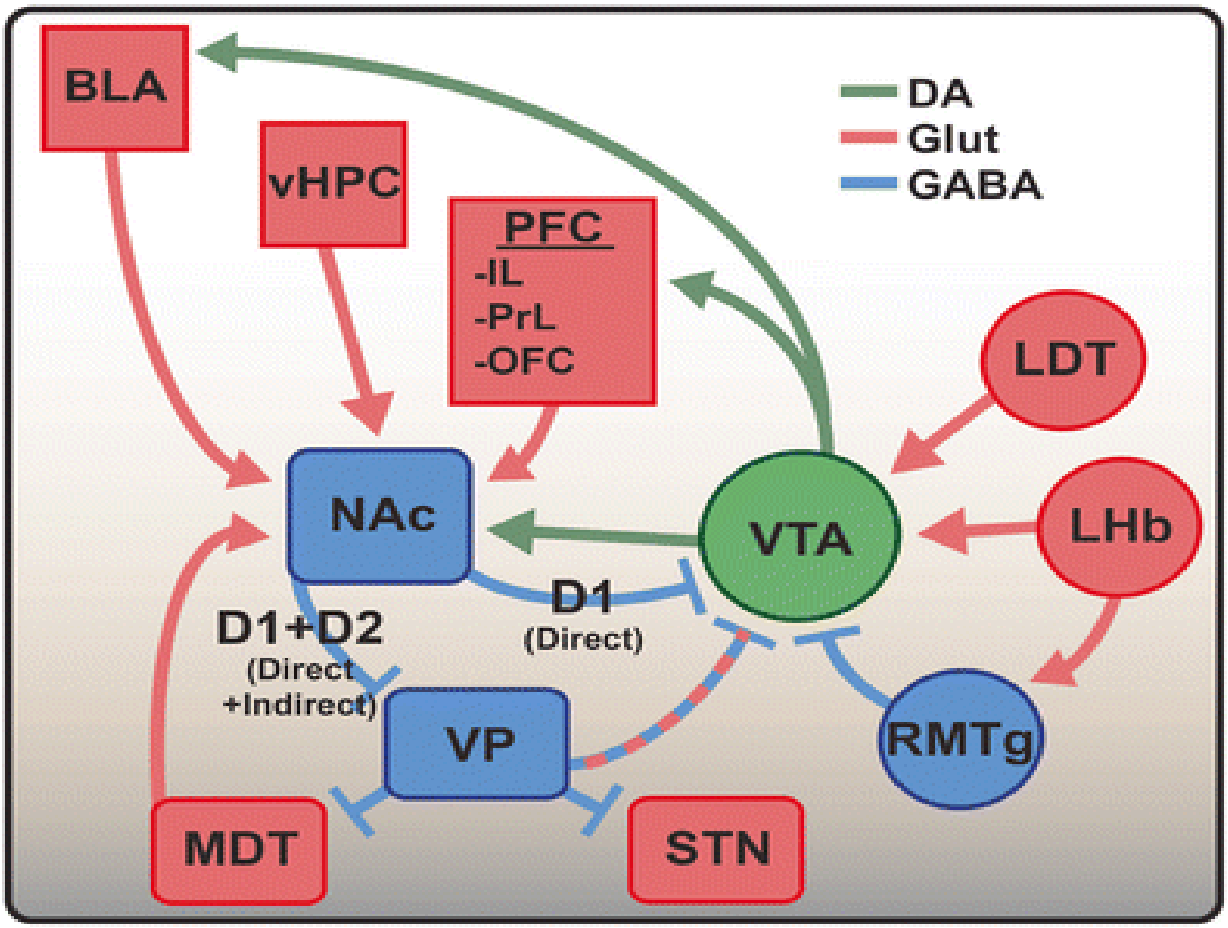

Figure 2: The reward circuitry showing major projections of GABAergic neurons in blue, glutamatergic neurons in red and dopaminergic neurons in green. Abbreviations: VTA, ventral tegmental area; LDT, laterodorsal tegmentum; LHb, lateral habenula; NAc, nucleus accumbens; vHPC, ventral hippocampus; PFC, pre frontal cortex, IL, infralimbic; PrL, prelimbic; OFC, orbitofrontal cortex; D1, dopamine type 1 receptor; D2, dopamine type 2 receptors; MDT, mediodorsal thalamus; RMTg, rostromedial tegmentum; STN, subthalamic nucleus; VP, ventral pallidum (NIDA, 2007).

2.7.1 The ventral tegmental area (VTA)

Ventral tegmental area (VTA) is a brain region, located in the midbrain, adjacent to the substantia nigra. It is comprised of several group of neurons located around the midline of the midbrain floor, with about 60-65% dopaminergic neurons, about 30-35% GABAergic neurons and 2-3% glutamatergic neurons (NIDA, 2007). The dopaminergic VTA neurons project to the NAc (mesolimbic pathway), PFC (mesocortical pathway), amygdala and hippocampus.

Glutamatergic neurons located in the laterodorsal tegmentum synapse onto VTA dopaminergic neurons, thereby promoting the release of dopamine in the NAc. Activation of this VTA-NAcc pathway increases reward behaviour. In contrast, activation of glutamatergic lateral habenula neurons that innervate VTA dopaminergic neurons that project to the mPFC, induces aversive behaviour. These two pathways therefore have a strong influence on the final behavioural outcome associated with drug intake. The VTA GABAergic interneurons controls dopaminergic activity by decreasing the firing rate of local dopaminergic neurons, thereby inhibiting the release of dopamine. Reductions in VTA GABAergic neuron activity has been linked to reward regulation, motivation, cognition and aversion (Bouarab *et al*., 2019).

2.7.2 The nucleus accumbens

The nucleus accumbens (NAcc) is a brain region found in the basal forebrain, rostral to the preoptic area of the hypothalamus. It forms a major component of the ventral striatum of the basal ganglia (Ikemoto, 2010). It is divided into two major areas, the shell and the central core. The shell is associated with the limbic system and plays an essential role in drug initiation within the reward circuitry. The central core on the other hand, is connected to the motor system (Malenka *et al*., 2009) and is said to be responsible for the hyperactivity seen in consumers of stimulants. NAcc is filled with GABAergic medium spiny neurons which receive glutamatergic input from the VTA. These neurons project to the pallidum (Ikemoto, 2010). The NAcc is the primary site responsible for reward mediation, reinforcement of learning, and motivation (Salamone *et al*., 2016; Wenzel *et al*., 2015), acquiring and eliciting conditioned behaviour, and increased sensitivity to drugs as drug seeking behaviour progresses (Kelley, 2004).

2.7.3 Hippocampus

Hippocampus is a C-shaped cortical structure located deep in the allocortex in the temporal lobe of the cerebrum, with neural projections into the neocortex (Amaral and Lavenex, 2006). It is one of the recognised brain parts that is proven to play an important role in SUD due to its role in memory and learning. Evidence shows that manipulation of cells located in the hippocampus alters the firing rates of VTA dopaminergic cells that modulate dopamine release in the NAcc (NIDA, 2007).

The hippocampus can be divided along the longitudinal axis into a dorsal and ventral lobe. The dorsal hippocampus (dHPC) is associated with episodic memory and associative learning (Squire, 2007), while the ventral hippocampus (vHPC) is involved in stress response modulation, motivation and regulation of emotions (Kjelstrup *et al*., 2008). The dHPC projects to the retrosplenial area of the anterior cingulate cortex, vHPC and to the VTA via the septum. The vHPC projects to the medial prefrontal cortex, hypothalamus, amygdala, bed nucleus of the stria terminalis, and VTA via the NAcc (Tannenholz *et al*., 2014). The ventral hippocampus projections to NAcc are glutamatergic and they allow the integration of spatial/contextual information to be associated with drug intake. Thus the connection between the vHPC and the NAcc acts to provide contextually relevant emotional information to influence goal directed behaviour.

2.7.4 Prefrontal cortex (PFC)

The prefrontal cortex (PFC) is a brain region located in the cerebral cortex covering the front part of the frontal lobe (Yang and Raine, 2009). It is divided into several sub-regions, which differ in their projections to the NAcc. The Infralimbic (IL) medial prefrontal cortex (mPFC) projects to the NAcc shell, while the prelimbic (PrL) mPFC projects to the NAcc core (NIDA, 2007). The PFC is mainly involved in the integration of information, rationalizations, decision making, intelligent thoughts, and even one's moral compass. The PFC therefore plays an important role in determining whether an addictive behaviour will be elicited (Yang and Raine, 2009).

2.7.5 Thalamus

The thalamus is a paired gray matter structure of the diencephalon (Savage *et al*., 1997), situated above the brain stem between the midbrain and the cortex with nerve fibres projecting outward towards the cerebral cortex (Sherman, 2006). It is composed of a variety of nuclei which serves unique roles, ranging from relaying sensory and motor signals to the regulation of consciousness and alertness. These nuclei are formed by both excitatory and inhibitory neurons (Stein *et al*., 2000). Direct activation of glutamatergic input from the thalamus, especially the midline thalamic nuclei such as the paraventricular nucleus (PVT), into the NAcc (PVT-NAcc pathways) usually

evoke an aversive response. Alterations in the PVT activity that disrupts this PVT-NAcc pathway, has been proven to alter drug related behaviour (NIDA, 2007).

2.7.6 Hypothalamus

The hypothalamus is a brain structure located just beneath the thalamus near the pituitary gland. It is normally considered part of the limbic system (Saper *et al*., 2005). The hypothalamus is composed of multiple subnuclei such as the paraventricular, dorsal and lateral hypothalamic areas. These areas innervate autonomic nuclei in the brain stem, for example; the rostral ventrolateral medulla, dorsal motor nucleus of the vagus, the intermediolateral column of the spinal cord and the nucleus of the solitary tract (Guyenet, 2006).

The hypothalamus regulates many fundamental processes such as body temperature, appetite, sexual behaviour and emotions. In contrast to the medial part of the hypothalamus, which is associated with aversion and displeasure, the lateral regions of the hypothalamus play an important role in regulating emotions such as experiencing pleasure (Virginia *et al*., 2016). The hypothalamus is also instrumental in the stress response. In terms of the drug addiction process, the hypothalamus is shown to be involved in the processing and storing of negative memories associated with drug withdrawal, positive memories associated with drug clues, and the pleasurable relief following unpleasurable experiences during drug withdrawal (Dileone *et al*., 2003). There is therefore no doubt about the importance of the hypothalamus in SUD.

2.7.7 Habenula

The habenula is a brain region situated at the caudal and dorsal part of the thalamus and lateral to the third ventricle. It forms part of the diencephalon and epithalamus (Hikosaka, 2010; Wise, 2004) and acts as a critical point of interaction between the brain stem and forebrain. The habenula receives afferents from both the limbic system and the basal ganglia. In return it contains serotonergic neurons that project to the raphe nuclei, and dopaminergic and glutamatergic neurons projecting to the substantia nigra and VTA. Together these brain regions regulate motivated and cognition behaviours, and play an important role in learning from aversive experiences and the

processing of unpleasant rewards (Cowen and Sherwood, 2013). For example, when experiencing an unpleasant reward such as drug withdrawal or punishment, the habenula sends this information (disappointing rewards) to the substantia nigra and VTA for processing, inhibiting the release of dopamine and serotonin. The mechanism by which the habenula mediates this inhibitory effect is either via direct activation of GABAergic projections (Balcita-Pedicino *et al*., 2011; Brown *et al*., 2017) or indirectly via the activation of GABAergic interneurons (Matzger *et al*., 2017). Thus disturbances in neuron projections to the habenula can result in behavioural disorders such as SUD (Mutsumoto and Hikosana, 2007).

2.8 Opioids and opioid receptors

The opioid receptor family consists of three classical receptors: mu (μ), delta (δ) and kappa (κ) opioid receptors. Opioid receptors are major targets for many drugs of abuse, with complex biological and pharmacological properties. They are distributed throughout the CNS and within peripheral tissues of both neural and non-neural origin (Cichewicz, 2004). Activation of opioid receptors have been closely related to analgesia, but also to tolerance and drug dependence (Manzanares et al., 1999, Pasternak, 2005, Demuth and Molleman, 2006). These receptors are G-protein-coupled and they often mediate their effects by inhibiting the release of neurotransmitters in the CNS (Cichewicz, 2004).

2.8.1 Endogenous opioids system and opioid disorder

The human body naturally produces its own opiate-like substances and uses them as neurotransmitters. These substances include endorphins, enkephalins, and dynorphin, and these are collectively known as endogenous opioids. Endorphin (ligand for μ receptors) shows analgesic properties and plays a role in respiratory inhibition and the decrease of heart rate. Dynorphin (ligand for κ receptors) also shows some analgesic property, but can induce anxiety. Enkephalins (ligand for δ receptors) shows no sign of analgesia and is more involved in protection against insults such as myocardial ischemia. In addition to modulating the body's reaction to painful stimuli, endogenous opioids also regulate vital physiological functions such as hunger, thirst, mood control and immune responses (Liang *et al*., 2016).

Exogenous opioids such as heroin and morphine bind to the same receptors as endogenous opioids exerting similar or exaggerated effects depending on the dose used. The euphoric effects of opiates appear to be mediated by mu and delta receptors and involves GABA-inhibitory interneurons of the ventral tegmental area. By attaching to their mu receptors, exogenous opioids reduce the amount of GABA released. Normally, GABA inhibits neuron firing in the ventral tegmental area, resulting in a diminished amount of dopamine released in the NAcc. By inhibiting this inhibitor, opiates ultimately increase the release and concentration of dopamine in the NAcc with a consequential increase in the level of pleasure experienced (Maurer *et al.,* 2006; Rook *et al*., 2006).

A number of cases of unintentional deaths and unwanted medical conditions have been associated with heroin use (Stam *et al.,* 2019; Love *et al.,* 2018; Darke and Zador, 1996). For instance, extensive damage to the structure and/or function of the lung (Megarbane and Chevillard, 2013) and kidneys (Singh *et al.,* 2013) has previously been reported. Significant morphological changes in the liver tissue such as vesicular changes in hepatocytes, fat changes, chronic hepatitis, and cirrhosis were also reported after intravenous heroin intake (Ilic *et al*., 2005). Of greater concern is the fact that heroin crosses the blood-brain barrier to interact with the central nervous system to cause alterations in neuron structure and functioning (Oldendorf *et al.,* 1972), thus complications such as cerebral oedema, neuron ischemia and neuronal loss have been observed (Buttner *et al.,* 2000). Neuroimaging studies have shown heroin use to be associated with cerebral atrophy and demyelination of white matter (Buttner *et al.,* 2000), while other studies have demonstrated neurovascular complications (Borne *et al.,* 2005), movement disorders (Brust, 2010) and damage to the cerebellum (Manto, 2012).

The mechanisms by which heroin mediates these toxic effects have been a thrust of investigation for many researchers. As such heroin has been shown (i) to induce a dose-dependent decrease in cell viability in PC12 cells (a cell line derived from rat adrenal medulla) (Oliveira *et al*., 2007); (ii) to promote caspase dependent mitochondrial apoptosis in primary cultured rat cortical neurons (Oliveira *et al.*, 2003), and (iii) to activate the oxidative stress-associated c-Jun-N-terminal kinase (JNK) pathway in primary cultured cerebellar granule cells (CGC) (Lai *et al.*, 2011). Other mechanisms by which heroin may generate damaging effects include disruption of

calcium homeostasis (Liu *et al.*, 2014) and epigenetic modification of genes associated with neuronal integrity (Kovatsi *et al.*, 2011). Interestingly reductions in mitochondrial DNA copy number, likely due to autophagy, have also been proposed as a mechanism for opiate-mediated detrimental effects (Feng *et al.*, 2013). It is therefore obvious that heroin has the ability to recruit a variety of molecular strategies to exert its toxic effects.

2.9 Cellular stress response

Cells respond to stress in a variety of ways, either by activating pathways that promote cell survival or by eliciting programmed cell death that eliminates damaged cells. The decisive ability of the cells to elicit protective pathways that favors cell survival or to engage destructive stress responses that favour cell death, depends on numerous factors including the nature, duration, magnitude and frequency of the stressor, as well as the cell type. The various processes involved in cellular stress responses serve the adaptive purpose of protecting a cell against unfavourable environmental conditions, both through short term mechanisms that minimize acute damage to the cell's overall integrity, and through longer term mechanisms which provide the cell a measure of resiliency against similar adverse conditions (Kültz, 2005).

2.10 *In vitro* methods used to assess neurotoxicity

Toxicity testing is an important approach used to assess the impact of chemicals on cells and to determine the extent of tissue damage caused by the toxic substance. A number of standardized protocols have been employed to assess the toxic effects of substances on cells and/or organ function (Polli, 2008). These include but are not limited to assays that measure cytotoxicity by measuring the impact on cell proliferation or cell death (Uysal *et al.*, 2018, Senthilraja and Kathiresan, 2015), assessment of the mode of cell death (Kroemer *et al.*, 2009) and/or the presence of deleterious processes such as oxidative stress (Schönhofen *et al.*, 2015). These cytotoxicity assays are widely used in fundamental research and in drug discovery to screen libraries for toxic compounds (Duzgunes, 2012). Cells exposed to a sublethal insult may either stop actively growing and dividing (a decrease in cell proliferation) or die instantly. Any of these responses can be measured individually or with multiplex assays, to monitor whole cells or subcellular components or organelles.

2.10.1 Determination of cell viability

2.10.1.1 Trypan blue exclusion method

Traditionally, the determination of cell growth as well as cell death can be conducted by counting viable and non-viable cells after staining them with a vital dye. Trypan blue staining is a simple way to evaluate cell membrane integrity (and thus assume cell wellness or death). However, this method is not very sensitive and cannot be adapted for high throughput screening as it fails to distinguish healthy cells from unhealthy alive cells with loss of cell function (Strober, 2001).

2.10.1.2 Mitochondrial enzyme activity

Other methods used for the assessment of cell viability are based on various cellular functions such as enzyme activity, cell membrane permeability, cell adherence, ATP production, co-enzyme production, and nucleotide uptake activity (Uysal *et al.,* 2018). Among them the reduction of certain compounds such as 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) (WST1) or (2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-carboxanilide-2H-tetrazolium, monosodium salt) (XTT), are of the most frequently used methods. The latter assays are based on the fact that living cells metabolize the compounds in their mitochondria to form insoluble coloured formazan crystals and this colour reaction can then be measured by spectrophotometry (Uysal *et al.,* 2018).

The MTT assay specifically measures dehydrogenase activity as an indicator of metabolically active mitochondria, thus cell viability, and requires cell lysis before absorbance can be measured. Healthy and rapidly growing cells normally exhibit high rates of MTT reduction to formazan, while dead or inactive cells fail to do so. The final product of MTT reduction is a purple coloured formazan salt that can be easily dissolved in dimethylsulphoxide (DMSO). Cell viability in the MTT assay, as quantified by formazan formation at 540 nm, is linearly related to the enzyme activity and therefore indirectly to the number of viable cells. High purple colour intensity denotes higher cell viability while a decrease in purple colour intensity signifies reduced cell number and thus cytotoxicity (Sambale *et al*., 2015).

The use of the MTT method does have limitations. Production of formazan salts is influenced by (i) the physiological state of cells and (ii) the variance in mitochondrial dehydrogenase activity in different cell types. Nevertheless, the MTT method of cell determination is useful in the measurement of cell growth in response to mitogens, antigenic stimuli, growth factors and other cell growth promoting reagents, cytotoxicity studies, and in the derivation of cell growth curves (Senthilraja and Kathiresan, 2015; Fotakis and Timbrell, 2006; Nezu *et al*., 1988)

2.10.1.3 Lactate dehydrogenase (LDH) activity

Lactate dehydrogenase is a stable cytosolic enzyme found in all cells with intact plasma membranes. Once the integrity of the cell membrane is compromised, the LDH enzyme is quickly released from cytosol into the cell culture supernatant (Luhr *et al.,* 2018). LDH cytotoxicity assays such as the CytoTox 96® cytotoxicity assay or cytotoxicity detection kit ^{PLUS}, are commonly used commercially available colorimetric assays employed to measure cellular damage (Decker and Lonhman-Matthes, 1988).

The assay involves a coupled two-step reaction. The first step involves the reduction of NAD^+ to $NADH/H^+$ by LDH when lactate is oxidized to pyruvate. In the second step, diaphorase (catalyst) transfers the H/H^+ from $NADH/H^+$ to catalyze the reduction of the tetrazolium salt to a highly coloured formazan solution, which strongly absorbs at 490-520 nm. Thus the amount of formazan formed is proportional to the amount of LDH released into the culture medium.

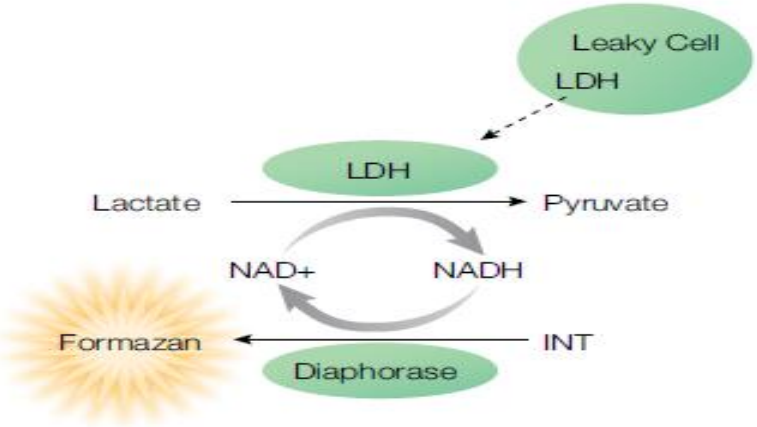

Figure 3: The two-step reaction as described in the cytotoxicity detection kit ^{PLUS} from Roche Diagnostics (SA).

2.10.2 Determination of the mode of cell death

Cytotoxicity often leads to the demise of cells. Cell death normally is a fundamental though complex process occurring under normal physiological as well as pathological conditions (Zhao *et al*., 2014). Like proliferation, migration and differentiation, cell death also plays a variety of roles in human development and survival. Its importance has been well recognized in processes such as the deletion of unwanted cells, recycling of biological macromolecules, maintenance of epithelial barrier function, elimination of abnormal cells and adaptive immunity (Daniel and Hockenbery, 2018). As cell death is considered as the basis of embryogenesis and metamorphosis, cell proliferation is similarly associated with growth. The maintenance of normal structure and function of every human tissue therefore results from a balance between cell production and cell death. This keeps the overall numbers of cells within physiologically appropriate ranges (Proskuryakov *et al.,* 2002). Common modes of cell death include necrosis, apoptosis and autophagy (Kroemer *et al.,* 2009).

2.10.2.1 Necrosis

Necrosis or uncontrolled cell death is usually observed in response to ischaemia or a lethal insult by toxins or other injuries (Li *et al*., 2013). Such a response often results in the unregulated destruction of cell components (Karch and Molkentin, 2015) and presents characteristically with a loss of membrane integrity, followed by cell swelling and lysis. These cellular changes usually elicit an inflammatory cell response by attracting nearby phagocytes to eliminate the dead cells by phagocytosis (Stenson and Ciorba, 2018; Sergey *et al*., 2003). However, microbial damaging substances released by leukocytes have the ability to create collateral damage to surrounding tissues. This excess collateral damage inhibits the healing process. Thus, untreated necrosis results in a build-up of decomposing dead tissue and cell debris at or near the site of the initial cell death (Stenson and Ciorba, 2018). A classic example is gangrene.

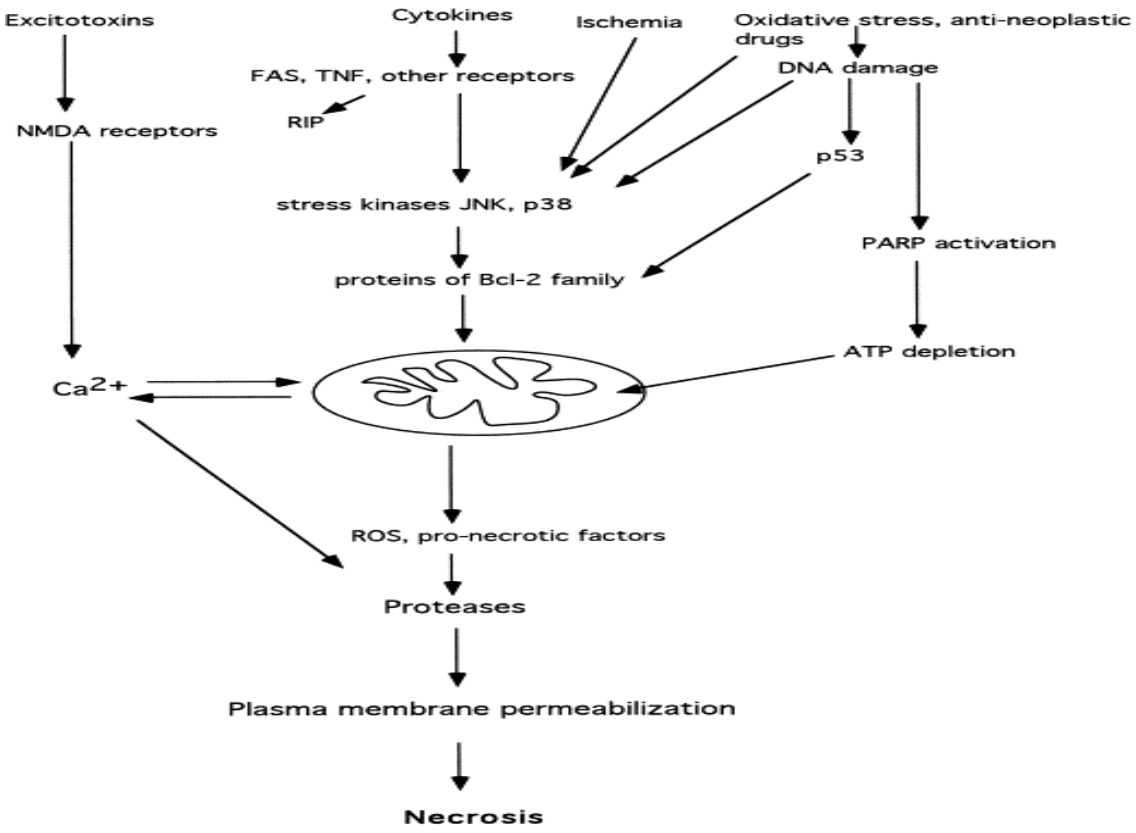

Figure 4: A possible molecular scenario of necrosis (Sergey *et al.*, 2003).

Loss of plasma membrane integrity can be assessed by using cell-impermeable dyes with reasonable accuracy. Usually the dye is permeable to dead cells and non-permeable to live cells. A variety of DNA-binding, impermeable fluorescent dyes exist that have been used to establish a necrotic mode of cell death. These include propidium iodide (PI, MW 668.4 Da) (Sawa and Domae, 2011), 7-amino actinomycin D (7-AAD) (Shibuya *et al*., 2003) or the Sytox® probes (Grootjans *et al*., 2016). The fluorescence intensity of these dyes increases markedly after DNA binding, thus eliminating the need for time-consuming washing steps. This fluorescence intensity shift upon DNA binding can be exploited in assays done in microtiter plates, as only dead cells are intensely fluorescent.

2.10.2.2 Apoptosis

Apoptosis or programmed cell death is a physiological mechanism, characterized by specific morphological and biochemical changes. Morphologically apoptosis is characterised by cell shrinkage, chromatin condensation, nuclear fragmentation, protein cleavage, membrane bleeding and phagocytosis (Kerr *et al*., 2013; Williamson, 2000). Apoptosis is a significant contributor to the morphologic and functional development of multicellular organisms. It is also involved in the pathogenesis of several diseases including degenerative diseases of the central nervous system (CNS) like Alzheimer's disease (Shimohama, 2000) or Parkinson's disease (Lev *et al.*, 2003), cancer (Ouyang *et al*., 2012) and immune system dysfunction (Eguchi, 2001).

Nevertheless, the biological importance of apoptosis is widely recognised as displayed in its involvement in tissue homeostasis of multicellular organisms (Harvey and Kumar, 1998). Apart from its importance in the development of multicellular organisms and in securing constancy of cell numbers for the different tissues, apoptosis is also involved in the deletion of damaged and / or dangerous cells such as autoreactive cells in the immune system. Infected cells are often eliminated by apoptotic mechanisms (D'Arcy, 2019; Elmore, 2007).

Given the role of apoptosis in the maintenance of tissue homeostasis, deletion of autoimmune cells and the removal of damaged cells, it comes as no surprise that dysfunctional apoptosis signalling is implicated in many pathological manifestations (Jan and Chaundhry, 2019). For instance, deficient apoptosis can lead to an accumulation of unwanted cells that are unable to respond normally to apoptotic stimuli. Diseases such as cancer and autoimmune disorders are characterized by this (Nicholson, 2000; Reed, 2002). It has been postulated that apoptosis play an important role in minimising the spread of viral infections (Thompson, 2001), hence abnormalities in apoptotic signalling may be a major reason why some viral infections are more intense than expected. In contrast, excessive apoptosis may also result in diseases where cells are inappropriately removed. Examples of such diseases include neurodegenerative disorders (Shimohama, 2000; Lev *et al.*, 2003).

Apoptosis follows a sequence of molecular events which are triggered by specific signals that instruct the cell to undergo cell death. As such apoptosis is a fundamental

eukaryotic biological process whereby individual cells die by activating their own genetically programmed cell death mechanisms. There are many factors which are involved in the activation, regulation and execution of apoptosis and related events, most of these factors are mostly proteins (Papaliagkas *et al.*, 2007). Apoptosis can be initiated by numerous factors such as oxidative stress, alkylating agents, ionizing radiation and chemotherapeutic agents, or by external factors such as the tumor necrosis factor superfamily of cytokines, the Fas ligand and the TNF-related apoptosis inducing ligand. These factors trigger the apoptotic process either through the intrinsic (Parone *et al.*, 2003) or the extrinsic cell death pathway (Hongmei, 2012).

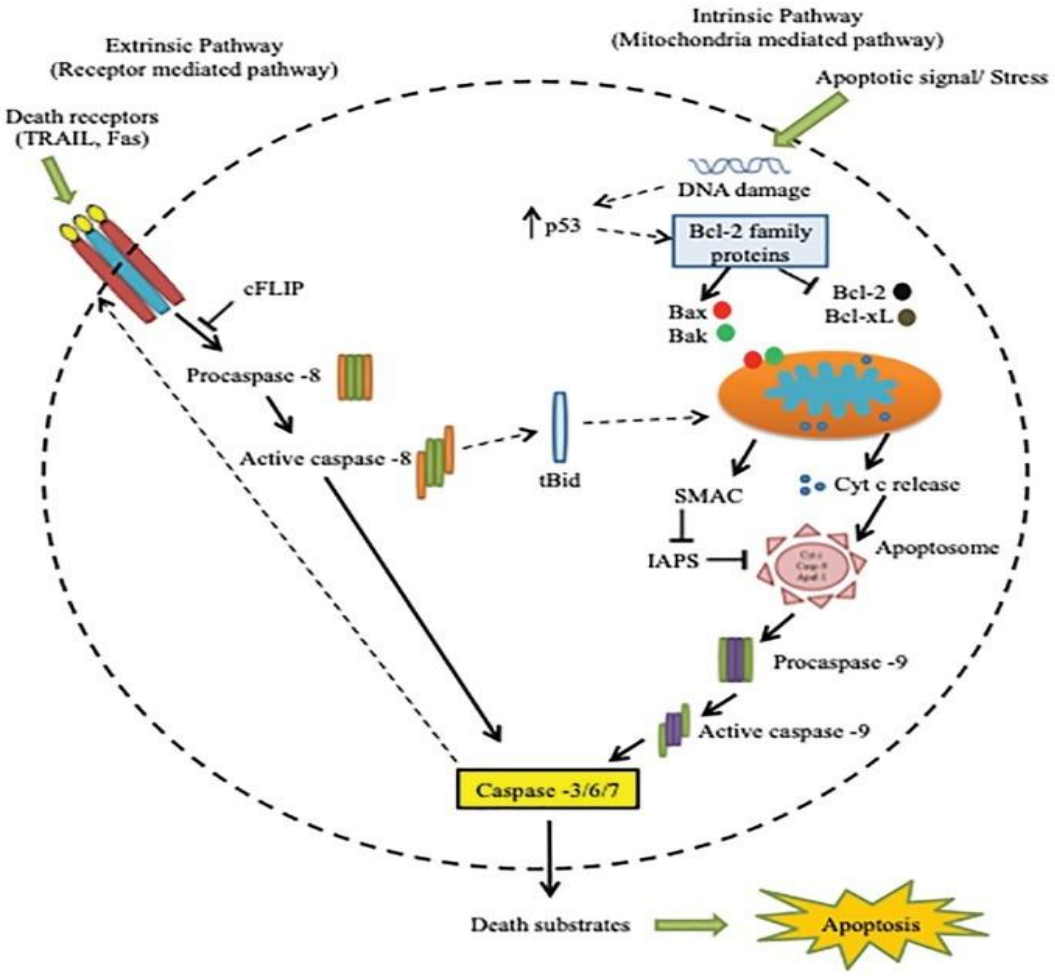

Figure 5: The apoptotic pathway. Apoptosis mainly consists of two main pathways, the intrinsic pathway which is mediated by the Bcl-2 family of proteins and the extrinsic pathway which is triggered by external stimuli or ligand molecules (Jan and Chaudhry, 2019).

Many intracellular death signals are communicated through the intrinsic cell death (or mitochondrial-mediated) pathway, such as DNA damage, oncogene activation, growth factor deprivation, endoplasmic reticulum (ER) stress, and microtubule disruption (Parone *et al.,* 2003). The key step in the intrinsic cell death pathway is permeabilization of the mitochondrial outer membrane, which has been identified as a 'point of no return' after which cells are committed to cell death (Enari *et al.*, 1996). Following mitochondrial permeabilization, the release of various proteins such as cytochrome C, from the mitochondrial inner-membrane, promotes caspase activation and apoptosis. Cytochrome c binds to apoptosis protease-activating factor-1 (APAF1), inducing its oligomerization and thereby forming a structure called the apoptosome. The apoptosome consequently recruits and activates an initiator caspase, caspase-9, which then cleaves and activates the executioner caspases, caspase-3 and -7, leading to apoptosis. This process is controlled by the Bcl-2 family of proteins (Jan and Chaudhry, 2019).

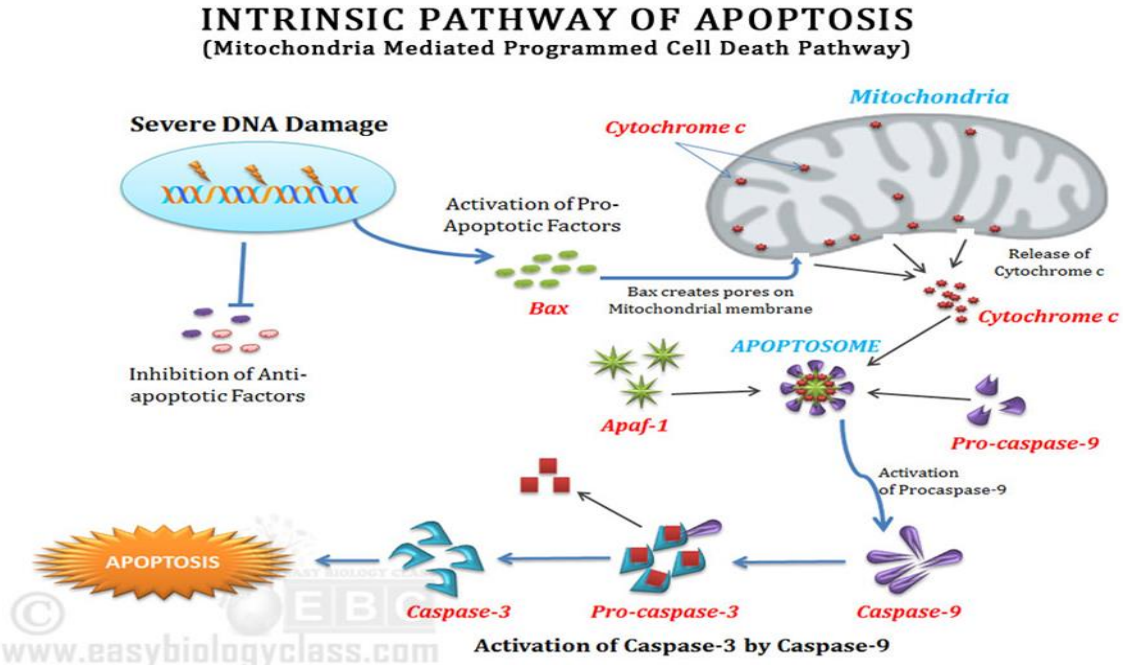

Figure 6: The intrinsic apoptotic pathway (Jan and Chaudhry, 2019).

The Bcl-2 family of proteins consists of a number of evolutionarily conserved proteins that share one or more of the Bcl-2 homology (BH) domains. The members of the Bcl-2 family can either promote or inhibit apoptosis, depending on the BH domains they contain. The anti-apoptotic members, such as Bcl-2 and Bcl-X (L), conserve all four BH domains; the pro-apoptotic members, such as Bax, Bak or Bad, always contain

the BH3 domain and may have lost other domains (Chipuk *et al.,* 2010). In response to a death stimulus BH3 proteins and pro- apoptotic factors, move to mitochondria where they counteract the stabilizing actions of anti-apoptotic factors.

There is a dynamic balance between anti-apoptotic and the pro-apoptotic proteins. When the intrinsic pathway is activated, some of the pro-apoptotic members will dimerise and form pores in the outer mitochondrial membrane. Destabilization of mitochondria by pro-apoptotic proteins causes release of mitochondrial proteins into the cytoplasm, leading to the previously described process of: release of APAF1 and cytochrome c, cytochrome C interaction with APAF1 which binds to caspase-9, and caspase-9 in turn cleaving and activating the executioner caspases (caspase-3, 6 and 7). Thus when these pro-caspases are activated, they act to cause rapid apoptotic cell death. Measuring the activity of members of the Bcl-2 family has therefore become a useful method to monitor apoptotic activity (Li *et al.*, 1997).

The tumour suppressor gene, p53, has also been shown to play a key role in apoptosis. The protein coded by the p53 gene belongs to a family of proteins that has three members including P53, P63 and P73. These proteins share about 60-70% amino acid identity between their DNA-binding regions. The activation of p53 can be induced following various stimuli such as DNA damage, ionizing radiation, UV irradiation, hypoxia, heat shock, oncogene activation, as well as cytotoxic drugs (Slee *et al.*, 1999). Apart from its involvement in apoptotic cell death, p53 is also involved in other physiological processes such as cell cycle arrest, DNA repair and cell differentiation (Ozaki *et al.*, 2011, Yeu *et al.*, 2017). p53 performs these functions through transcriptional activation of specific target genes that carry p53 DNA binding sites.

Under normal cellular function, the expression of p53 is deficient, however, extra and intracellular stress stimulate p53, resulting in its excess in the cell nucleus. Thus following this apoptotic stimulus, activated p53 binds to the Bax gene promoter and directly trans-activates the transcription of this pro-apoptotic gene. The Bax promoter can be activated by wild-type but not mutant p53, which forms a link between p53 expression, its mutational status, and alterations in Bax expression (Chi, 2014). Excess p53 can be used as detector for successful apoptotic cell death. Expression of Bcl-2 family proteins together with p53 expression, can therefore provide a strong

indication of apoptosis. The expression profiles of these genes or gene products can be measured with either an ELISA kit, by real-time polymerase chain reaction (RT-PCR) or by immunochemical techniques (Absalan *et al*., 2012).

Activation of the extrinsic cell death pathway occurs following the binding of ligands such as Fas, TNFR1, or TRAIL to their corresponding cell surface “death receptors”. These death receptors have two distinct signalling motifs: death domains and death effector domains that allow them to interact and recruit other adaptor molecules, such as FAS-associated death domain protein and caspase-8, which can then directly cleave and activate caspase-3 and caspase-7, leading to apoptosis (Hongmei, 2012).

The caspases are a family of cysteine-aspartate proteases involved in the restricted proteolysis of over 400 proteins. The family is made up of highly conserved cysteine proteases that play an essential role in apoptosis. Mammalian caspases can be subdivided into three functional groups: initiator caspases (caspase-2, -8, -9 and -10), executioner caspases (caspase-3, -6 and -7), and inflammatory caspases (caspase-1, -4, -5, -11 and -12) (Salvesen, 2002). Initiator caspases initiate the apoptosis signal while the executioner caspases carry out the mass proteolysis that leads to apoptosis. Inflammatory caspases do not function in apoptosis but are rather involved in inflammatory cytokine signalling and other types of cell death such as pyroptosis. Initially synthesized as inactive pro-caspases, caspases become rapidly cleaved and activated in response to death receptors and apoptosome stimuli. Caspases will then cleave a range of substrates, including downstream caspases, nuclear proteins, plasma membrane proteins and mitochondrial proteins, ultimately leading to cell death (Papaliagkas *et al.*, 2007; Salvesen, 2002).

Substrate-based assays are commonly used to assess caspase activity. The biochemical substrates usually consist of short peptides that contain specific cleavage sequences that are recognized by caspase enzymes. These peptides are covalently attached to a colorimetric or fluorogenic detection probe. Upon cleavage of the substrates by the cognate caspase, the colorimetric or fluorogenic compound is liberated, producing an increase in absorbance (colorimetric substrate) or fluorescence (fluorogenic substrate). The resulting signal is proportional to the amount of caspase activity present in the sample; however, many of the cleavage sites are similar and can be cleaved by more than one of the caspases. For example, caspase-

3 and -7 have very similar cleavage sites. In these situations, the use of specific activators and/or inhibitors of caspase activity is essential to ensure determination of the correct caspase activity (Allombert-Blaise *et al.*, 2003, Qi *et al.*, 2003).

2.10.2.3 Autophagy

Autophagy is a highly conserved cytoplasmic degradation process that allows the orderly degradation and recycling of intracellular components, including soluble proteins, aggregated proteins, organelles, macromolecular complexes, and foreign bodies (Mizushima, 2005). During this process the isolation membrane called a phagophore encloses a portion of cytoplasm forming a double-membrane vesicle called an autophagosome (Mizushima and Komatsu, 2011). Autophagosomes are then trafficked along microtubules in a dynein-dependent manner towards the microtubule organizing center, where they fuse with lysosome to form autolysosomes. Lysosome enzymes then digest the contents of the autolysosomes (Renna *et al.*, 2010).

Autophagy is involved in a number of physiological processes, including clearing of damaged organelles, prevention of necrosis (Glick *et al.*, 2010), intracellular quality control, prevention of cellular ageing, cell differentiation and development, cell death, innate and adaptive immunity, and most importantly, cellular homeostasis, by allowing recycling of long-lived proteins and organelles (Schlafli *et al.*, 2015). Its importance is also implicated during starvation where amino acids produced following protein digestion can be re-utilized for the formation of new proteins. As such autophagy maintains cytosolic protein levels and organelle homeostasis (Yoshii and Mizushima, 2017). However, autophagy has also been implicated in pathological conditions such as cancer, neurodegenerative disorders and inflammatory bowel disease (Chen *et al.*, 2014). As such detection of autophagic markers eg. LC3 and p62, has become very useful in toxicity studies to determine drug-induced autophagic cellular damage or death.

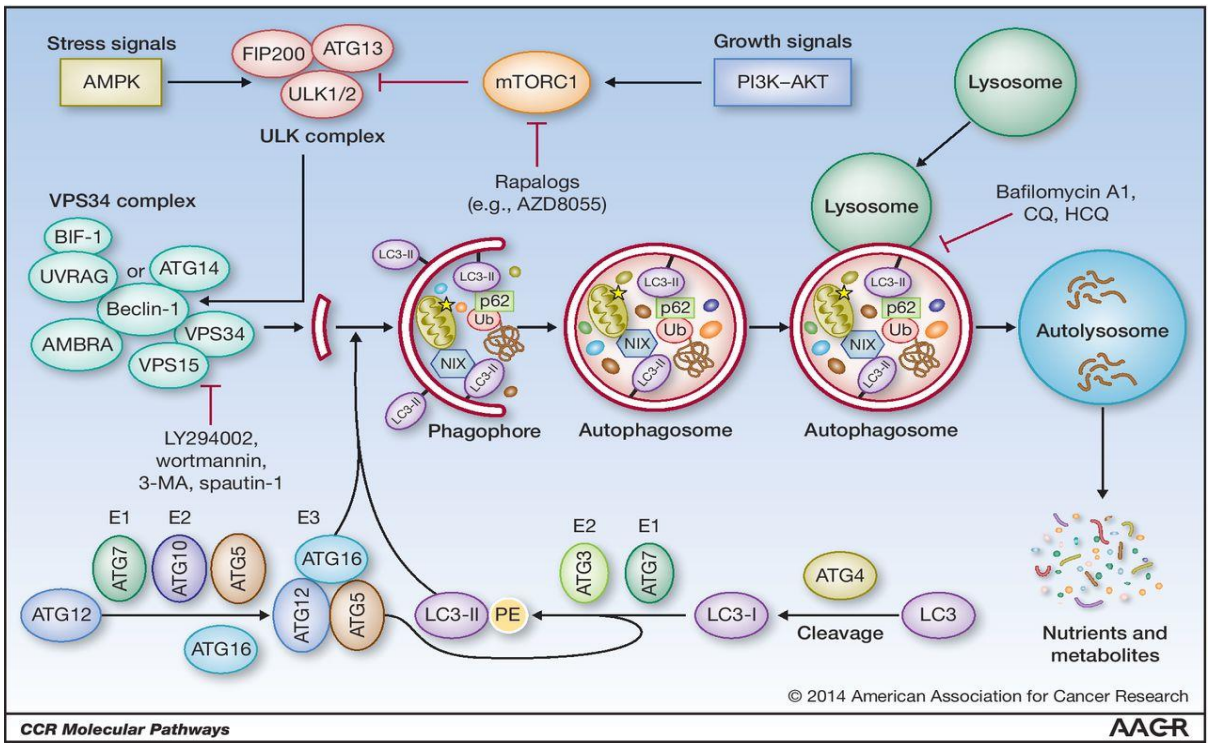

Figure 7: An illustration of the process of autophagy (Cicchini *et al.*, 2015).

Microtubule-associated protein light chain 3 (LC3) is considered one of the definitive markers of autophagy, and its use is widespread in cell culture studies throughout the world (Ma *et al.*, 2019; Klionsky *et al.*, 2016). Despite its popularity, there are several considerations when employing LC3 antibodies in immunoassays, and in particular Western blots. LC3 play a critical role in autophagosome biogenesis and closure, and is expressed as a propeptide. Initiation of autophagy causes the conversion of LC3-I to LC3-II via the addition of a phosphatidylethanolamine (PE) group to the C terminus (Tanida *et al.,* 2008). On an SDS-PAGE gel LC3-I migrate to around 16-18 kDa while LC3-II migrates faster to around 14-16 kDa. Thus the PE group increases the rate of band migration in an SDS-PAGE gel, likely due to its hydrophobic nature; this modification commonly manifests as the appearance of a doublet in a Western blot. The lipophilic character of the PE group also facilitates the insertion of LC3-II into the membranes of autophagosomes, and as a result LC3-II is degraded as autophagosomes are metabolised. It is common practice to consider an increase in LC3-II band intensity and a decrease in LC3-I expression as the hallmark of autophagy, but increases in LC3-II can be caused by enhanced autophagosome synthesis or reduced autophagosome recycling. Interpreting LC3 staining observations should therefore be done with caution (Rodríguez-Arribas *et al.,* 2017).

Apart from immunoblotting and immunoprecipitation, immunofluorescence can also be used to monitor LC3 (Tanida *et al*., 2008).

p62, is a multifunctional classical receptor of autophagy, located throughout the cell. It is involved in the proteasomal degradation of ubiquitinated proteins involved in many signal transduction pathways, including the Keap1–Nrf2 pathway. The role of p62 in autophagy is widely recognised. This includes its involvement during the delivery of ubiquitinated cargoes for autophagic degradation via the C-terminal UBA domain or the LIR domain. This process is promoted by the PB1 domain. The activation and inhibition of these proteins play an important role in regulating autophagy (Tanida and Waguri, 2010).

Manipulation of cellular levels of p62 changes the quantity and location pattern of ubiquitinated proteins with a considerable impact on cell survival. This was seen in some cell lines where overexpression of p62 enhanced protein aggregation and this had a protective effect on cell survival (Schlafli *et al.,* 2015). Interestingly, p62 deletion results in the impaired formation of the LC3-II aggresome and autophagosome, exacerbating cell injury and lowering cell viability. Low levels of p62 is therefore associated with enhanced autophagic activity as reflected by an increase in the conversion rate of LC3-I to LC3-II and in the amount of multilayered autophagosomes (Stern *et al.,* 2012). Detailed methods used to assess autophagy markers, LC3 and p62 have been previously published (Schlafli *et al*., 2015; Deng *et al*., 2013; Stern *et al.*, 2012; Kissiotis *et al.*, 2009).

Bulk autophagy is further characterized by the sequestration of large portions of cytoplasmic proteins into autophagosomes. LDH is a stable and highly abundant cytosolic enzyme that is non-selectively enwrapped into autophagosomes during autophagy. Its sequestration can therefore be used as an indication of bulk autophagy. Comparing the amount of LDH sequestrated to the LDH pool in the cytosol allows for a good quantitative analysis of the autophagic process, commonly referred to as autophagic flux (Luhr *et al*., 2018). The LDH amounts in the two fractions can also be determined by either the enzymatic measurement of NADH decline or by LDH levels by Western blot (Morten *et al*., 2018). Defects in autophagic flux have been associated with many disorders including neurodegeneration, cancer, cardiovascular and immune diseases (Zhang *et al*., 2013).

2.10.3 Oxidative stress

Oxidative stress is a state of disequilibrium between the production of reactive oxygen species (ROS) such as the superoxide radical, hydrogen peroxide and the hydroxyl radical, and the ability of cellular antioxidant enzyme defence mechanisms to neutralize these ROS. This disequilibrium in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell including proteins, lipids and DNA. For instance, oxidative stress from oxidative metabolism can cause lipid peroxidation as well as base damage and strand breaks in DNA (Dalle-Donne *et al.*, 2003). Thus the generation of ROS and the resulting oxidative stress have been suggested to be a common mechanism inducing toxicity that has been implicated in the onset and progression of several diseases such as cancer and neurodegeneration (Schönhofen *et al*., 2015).

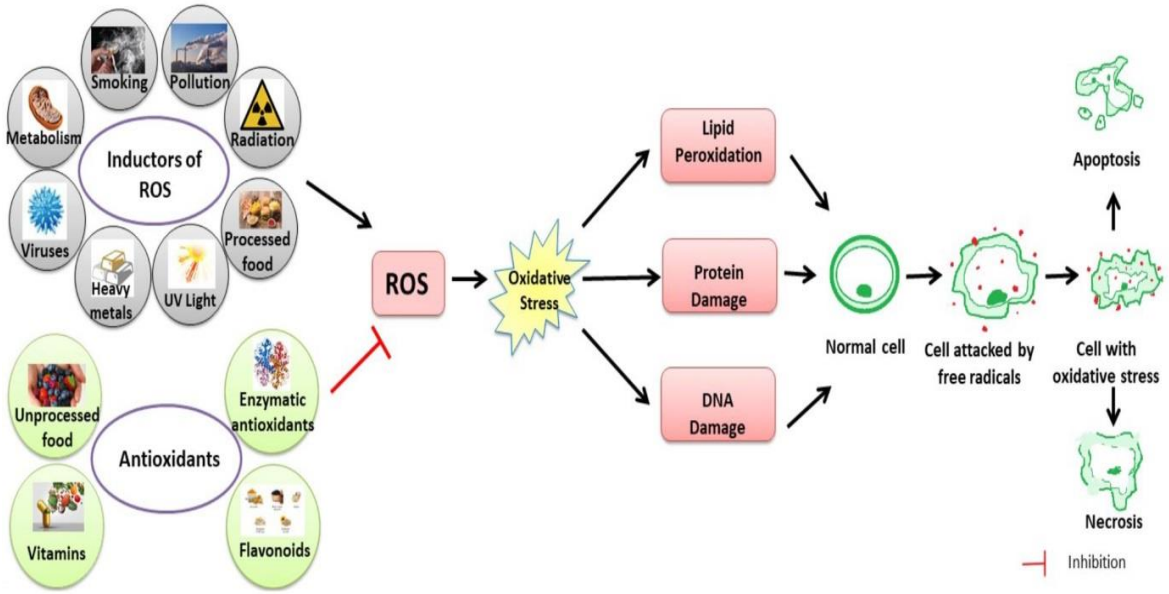

Figure 8: A schematic diagram indicating the sources of ROS and the induction of oxidative stress and cell death (Da Pozzo *et al*., 2018).

2.11 Summary

This literature review presented epidemiological data about the dire status of drug use in the world, Africa and in particular South Africa, with a focus on the use of nyaope amongst the youth in socio-economically deprived communities. Chemical analysis of nyaope indicates that it is a concoction of many substances with the main ingredient

being heroin. Some focus on heroin toxicity has therefore been provided. Since heroin toxicity may lead to cell death, some of the mechanisms / modes of cell death have also been discussed.

Aim and objectives of the present study

The overall aim of this study therefore was to investigate the toxic effects of nyaope on liver and brain tissue. This was done in order to demonstrate peripheral and central deleterious effects following nyaope consumption. Hepatocytes (HepG2 cells) and neuroblastoma (SH-SY5Y) cells were used for *in vitro* characterization of nyaope-induced harmful effects. *In vivo* experiments were subsequently performed to see whether some of the effects could be repeated in a whole body system.

Our specific objectives for the study were

a. To assess whether nyaope was toxic to hepatocytes. Here HepG2 cells were exposed to nyaope and evaluated microscopically together with the trypan blue exclusion assay.
b. To assess whether nyaope was toxic to neurons. Here neuroblastoma cells were exposed to various concentrations of nyaope for different time periods to determine the optimal conditions at which nyaope toxicity occurs. MTT assays were done to assess cell viability after nyaope exposure.
c. To determine the mode of cell death induced by nyaope. Here we determined whether the drug activated necrotic, apoptotic, and/or autophagic modes of cell death. For necrosis the levels of lactate dehydrogenase in the culture medium was measured. For apoptosis we measured the expression of Bcl-2 and Bax. For autophagy the expression levels of LC3 and p62 were determined.
d. To establish whether some of these effects also occurred *in vivo*, we measured Bcl-2, Bax, LC3 and p62 in the liver and frontal cortex of nyaope-treated rats.

Chapter 3: Methodology

The consumption of nyaope by the youth of especially lower income communities has been escalating at a worrying rate. Despite this alarming statistic, research investigating the deleterious effects of nyaope has been limited. The neurotoxic effects of heroin are better known. These include cerebral atrophy, neurovascular complications, demyelination of white matter, respiratory depression and addiction (Borne et al., 2000; Buttner et al., 2000). Since heroin is the main constituent of nyaope, together with other components such as detergents, antiretroviral drugs and even anti-depressants, the present study set out to firstly characterise the toxic effects of nyaope using two cell culture systems, a liver cell line (HepG2 cells) and a neuronal cell line (SH-SY5Y neuroblastoma cells). The second part of the study investigated whether some of the findings obtained in the *in vitro* experiments could be replicated *in vivo*.

3.1 *In vitro* experiments

3.1.1 SH-SY5Y and HepG2 cell lines

Neuroblastoma (SH-SY5Y) cells and a hepatocyte cell line (HepG2 cells) were chosen as a model to study the potential toxic effects of nyaope on the brain and liver respectively. The SH-SY5Y neuroblastoma cell line is a thrice-subcloned cell line derived from the SK-N-SH neuroblastoma cell line. The parental cells, were isolated from a bone marrow aspirate of a four-year-old female with neuroblastoma. These cells express dopaminergic markers (Xie *et al*., 2010). The HepG2 cell line is an immortal cell line derived from liver tissue of a 15-year-old Caucasian male from Argentina with well-differentiated hepatocellular carcinoma. This cell line was successfully used as a model system to study liver metabolism and the toxicity of xenobiotics (Sundermann *et al*., 2004).

3.1.2 Cell maintenance

SH-SY5Y and HepG2 cells (Cellonex^{TM}, South Africa) were cultured in T75 cell culture flasks. The SH-SY5Y cells were cultured with a 1:1 ratio of Dulbecco's Modified Eagle's Medium (DMEM) and Ham’s F-12 nutrient mixture, supplemented with 10% foetal calf serum (FCS) (Hyclone, Sigma-Aldrich, Germany). HepG2 cells were

cultured with DMEM, supplemented with 10% FCS (Hyclone, Sigma-Aldrich, Germany). The cells were maintained at 37°C in a humidified incubator with 5% CO_2. Cells were passaged at 80-90 % confluence.

3.1.3 HepG2 cells morphology assessed using a ZOE microscope

The HepG2 cells were treated with nyaope at concentrations of 6 and 10 mg/ml, and then incubated for 24 hours. Untreated cells were used as controls. After 24 hours, the cells were viewed under a ZOE microscope (ZOE Fluorescent Cell Imager, Bio-rad, SA) under 40× magnification. The usefulness of this approach is that it allows the rapid inspection and capturing of unstained live cells under magnification.

3.1.4 Trypan blue exclusion method for cell viability

Dye exclusion methods such as trypan blue, are simple and rapid traditional methods that are commonly used to measure cell proliferation and viability. The methodology is based on the principle that viable cells have intact cell membranes whereas non-viable cells have damaged cell membranes which allow the uptake of dyes resulting in cell staining (Mascotti *et al*., 2000). The procedure entails the following: 1×PBS (137 mM NaCl, 2.7 mM KCl, Na_2HPO_4 and KH_2PO_4, LasecSA), 80 µL trypan blue reagent (BioReagent, Sigma-Aldrich, Germany) and 20 µL of $2x10^6$ cells/ml was prepared, from which 10 µL of this cell/trypan blue reaction mixture was pipetted onto a haemocytometer slide (Abcam, Biocom, RSA). Cells were evaluated under an inverted light microscope (Olympus, Model CKX31, Europe).

For the experiment, HepG2 cells were exposed to increasing concentrations of nyaope (1 mg/ml, 3mg/ml, 6mg/ml, and 10 mg/ml) and incubated for 24 hours. Following incubation, cells were transferred to the haemocytometer slide to perform the trypan blue exclusion test. Cells that stained blue were considered dead, while cells that remained unstained by the dye, were considered viable. Cell viability was subsequently calculated as follows:

$$Viable\ cell\ count\ \ (live\ cells\ per\ mL) = \frac{no\ of\ live\ cells}{no\ of\ large\ corner\ square} \times dilution\ factor$$

$$Dead\ cell\ count\ \ (dead\ cells\ per\ mL) = \frac{no\ of\ dead\ cells}{no\ of\ large\ corner\ square} \times dilution\ factor$$

$$Percentage\ viability = \frac{no\ of\ viable\ cells}{total\ no\ of\ cells}$$

3.1.5 Cell viability detection by 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

The MTT assay (Roche, South Africa) provides another method to assess the viability of cells. It has high sensitivity and is based on the conversion of the yellow tetrazolium salt (MTT) into purple formazan crystals by cells that are metabolically active (Uysal *et al*., 2018).

In the present study cells were seeded in 96 well plates 24 hours prior to treatment with nyaope. Culture medium was aspirated and replaced with colourless Roswell Park Memorial Institute – 1640 medium (RPMI-1640, Gibco, South Africa) containing nyaope at different concentrations (0.625 µg/µl, 1.25 µg/µl, 2.5 µg/µl, 5 µg/µl and 10 µg/µl). Untreated cells (not exposed to nyaope) were used as negative controls. The cells were exposed to nyaope for various time intervals (1 hour, 6 hours and 24 hours) at 37^0 C with 5% CO_2. At the end of the exposure period, 0.5mg/ mL MTT solution was added to each well and incubated for 4 hours at 37°C. Medium was then discarded and replaced with dimethyl-sulfoxide (DMSO) to solubilise the formazan crystals. The cells were then incubated at room temperature for 1 hour. Absorbance was measured at 595 nm using a microplate reader (Multiscan FC, Thermo Scientific, Life Technologies, South Africa) and plotted using GraphPad Prism software (version 9.02). Data were plotted as the percentage of cell viability versus concentration. All drug concentrations and times were tested in triplicate and experiments were repeated twice. Percentage cell viability was calculated as follows:

$$percentage\ cell\ viability = \frac{corrected\ absorbance\ (treated)}{corrected\ absorbance\ (control)} \times 100$$

$$corrected\ absorbance = absorbance\ (treated\ or\ control) - absorbance\ (blanks)$$

3.1.6 Necrotic cell death detection by lactate dehydrogenase (LDH) assay

The LDH assay (Roche, South Africa) quantifies cytotoxicity by measuring lactate dehydrogenase enzyme (LDH) activity released from damaged cells. Lactate dehydrogenase is a cytosolic enzyme present in many different cell types. When the plasma membrane is damaged, LDH leaks into the extracellular space. In the case of a cell culture system, this will be into the cell growth medium.

In this experiment three controls were included in each experimental setup to ensure accurate determination of cytotoxicity. The first measure was a background control which determined the LDH activity contained in the assay medium. The absorbance values obtained from this control were subtracted from all other absorbance values. Secondly a low control was used to determine the LDH activity released from untreated normal cells (spontaneous LDH leakage) and thirdly, a high control was used to determine the maximum releasable LDH activity from the cells (maximum LDH release).

Cells were seeded in 96 well plates 24 hours prior to treatment with nyaope. Culture medium was aspirated and replaced with colourless RPMI-1640 (Gibco, Thermo Fischer Scientific, U.K.) containing nyaope at differing concentrations (0.625 µg/µl, 1.25 µg/µl, 2.5 µg/µl, 5.0 µg/µl and 10.0 µg/µl). Untreated cells were used as 'low controls', while cells exposed to lysis buffer where used as 'high controls'. The cells were exposed to nyaope at time intervals of 1 hour, 6 hours and 24 hours at 37°C with 5% CO_2. Following exposure, 5µl of lysis buffer was added to 'high controls' to enhance LDH leakage. At the end of exposure period, 10 µl of reaction mixture was added to each well and incubated for 10 minutes. 50 µl of stop solution was then added. Absorbance was measured at 492 nm using a microplate reader (Multiscan FC, Thermo Scientific, Life Technologies, South Africa).

3.1.7 Comparative gene expression using real time polymerase chain reaction (PCR)

A comparative gene expression approach was adopted in order to ascertain the presence of apoptotic and/or autophagic modes of cell death induced by nyaope. Understanding the mode of cell death is a vital component in biological research (Stenson and Ciorba, 2018) as it identifies potential opportunities for therapeutic intervention.

3.1.7.1 RNA extraction from SH-SY5Y cell lines

Extraction and purification of RNA were carried out using Illustra RNAspin mini RNA isolation kit (GE Healthcare, Sigma-Aldrich, South Africa) according to the manufacturer's instructions. In preparation for cell extraction, cells were seeded in 12-well plates and exposed to nyaope at different concentrations (0.625 µg/µl, 1.25 µg/µl, 2.5 µg/µl, 5 µg/µl and 10 µg/µl) at given time intervals (1 hour and 24 hours). Following incubation, cells were lysed and transferred to centrifuge tubes for commencement of the extraction process. For RNA extraction from the cells, firstly the RNAlater was added to the tubes, then 350 µl lysis solution followed by 3.5 µl *B*-mercaptoethanol. Eluted RNA from cells was subsequently utilized for first-strand cDNA synthesis. RNA was reverse transcribed using Superscript IV VILO reverse transcription kit as per manufacturer's instructions (Catalogue #11756050, Thermo Scientific, U.K.).

3.1.7.2 Taqman® PCR

The PCR reactions were performed using assay-on-demand TaqMan® fast advanced PCR master mix (Thermo Scientific, U.K.). Expression of mRNA for apoptotic (Bax, Bcl-2) and autophagic (LC3, p62) markers and reference gene (18S ribosomal RNA) (TaqMan® assays ID; Hs99999901-S1), was detected in duplex reactions by comparative RT-PCR with the following TaqMan® probes; Bax (TaqMan® assay ID; Hs00180269_m1), Bcl-2 (TaqMan® assay ID; Hs04986394-s1), p62 (TaqMan® assay ID; Hs02621445_s1) and LC3 (TaqMan® assay ID, Hs01076567_g1) (Thermo Scientific, U.K.). Thermocycling was performed using a 96 well plate format on a StepOne Plus Real Time Thermocycler (Thermo Scientific, Life Technologies, Carlsbad, USA). The reaction mixture contained 1 µl cDNA (concentration > 1000ng/uL), 5 µL TaqMan® fast advanced PCR master mix, 0.5µL target probe, 0.25µL reference probe, and 3.25µL dH_2O was added to each well, with a total volume of 10 µL/well. The plate was sealed and centrifuged briefly before PCR. The reactions were performed in duplicate under the following conditions: 95˚C for 3 min for initial denaturation followed by 40 cycles of 95˚C for 15 seconds and 60˚C for 1 min for primer annealing, extension and fluorescence detection. Calculations for gene expression analysis were performed using Excel software (Microsoft Office, 2016) according to the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001). Briefly, the PCR products were detected by measuring the emitted fluorescence (Rn) at the

end of each reaction cycle and average threshold cycle (CT) values were calculated for subsequent expression analysis. These Ct values were first normalized with that of 18S rRNA in the same sample and then expressed as fold changes with control. The Ct corresponds to the number of cycles required to detect a fluorescence signal above the baseline. The relative quantification units (RQ units=$2^{-\Delta\Delta Ct}$), representative of the normalized expression of the target genes, were calculated for each sample. ΔΔCt is the difference between the ΔCt value of a treated sample and the ΔCT for the control sample, whereas ΔCt is the difference between the Ct value of the target gene (Bcl-2, Bax, p62, LC3) and the Ct of the endogenous reference gene (Livak and Schmittgen, 2001).

3.2 *In vivo* experiment

An *in vivo* experiment was conducted to see whether some of the *in vitro* findings could be replicated within a whole animal system. Rats were subsequently treated with nyaope and their liver and prefrontal cortex dissected for the determination of expression levels of Bcl-2, Bax, p62 and LC3.

3.2.1 Animal treatment

A total of 12 Wistar rats, weighing 150-200g, were obtained from the Central Animal Service facility at the University of the Witwatersrand. The animals were housed two per cage in this facility and maintained under standard conditions (room temperature of 25°C, balanced diet, and constant light 12h/ 12h rhythm). The 12-hours day/night cycle was set at 06h00 lights on and 18h00 lights off. The rats had free access to food and water throughout the experiment. Ethical approval for this experiment was obtained from the Animal Research Ethics Committee of the university (Clearance certificate number 2018/09/40C).

Of this group of animals 6 rats were injected intra-peritoneally every morning with 6 mg/kg nyaope for 3 days. Control animals (n=6) received equivalent saline injections. On day 3, the rats were briefly anaesthetised by isoflurane exposure, prior to being decapitated. The liver and prefrontal cortex tissues were collected and stored in RNAlater.

3.2.2 Comparative gene expression using real time polymerase chain reaction (PCR)

3.2.2.1 RNA extraction from tissue samples stored in RNAlater

For RNA extraction the RNAlater was first removed prior to adding 350 µl lysis solution and 3.5 µl *β*-Mercaptoethanol into a centrifuge tube containing up to 30 mg tissue. Eluted RNA from the tissue was utilized for first-strand cDNA synthesis. RNA was reverse transcribed using Superscript IV VILO reverse transcription kit as per manufacturer's instructions (Catalogue #11756050, Thermo Scientific, U.K.).

3.2.2.2 Taqman® PCR

For quantification of apoptotic and autophagic markers, the procedure in 2.2.7.2 was followed. In this animal experimental model, the following TaqMan® probes were used; Bax (TaqMan® assay ID; Hs00180269_m1), Bcl-2 (TaqMan® assay ID; Hs04986394-s1), p62 (TaqMan® assay ID; Hs02621445_s1) and LC3 (TaqMan® assay ID, Hs01076567_g1) (Thermo Scientific, U.K.)

3.3 Statistical analysis

The data are expressed as mean ± SEM of n samples. Statistical analysis was performed with GraphPad Prism version 9.02. The Shapiro-Wilk test was used to establish the distribution of data. For the *in vitro* experimental data one-way analysis of variance (ANOVA), for comparison of various concentrations of nyaope, was used to identify significant differences between the different groups. This test was followed by Dunnett's multiple comparison test. For the *in vivo* experiment the t-test was used to compare control animals with nyaope treated animals. A probability value of $p < 0.05$ was considered statistically significant.

Chapter 4: Results

HepG2 and SH-SY5Y cells were exposed to various concentrations of nyaope for different time periods. The potential harmful effects of these exposures on the cell viability and integrity were subsequently assessed by means of microscopic evaluations (HepG2 cells) and cell viability assessments (trypan blue exclusion for HepG2 cells and MTT assays for SH-SY5Y cells). The possible mechanisms of cell death (necrosis, apoptosis and autophagy) of SH-SY5Y cells were also investigated. Lastly, an animal study was conducted to reproduce some of the *in vitro* results in an *in vivo* system.

4.1 In vitro study

4.1.1 Microscopic evaluation of cell well-being following exposure to nyaope

The potential toxic effects of nyaope were initially assessed by evaluating its impact on cell morphology. HepG2 cells were treated with 6 or 10 µg/µL nyaope for 24 hours and cellular morphology was evaluated using a ZOE™ microscope (ZOE™ Fluorescent Cell Imager, Biorad, SA). This microscope allows the assessment of unstained, live cells. Untreated cells served as controls. Control cells maintained their characteristic morphology and symmetry. Control cells remained adherent to the culture vessels for the entire duration of the experiments. In contrast to controls (Figure 9A), cells treated with nyaope (Figure 9B) show morphological alterations such as an increased number of diffused rounded cells, as well as an increase in the number of cells suspended in the culture medium that lost adherent propoerties. These alterations observed after nyaope treatment, were more pronounced in cells exposed to 10 µg/µL (Figure 9C).

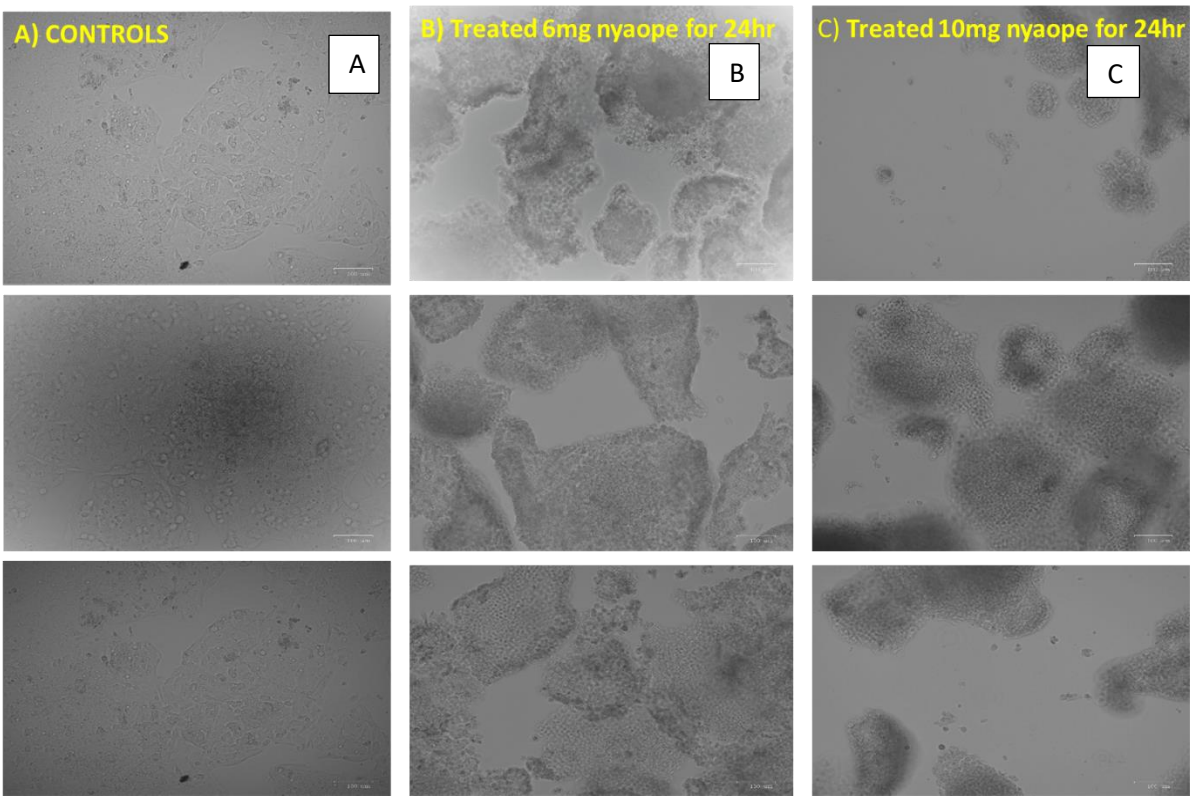

Figure 9: Three independent viewing fields showing the effect of nyaope treatment on the general morphology of HepG2 cells. The HepG2cells were either untreated (Controls; Panel A) or treated with 6 µg/µL (Panel B) or 10 µg/µL (Panel C) of nyaope respectively, for 24 hours. Unstained images were taken with a ZOE™ microscope (ZOE™ Fluorescent Cell Imager, Biorad, SA)., captured at 40X magnification.

4.1. 2 Viability of HepG2 cells following nyaope exposure

HepG2 cells were exposed to increasing concentrations (1.0 mg/mL, 3.0 mg/mL, 6.0 mg/mL and 10 mg/mL) of nyaope for 24 hours. Following exposure, the toxic effects of nyaope were evaluated using the trypan blue exclusion assay. The effect of nyaope on cell viability was shown to be dose dependent. A concentration of 1 mg/kg of nyaope significantly reduced the viability of HepG2 cells by 20%. The reduction was more pronounced when the cells were exposed to higher concentrations of nyaope, i.e. 3.0 mg/kg (more than 51% reduction), 6.0 mg/kg (more than 80% reduction) and 10.0 mg/kg (nearly 100% reduction) (Figure 10).

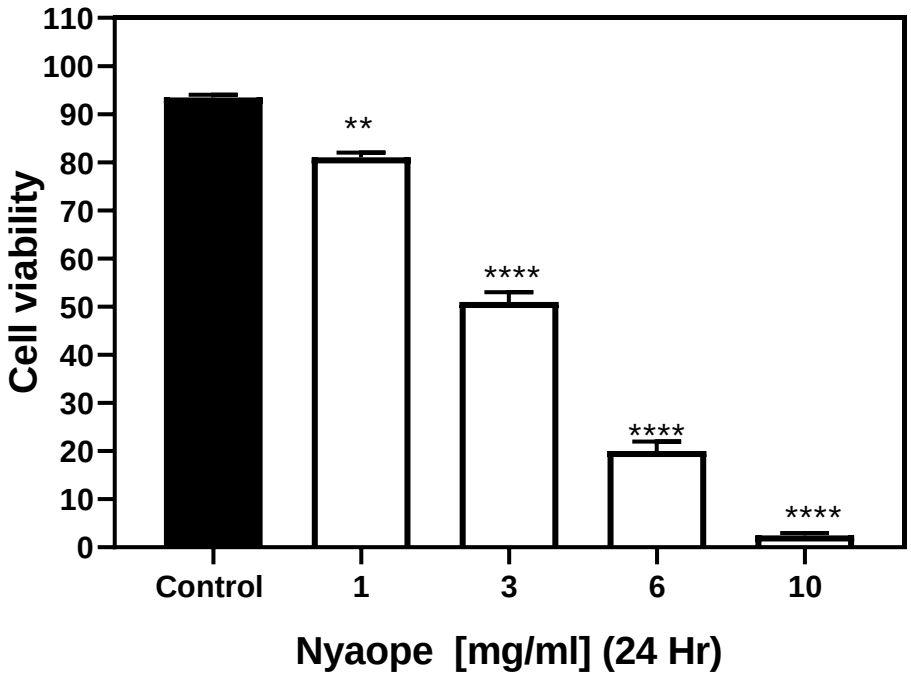

Figure 10: Cell viability of HepG2 cells following exposure to various concentrations of nyaope, as determined by the trypan blue exclusion assay. Results are expressed as mean ±SD from 2 independent experiments done in triplicate. ****p<0.0001 and **p < 0.01 in comparison to Control (ANOVA followed by Dunnett's multiple comparison test).

4.1.3 Viability of SH-SY5Y cells following nyaope exposure

SH-SY5Y cells were exposed to increasing concentrations of nyaope (0.63 µg/µL, 1,25 µg/µL, 2.5 µg/µL, 5.0 µg/µL and 10.0 µg/µL) for 1, 6 and 24 hours. At all-time points tested, exposure to nyaope induced a significant decrease in cell viability in SH-SY5Y cells (Figure 11). There was no significant difference in cell viability between cells that were exposed to 0.63, 1.25 and 2.5 µg/µL of nyaope for 1 hour when compared to controls. Greater concentrations (5.0 and 10.0 µg/µL) of nyaope did reduce the number of viable cells significantly ($p<0.01$ compared to control). Following 6 hours of incubation time with nyaope, significant decreases in the number of metabolically active cells was observed, with about 42% active cells after exposure to 0.63 µg/µL nyaope ($p<0.05$ compared to control), 65% when 2.5 µg/µL nyaope was used ($p<0.01$ compared to control), and 14% ($p<0.001$ compared to control) and 12% ($p<0.001$ compared to control) recorded for 5 and 10 µg/µL respectively. Surprisingly at a

concentration of 1.25 µg/µL nyaope, no significant differences were observed. A similar dose-dependent reduction in cell viability was observed when the cells were exposed to 10 µg/µL for 24 hours. At this time interval all concentrations of nyaope caused a significant decrease in the number of viable cells (Figure 11). Of note, the data showed that at concentrations of 5.0 and 10.0 µg/µL, nyaope seemed to have achieved its maximum effect with respect to cell viability at all time periods.

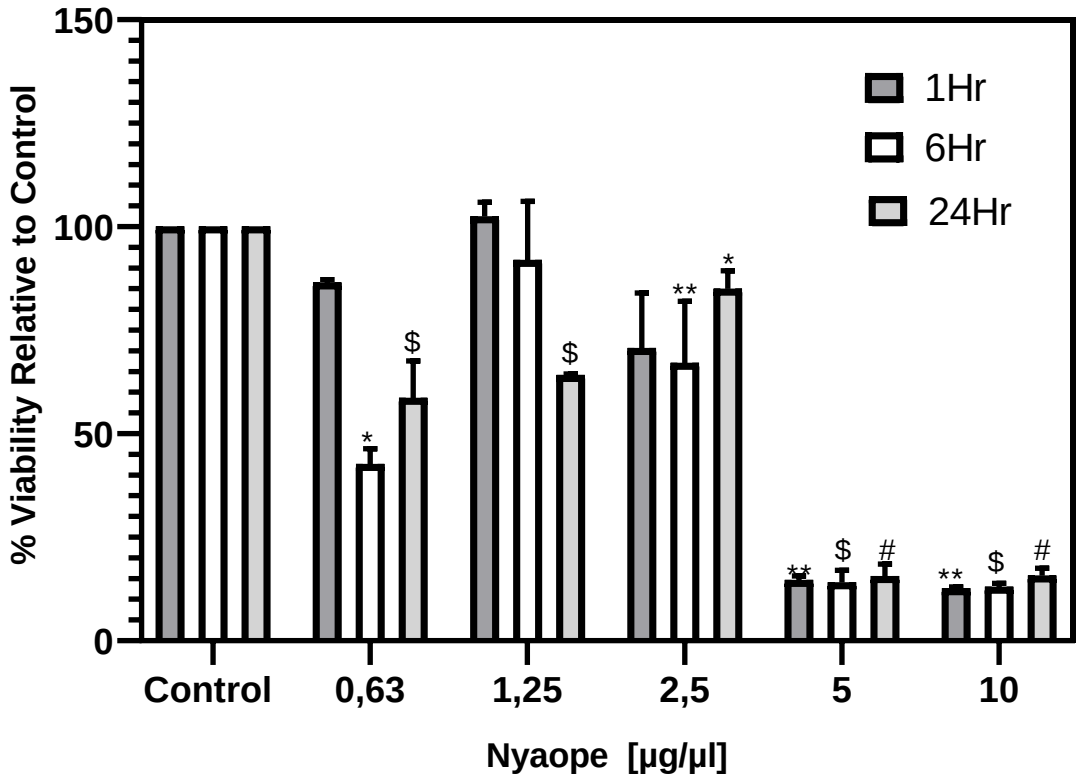

Figure 11: The effect of nyaope on cell viability. SH-SY5Y cells were exposed to increasing concentrations of nyaope for 1, 6 and 24 hours. Cell viability was measured by MTT assay. The results were normalised against control (untreated group), which was set at 100 %. Results are expressed as mean ±SD from 3 independent experiments done in triplicate. ****p<0.0001 (#), ***p < 0.001 ($), **p < 0.01 and*p < 0.05 compared to control (ANOVA followed by Dunnett's multiple comparison test).

4.1.4 Necrotic cell death

In order to assess the mechanism of nyaope toxicity and cell death, the concentration of LDH was measured in the culture medium using the LDH detection kit^{PLUS}. The results show that there were no significant differences in LDH levels in the culture

medium of cells exposed to 0.63 µg/µL, 1.25 µg/µL or 2.5 µg/µL of nyaope at all three time points measured, when compared to control (Figure 12). Exposing cells to 5 µg/µL and 10 µg/µL of nyaope caused significant increases in the LDH level in the culture medium at all time periods, except at 1 hour of 5 µg/µL nyaope exposure, when compared to control (Figure 12). The LDH release induced by higher nyaope treatment was shown to be time dependent as higher LDH levels were recorded at 5 µg/µL and 10 µg/µL as exposure time progressed from 1 hour to 6 hours to 24 hours (Figure 12).

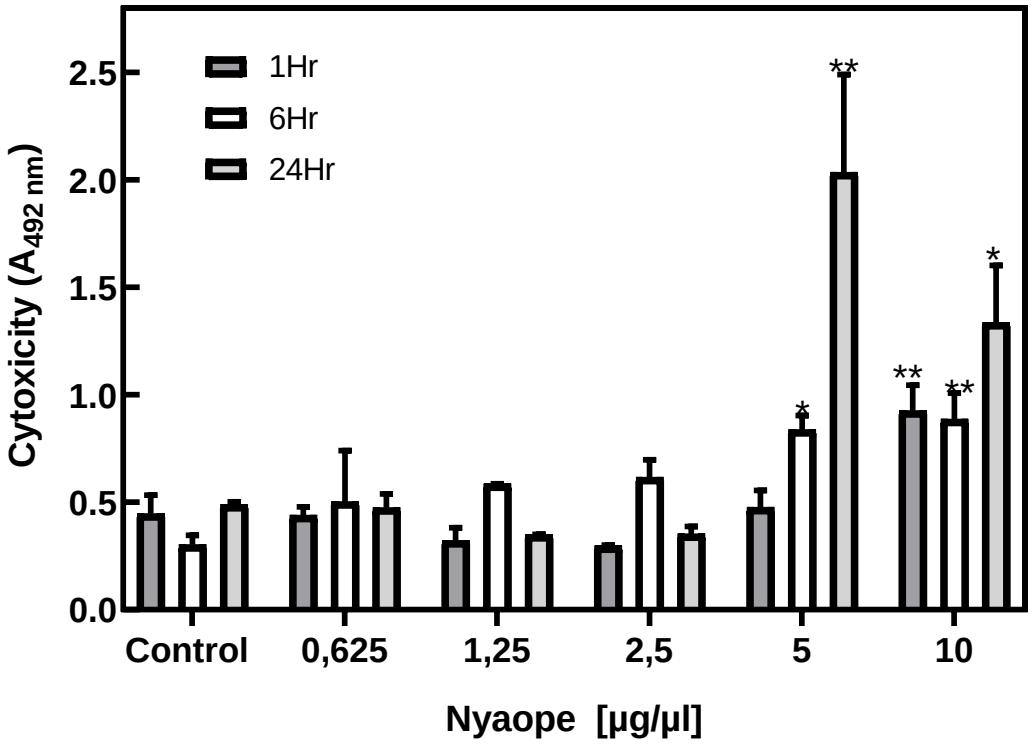

Figure 12: Cell death by necrosis as measured by LDH detection in the cell culture medium. SH-SY5Y cells were exposed to increasing concentrations of nyaope for 1, 6 and 24 hours. Results are expressed as mean ±SD from 2 independent experiments done in triplicate. **p < 0.01 and *p < 0.05 when compared to control (ANOVA followed by Dunnett's multiple comparison test).

4.1.5 Apoptosis

Cell death by apoptosis was assessed by quantifying the mRNA expression of the pro–apoptotic protein, Bax and the anti-apoptotic protein, Bcl-2, at two time periods of exposure (1 hour and 24 hours), to increasing nyaope concentrations. The results

show that exposure of SH-SY5Y cells to 0.63 µg/µL, 1.25 µg/µL or 2.5 µg/µL of nyaope had no significant effect on Bax expression levels at both 1 hour (Figure 13A) and 24-hour (Figure 13B) time points. However, exposure to 5 µg/µL and 10 µg/µL of nyaope significantly reduced Bax expression levels at both time points. At the 1-hour time point the significance was $p<0.001$ (Figure 13A) and at 24 hours it was $p<0.05$ (Figure 13B) when compared to control.

Similar to Bax expression, exposing SH-SY5Y cells to the lower concentrations of nyaope (0.63 µg/µL, 1.25 µg/µL and 2.5 µg/µL) had no significant effect on Bcl-2 expression levels at either of the time intervals. Again exposing the cells to 5 µg/µL and 10 µg/µL of nyaope yielded significant effects at both 1 hour and 24 hours. At 1 hour both 5 µg/µL and 10 µg/µL of nyaope significantly stimulated the expression levels of Bcl-2 ($p<0.001$ and $p<0.01$ respectively; Figure 13C), while at 24 hours this stimulation was even further enhanced (Figure 13D). Calculation of Bax/Bcl-2 expression ratios showed that exposure of SH-SY5Y cells to the lower concentrations of nyaope (0.63 µg/µL, 1.25 µg/µL and 2.5 µg/µL) generated results comparable to controls, while the higher concentrations of nyaope (5 µg/µL and 10 µg/µL) yielded expression ratios markedly lower than controls. This pattern of expression ratio was similar at 1 hour (Figure 13E) and 24 hours (Figure 13F).

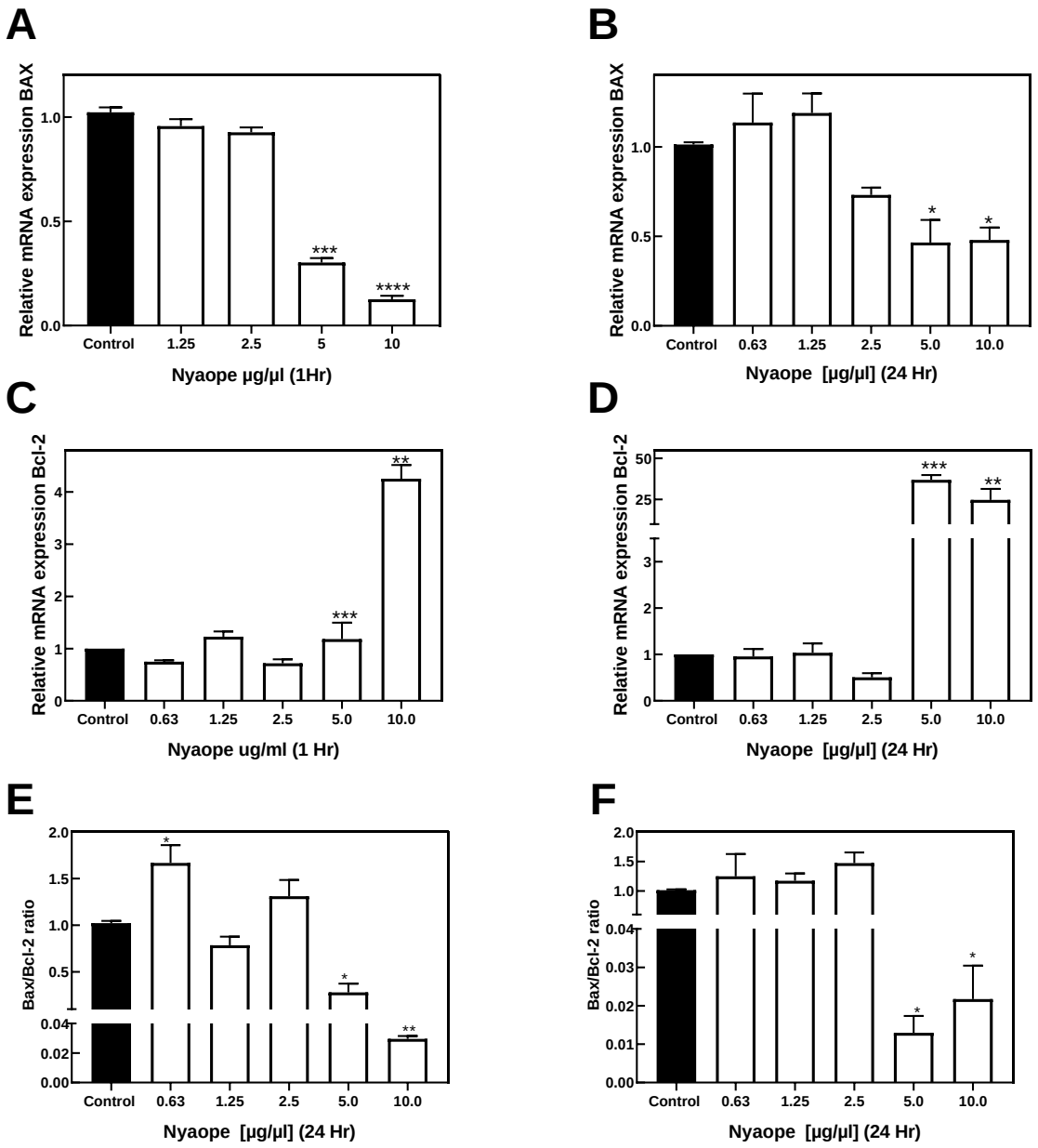

Figure 13: mRNA expression of Bax and Bcl-2 proteins in SH-SY5Y cells following exposure to increasing concentration of nyaope for 1 hour and 24 hours. The mRNA expression levels were examined by RT-PCR and were normalised against the housekeeping gene, 18s rRNA. Expression levels (2^-ΔΔCt) were also normalised against control (gene expression values obtained in the absence of nyaope) and this was set as 1 (100%). Results are expressed as mean ±SD from 2 independent experiments done in triplicate. ****p< 0.0001, ***p<0.001, **p < 0.01 and *p < 0.05 when compared to control (ANOVA followed by Dunnett's multiple comparison test)

4.1.6 Autophagy

LC3 and P62 are death receptor markers used to monitor the autophagy process. To assess if exposure of SH-SY5Y cells to various concentration of nyaope lead to autophagy, the expression levels of LC3 and P62 were analysed against 18S rRNA both in control and treated cells.

4.1.6.1 p62 mRNA expression level

At the 1-hour time point significant decreased p62 mRNA expression levels were observed when SH-SY5Y cells were exposed to nyaope at all concentrations tested except when exposed to 10 µg/µL ($p<0.05$; Figure 14A). There was no significant difference in p62 expression levels between cells exposed to 10 µg/µL nyaope and control (Figure 14A). This pattern of p62 expression levels were different at the 24 hours' time interval. Here contrasting results were found with nyaope concentrations of 0.63 µg/µL, 1.25 µg/µL and 2.5 µg/µL yielding p62 expression levels comparable to control, 5 µg/µL of nyaope reducing expression level of p62 compared to control but was not found to be significant ($p<0.303$), while 10 µg/µL of nyaope stimulated the expression of p62 significantly when compared to control ($p< 0.05$; Figure 14B).

4.1.6.2 LC3 mRNA expression level

The overall assessment of LC3 mRNA expression levels showed that nyaope induced a reduction in LC3 levels as its concentration increased. This pattern of decreasing LC3 expression levels were comparable at the two time points studied (Figures 14C and 14D).

After 1-hour exposure, there was no significant difference between untreated cells and cells exposed to nyaope at concentrations of 0.63 µg/µL, 1.25 µg/µL and 2.5 µg/µL ($p> 0.05$, Figure 14C). This result was accompanied by a significant decrease in LC3 expression level as the concentration of nyaope increased to 5 µg/µL and 10 µg/µL when compared to control ($p<0.05$; Figure 14C). After 24 hours of nyaope exposure SH-SY5Y cells displayed a dose-dependent decrease in LC3 expression (Figure 14D) with the reduction in LC3 expression levels reaching significance at nyaope concentrations of 2.5 µg/µL ($p< 0.05$), 5 µg/µL ($p<0.05$) and 10 µg/µL ($P<0.0001$)

respectively (Figure 14D. This data showed that higher concentration of nyaope lead to greater LC3 degradation than when lower concentrations are used.

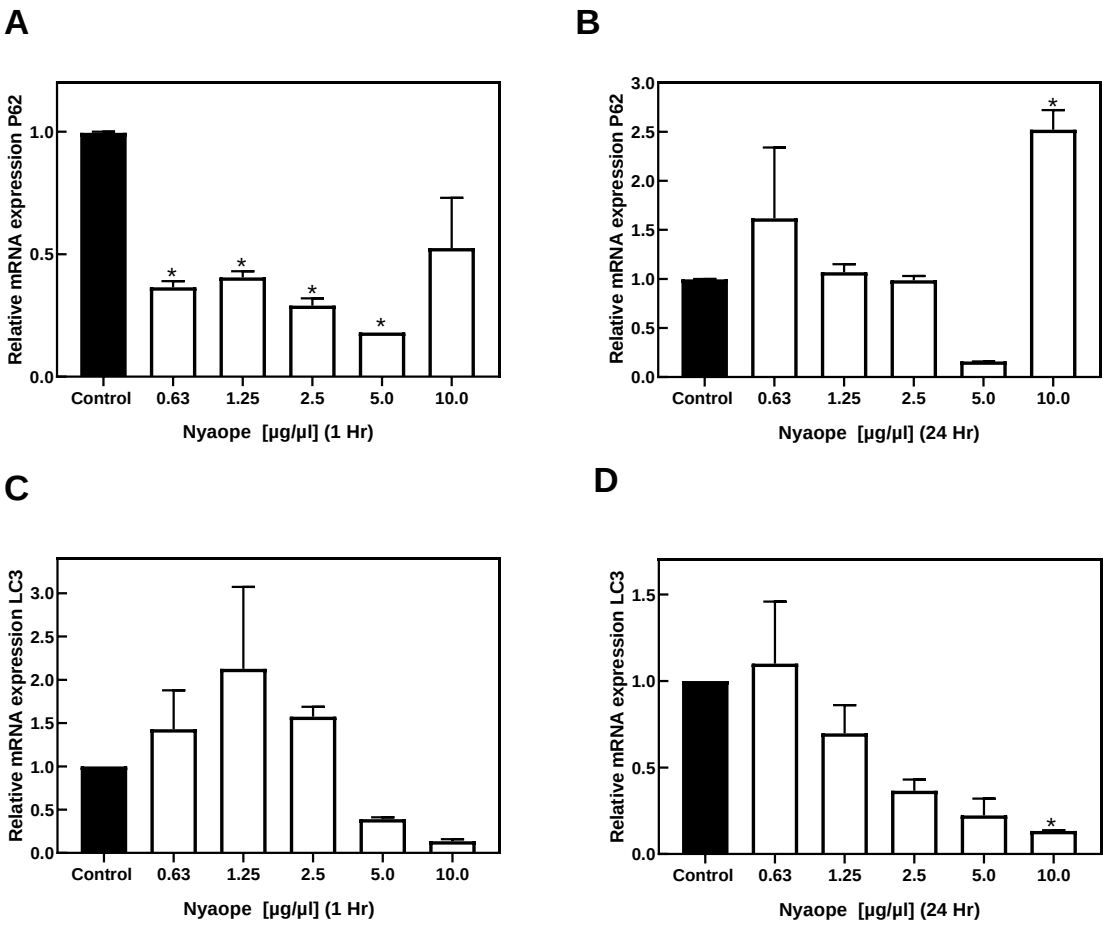

Figure 14: mRNA expression of p62 (A and B) and LC3 (C and D) in SH-SY5Y cells was examined at 1 hour and 24 hours after exposure to increasing concentrations of nyaope. The mRNA expression levels of these autophagy markers were normalised against the house keeping gene (18s rRNA). Expression levels (2^-ΔΔCt) were also normalised against control (gene expression values obtained in the absence of nyaope), set as 1 (100%). Results are expressed as mean ±SD from 2 independent experiments done in triplicate. ****p < 0.000 and *p < 0.05 compared to control (ANOVA followed by Dunnett's multiple comparison test).

4.2 *In vivo* study

To explore the toxicity of nyaope *in vivo*, Wistar rats were given intra-peritoneal injections of either nyaope (6 mg/kg) or saline for 3 days. On day 3 the rats were briefly anaesthetised, decapitated and their liver and prefrontal cortex dissected for the determination of the apoptotic (Bax and Bcl-2) and autophagic (p62 and LC3) markers. These experiments were done to see if the findings of the *in vitro* study were mirrored in an *in vivo* system.

4.2.1 Apoptosis

Treating rats for 3 days with 6 mg/kg nyaope significantly reduced the expression level of Bax in the prefrontal cortex ($p<0.05$; Figure 15A), whereas the expression of Bcl-2 was not significantly affected (Figure 15C). In contrast, the expression level of Bax was not significantly affected by nyaope in the liver (Figure 15B), while that of Bcl-2 was significantly decreased ($p<0.05$; Figure 15D). Interestingly the Bax/Bcl-2 ratio was significantly lower in the prefrontal cortex of treated animals ($p<0.05$; Figure 15E), while in the liver no significant differences were observed between nyaope-treated and saline-treated animals (Figure 15F).

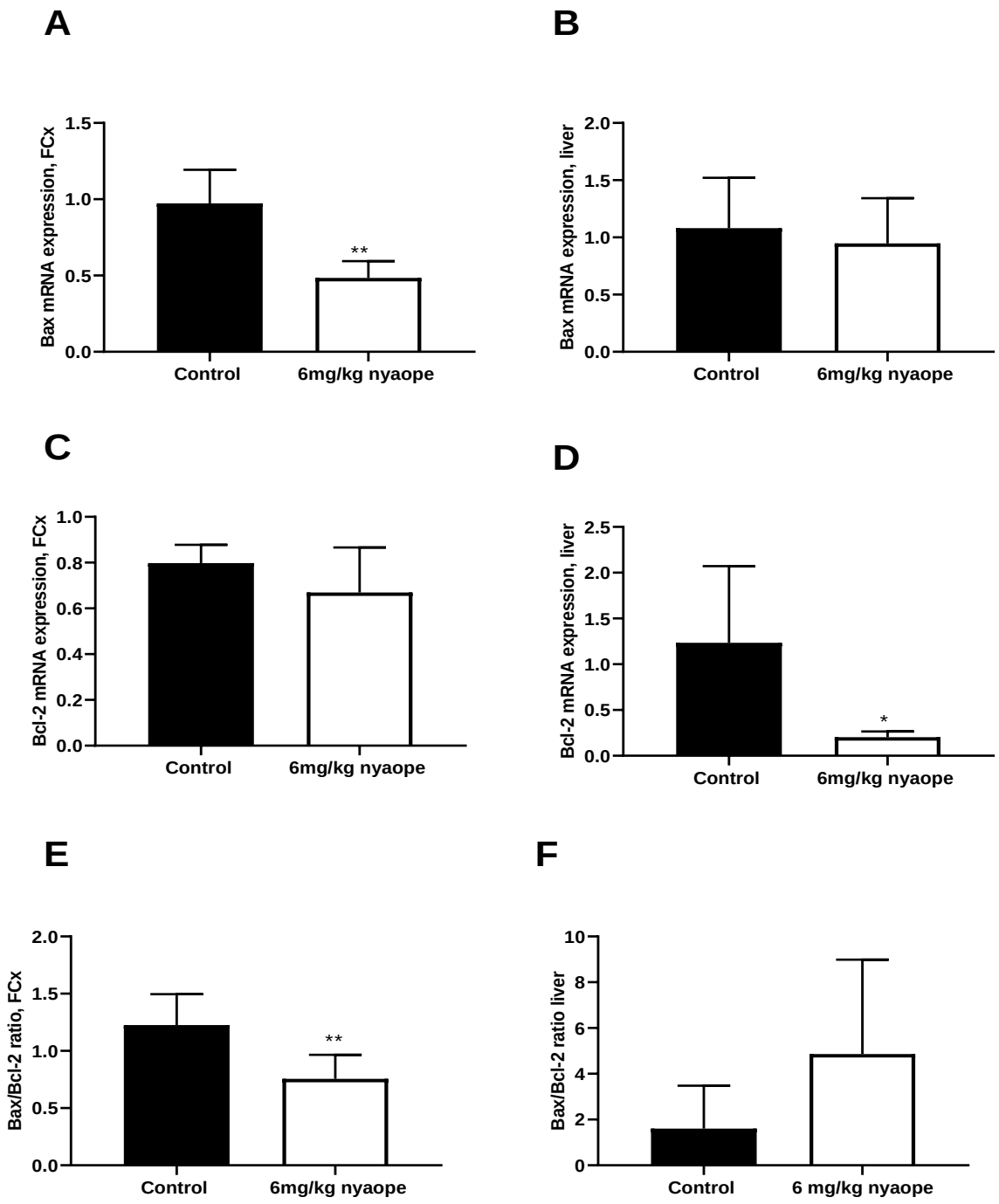

Figure 15: Relative mRNA expression of Bax and Bcl-2 in saline-treated (control) and nyaope-treated Wistar rats. The mRNA expression level of these apoptotic markers were normalised against the house keeping gene (TBP). Expression levels (2^-ΔΔCt) were also normalised against control (gene expression values obtained in the absence of nyaope), set as 1 (100%). Results are expressed as the mean ±SD of 6 rats per group. **p < 0.01, *p < 0.05 compared to control (t-test).

4.2.2 Autophagy

In order to further characterise th e mode of cell death involved in the toxicity of nyaope *in vivo*, the expression levels of p62 and LC3 from the liver and prefrontal cortex were quantified. We observed that nyaope significantly decreased the expression levels of p62 in both the liver ($p<0.05$; Figure 16A) and the FCx ($p<0.05$; Figure 16B), while the decrease in LC3 expression was not significant in any of the two tissues measured (Figures 16C and 16D).

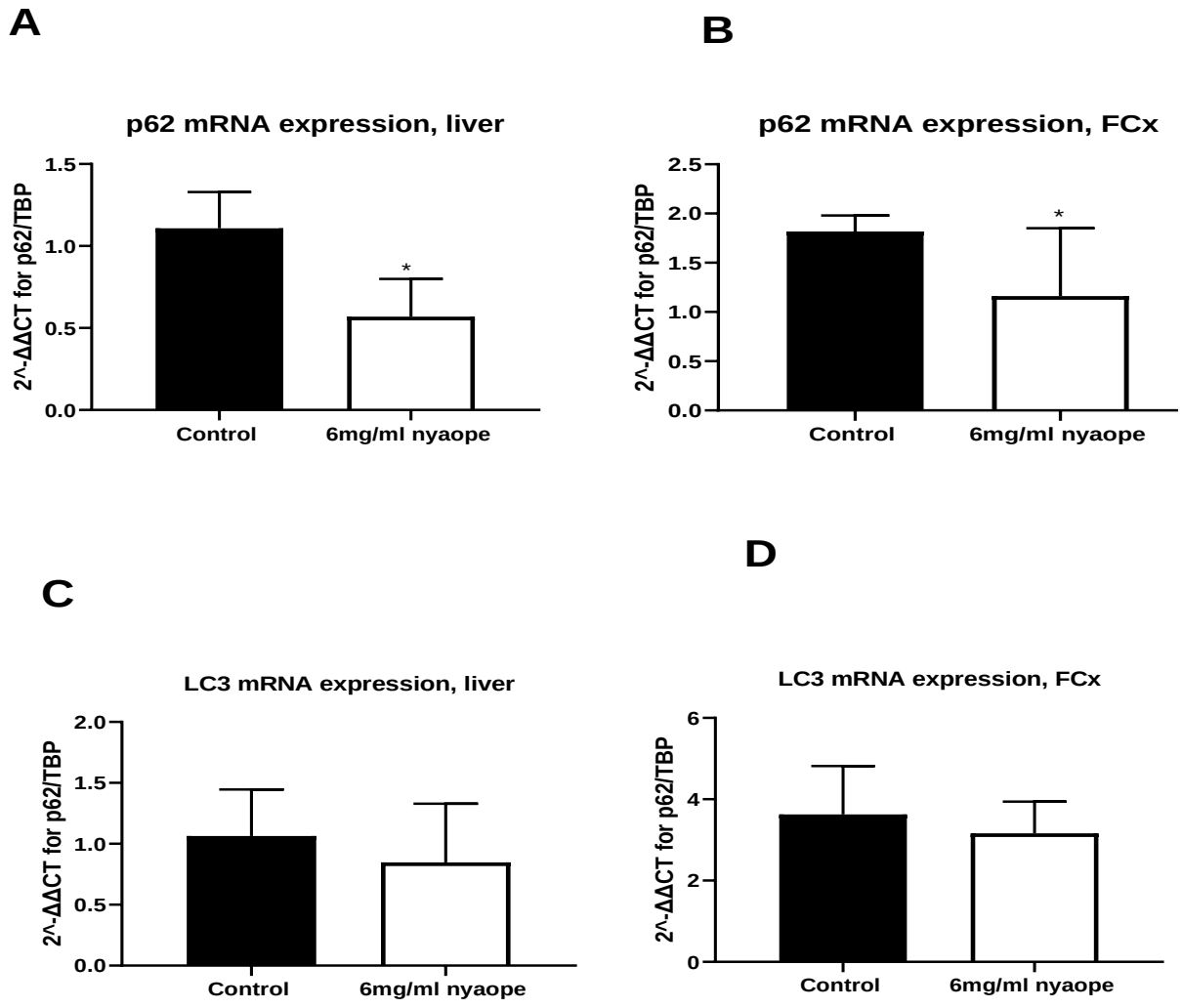

Figure 16: Relative mRNA expression of p62 and LC3 in the liver and prefrontal cortex of saline-treated (Control) and nyaope-treated Wistar rats. The mRNA expression level of these autophagy markers were normalised against the house keeping gene (TBP). Expression levels (2^-ΔΔCt) were also normalised against control (gene expression values obtained in the absence of nyaope), set as 1 (100%). Results are expressed as mean ±SD of 6 rats per group. *$p < 0.05$ compared to Control (t test).

Chapter 5: Discussion

The prevalence of substance use and substance abuse continues to escalate at an alarming rate worldwide (World Drug Report, 2017). In South Africa, the consumption of illicit substances matches that of other countries (Mokwena, 2016) with socially disadvantaged communities being mostly affected (Khine *et al*., 2015; Mokwena and Huma, 2014). One of the drugs commonly used in these communities is nyaope (Morgan *et al.,* 2019; Mokwena 2015), a cocktail street drug containing large amounts of heroin (Mokwena, 2015; Khine *et al*., 2015).

The aim of the present study was therefore to investigate the toxic effects of nyaope. The liver and the brain were the two chosen organs of interest as one of the main functions of the liver is drug metabolism, while the effects of nyaope on the brain is clearly evidenced by the impact on the behavior of the consumer eg. hallucinations, euphoria, as well as the presence of mental disorders such as depression and anxiety (Morgan *et al.*, 2019).

The study design included both *in vitro* and *in vivo* experiments. HepG2 cells were used as surrogate liver cells and SH-SY5Y neuroblastoma cells represented neuronal tissue. These cells were treated with increasing concentrations of nyaope and the effects thereof on cell viability and cell death were subsequently investigated at various time points.

For the *in vivo* experiments, Wistar rats received intraperitoneal injections of nyaope for 3 consecutive days. On day 3 liver tissue and the prefrontal cortex were dissected and subsequently analyzed for the presence of cell death.

5.1 *In vitro* study

5.1.1 Microscopic evaluation of cell well-being following exposure to nyaope

A robust microscopic approach was adopted to evaluate the general well-being of HepG2 cells after they were exposed to 6 mg/kg and 10 mg/ml nyaope for 24 hours. Images were taken with a ZOE microscope that facilitated the capture of live, unfixed, unstained cells and allowed for raw visualization of changes in cell confluence and morphology. In the present study, visual fields of untreated cells showed a high degree

of confluence of adherent cells, growing as monolayers. These morphological aspects were in line with a previous study reporting similar characteristics of healthy HepG2 cells in culture (Babensee *et al*., 1992). Exposure to nyaope resulted in a significant decrease in cell confluence and affected cell adherence. Most of the cells were floating rather than adherent to the cell culture vessels following exposure to nyaope. The cells also displayed irregular shapes. These findings were consistent with other research whereby the authors exposed HepG2 cells to silver nanoparticles, which induced abnormal cellular morphology characterised by cell shrinkage and asymmetrical shapes (Kawata *et al.*, 2009). These observations suggested that the addition of nyaope or any toxic insults to the culture medium create an unfavourable environment for the growth of HepG2 cells.

5.1.2 Nyaope reduced cell viability of HepG2 and SH-SY5Y cells

Two methods were used to assess the viability of cells following nyaope treatment. For HepG2 cells the trypan blue exclusion method was used, while MTT assays were used for neuroblastoma cells. The trypan blue exclusion method is based on the principle that injured cells have impaired cell membranes that allows the entry of the colour reagent, trypan blue, to enter cells, while viable cells with intact cell membranes will prevent this entry (Strober, 2001). HepG2 cells displayed increasing trypan blue staining when exposed to escalating concentrations of nyaope for 24 hours. This implied that nyaope was toxic to the HepG2 cells and decreased its viability in a concentration dependent manner. The MTT assay revealed that lower concentrations of nyaope (<2.5 µg/µL) was not as effective in decreasing the viability of SH-SY5Y cells as the higher concentrations (≥5 µg/µL). This result confirmed the harmful effects of nyaope albeit at concentrations greater than 5 µg/µL.

The mechanism of cell toxicity induced by nyaope compounds is unknown. It may therefore be attributed to heroin itself, to other drugs present in the nyaope cocktail or a combination thereof. Insights into the toxic mechanisms of heroin (Cunha-Oliveira *et al.,* 2010), caffeine (Górska *et al*., 2018), and ARVs (Möller *et al*., 2018) may subsequently offer a possible explanation for the observed nyaope-induced toxic effects.

Heroin acts on the opioid system located in the central nervous system to exert its neurotoxic effects (Dickson *et al*., 2010). Heroin has been shown to decrease the

activity of the enzymes superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx). Inhibition of these enzymes leads to oxidative stress resulting in DNA damage, protein oxidation and lipid peroxidation (Cunha-Oliveira *et al*., 2010).

There is limited information on the actual toxic risk associated with caffeine consumption. Caffeine, in low doses (<200 mg), was reported as a neuro-stimulant but in larger quantities it was associated with anxiety, tremor, hallucination and convulsion (Davies *et al*., 2012). Caffeine acts via adenosine receptors to inhibit phosphodiesterase functioning. This leads to the release of calcium from intracellular stores and the activation of oxidative stress pathways (Myers *et al*., 1999; Ermak and Davies, 2002). It is therefore possible that some of the cell death identified with nyaope treatment could have been due to caffeine-induced calcium overload, mitochondrial dysfunction and consequent oxidative stress.

Recent studies in our laboratory have shown that ARVs such as tenofovir and efivarenz, may have deleterious effects on the brain. In these studies, the authors demonstrated the ARV's to induce inflammatory responses (Zulu *et al.,* 2018) and lipid peroxidation (Zulu *et al.,* 2020).

Since nyaope is a mixture of many substances, it is very likely that the observed decreases in cell viability in our experiments, resulted from the synergistic effect of all the constituents. However, the high amount of heroin in nyaope suggests that this drug may be a significant contributor to obtained toxic effects. In view of the cell viability results, subsequent experiments were conducted in order to identify the mechanism of cell death following nyaope exposure.

5.1.3 Cell death

In the current study we investigated whether nyaope induced cell death by necrosis, apoptosis and/or autophagy.

5.1.3.1 Necrotic cell death

Necrosis, following a toxic insult, is characterised by cell swelling, loss of cell membrane integrity and eventual cell lysis (Stenson and Ciorba, 2018; Sergey *et al*., 2003). During necrosis therefore, intracellular contents leak into the extracellular space. Our results show a significant difference in the release of LDH into the culture

medium of nyaope-treated cells when compared to untreated controls. Interestingly, this difference was only observed after the cells were incubated with the higher concentrations of 5 µg/µL and 10 µg/µL nyaope. At these concentrations of nyaope the membranes of the cells must have been compromised, resulting in a significant increase in LDH leakage. Our data is in line with an earlier study which demonstrated greater rat cortical neuron membrane damage at high concentrations of street heroin (> 427 µg/mL) (Cunha-Oliveira *et al*., 2007), the main nyaope ingredient. Moreover, the significant increase in LDH levels in the culture medium of cells exposed to high concentration of nyaope, was time-dependent. LDH activity in the culture medium increased as the incubation time was lengthened from 1 to 24 hours. Our results therefore suggest that nyaope-induced cell death can at least in part be ascribed to the involvement of necrosis.

5.1.3.2 Apoptotic cell death

Cell death by apoptosis induced by drug of abuse has been documented previously (Cunha-Oliveira *et al*., 2008; Krasnova *et al*., 2005; Oliveira *et al*., 2003; Perlman *et al*., 1999). This programmed type of cell death can occur through the activation of either an intrinsic or an extrinsic pathway that culminate in the activation of a common executioner/death pathway. In this study, we investigated the role of the intrinsic apoptotic pathway in nyaope toxicity by assessing the mRNA expression level of two proteins namely Bcl-2 (an apoptotic suppressor) and Bax (an apoptotic promotor).

Nyaope concentrations of 2.5 µg/µl or less caused no significant difference in the mRNA expression levels of either Bax or Bcl-2, whereas nyaope concentrations of 5 µg/µL or higher resulted in a significant decrease in mRNA expression level of Bax, while the mRNA expression levels of Bcl-2 were significantly stimulated. Previously it was reported that upregulation of Bax protein and down regulation of Bcl-2 protein was positively linked to an apoptotic form of cell death (Naseri *et al*., 2015). However, our experiments yielded opposite results suggesting that it was unlikely that nyaope induced this mode of cell death.

The ratio of Bax/Bcl-2 influences the ability of a cell to respond to apoptotic stimuli. A greater Bax/Bcl-2 ratio indicates a greater vulnerability of cells to undergo apoptosis, whereas a low ratio is associated with cell resistance to apoptotic stimuli (Perlman *et al*., 1999). Again nyaope-treated cells showed a decrease in Bax/Bcl-2 ratio and hence

a higher resilience against apoptotic cell death. This observation therefore supported the notion that apoptosis was not one of the modes of cell death employed by nyaope to achieve cell death.

As stated earlier the mechanism of cell death induced by nyaope *in vitro* is unknown. Nonetheless cell death induced by psycho and non-psycho-stimulant drugs of abuse, including heroin, has been described previously (Cunha-Oliveira *et al*., 2008). Heroin was shown to promote caspase dependent mitochondrial apoptosis in primary cultured rat cortical neurons (Oliveira *et al*., 2003), release cytochrome c from mitochondria (Cunha-Oliveira *et al*., 2007), activate the executioner pathway by stimulating caspase enzymes (Oliveira *et al*., 2003) and increase Bcl-2/Bax ratio (Cunha-Oliveira *et al*., 2007; Krasnova *et al*., 2005; Mao *et al*., 2002; Imam *et al*., 2001). All these mechanisms lead to apoptosis, hence the lack of apoptotic involvement in nyaope-treated cells (with its high heroin content) was rather surprising. It may therefore be possible that the other components contained within nyaope could have interfered or counteracted the effects of heroin with respect to apoptosis. Since Bcl-2 overexpression was shown to protect mesencephalic immortalized cells from methamphetamine-induced apoptosis (Cadet *et al*., 1997), it may also be possible that the increased Bcl-2 expression in our nyaope-treated animals could have offered protection against nyaope-induced apoptosis.

5.1.3.3 Autophagic cell death

Autophagy is an important physiological process for the well-fare of living beings as it regulates the clearing of damaged organelles, prevents self-destruction (Glick *et al.*, 2010) and maintain cellular homeostasis by allowing recycling of long-lived proteins and organelles (Schlafli *et al.*, 2015; Yoshii and Mizushima, 2017). p62, a multifunctional classical receptor of autophagy, is one of the pivotal proteins in the regulation of the autophagy. It participates in the central process of proteasomal degradation of ubiquitinated proteins by facilitating the delivery of ubiquitinated cargoes to the phagolysosome (Tanida and Waguri, 2010). p62 has subsequently been used as a strong indicator of the autophagic process.

Microtubule-associated protein light chain 3 (LC3) is another protein considered as a definitive marker of autophagy and has been used as such in a number of studies (Ma *et al.*, 2019; Klionsky *et al.*, 2016). LC3 play a critical role in autophagosome

biogenesis and turnover. During autophagy the propeptide LC3-I is converted to LC3-II. Usually an increase in LC3-II band intensity and a decrease in LC3-I expression is considered as a hallmark of autophagy, but increases in LC3-II can be caused by enhanced autophagosome synthesis or reduced autophagosome recycling. Interpreting LC3-I and LC3-II related observations should therefore be done with caution (Rodríguez-Arribas *et al.,* 2017). In view of the potential controversial data obtained when LC3-I and LC3-II is determined, some researchers (including ourselves) chose to measure the levels of total LC3. This approach is considered acceptable as increased expression of LC3 has been associated with elevated autophagic activity (Huang *et al.*, 2015).

Since p62 binds to LC3 at the level of the autophagosome, the measurement of both proteins was expected to provide a reliable indication of autophagy. In the present study low concentrations of nyaope caused a significant decrease in p62 mRNA expression levels after 1-hour exposure. At this time point no significant differences were found in LC3 mRNA expression levels at all concentrations of nyaope studied. When cells were exposed to nyaope for 24 hours, a significant increase in p62 mRNA expression was observed for the 10 µg/µL nyaope concentration, while the expression levels of LC3 remained reduced. These observations suggest that the autophagy process could have been partially initiated at the 24-hour time point (ubiquination of damaged proteins that require p62 binding), but that other processes in the autophagic pathway (eg. synthesis of autophagosome that requires LC3 binding) have not yet commenced. It is therefore possible that our data reflect early signs of autophagy.

These findings complemented previous studies reporting morphine-induced autophagy (Hayashi *et al*., 2014; Feng et al., 2013). It has been shown that morphine, a heroin metabolite, induces autophagy in hippocampal neurons through the activation of µ-opioids receptors (Zhao *et al*., 2010). Interestingly reductions in mitochondrial DNA copy number have also been proposed as a mechanism for opiate-mediated autophagy in hippocampal tissue (Feng *et al*., 2013). It therefore appears that nyaope, with its high heroin content, is equipped to induce autophagy.

In an interesting experiment Pietrocola and co-workers (2014) administered coffee to mice and investigated the effects thereof on autophagy. These authors reported an increase in LC3 lipidation thereby promoting insertion of LC3 into the membrane of the

autophagosome. This occurred in conjunction with a decrease in the abundance of p62 i.e. a reduction in the sequestosome (Pietrocola *et al*., 2014). It is therefore not inconceivable that nyaope could have yielded similar opposite outcomes with respect to LC3 and p62 expression levels in the current study, again pointing towards autophagic processes at play.

5.2 *In vivo* study

5.2.1 Presence of Apoptosis

Our findings indicate that in the prefrontal cortex treating rats for 3 days with nyaope resulted in a significant decrease in the mRNA expression of Bax with no changes in Bcl-2 mRNA expression. With Bax being considered a pro-apoptotic protein, the decrease in its levels suggests that it is unlikely that apoptotic mechanisms were activated in the prefrontal cortex of nyaope-exposed animals.

Contrasting observations were obtained for the liver where nyaope-treated animals had significantly low Bcl-2 mRNA expression levels and Bax mRNA expression levels comparable to control animals. This result suggested that the intrinsic apoptotic pathway could have been stimulated since the Bcl-2 protein normally functions as an anti-apoptotic factor. Thus reducing the suppressor effects of Bcl-2 may have eased the “brake” on the intrinsic pathway and thereby promote apoptotic activity in the liver.

Collectively, the observations above indicate that the sensitivity of the two tissue types studied (prefrontal cortex and liver) is different to the induction of apoptosis by nyaope. This notion is supported by the low Bax/Bcl-2 ratio for the prefrontal cortex indicating greater resistance and the higher Bax/Bcl-2 ratio for the liver indicating greater vulnerability (Perlman et a., 1999).

Chronic exposure to opioid drugs was shown to interfere with cognitive functioning of rats through the activation of apoptotic pathways (Garcia-Fuster *et al*., 2003; Tramullas *et al*., 2008). Heroin-focused research has shown that this opioid can activate caspases in the rat frontal cortex (Warren et al., 2007), while another study found heroin to increase Bax mRNA expression and decrease Bcl-2 mRNA expression in the hippocampus (Wang and Han,2009). These reports, in contrast to the findings of the current studying, seem to suggest that the activation of apoptosis in animals may

be drug and body organ specific. And within a particular body organ, the initiation of apoptotic pathways may even be area dependent (prefrontal cortex vs hippocampus). It is therefore plausible that nyaope, with its cocktail composition, may evoke varied apoptotic responses, when administered to animals.

5.2.2 Presence of Autophagy

The effects of nyaope were further explored by quantifying the mRNA expression levels of autophagy markers in the frontal cortex and liver of Wistar rats. The expression levels of p62 were significantly decreased in both the liver and frontal cortex, while the expression levels of LC3 were similar to that of control animals. These findings were in line with the results obtained from our *in vitro* study showing a downregulation of p62 mRNA expression and no change in LC3 expression at low concentrations of nyaope. The *in vivo* results therefore validated the *in vitro* data with respect to the potential of nyaope to induce autophagy.

Autophagy is usually initiated by metabolic stress, starvation, hypoxia and toxic insults, and plays an important role in the removal of intracellular aggregates and cytoplasmic organelle (Glick *et al*., 2010). Exposure to heroin was shown to promote autophagy in rats (Feng *et al*., 2012) and cause alterations in autophagy-related genes in the striatum of monkeys (Choi *et al*., 2020). Our nyaope results can be aligned to these findings.

Clinically, alterations have been reported in brain morphology of consumers of nyaope (Ndlovu *et al.*, 2021). These included extensive grey matter atrophy in the right hemispheric medial orbitofrontal, rostral middle frontal, superior frontal, superior temporal and supramarginal gyri. Cortical abnormalities in the prefrontal, temporal and parietal regions were also associated with nyaope intake. These regions are mostly involved in impulse control, decision making, social and self-perception and memory (Nhanisi *et al*., 2021). Interestingly, there is a large overlap in the pattern of cortical abnormalities in brain regions affected by nyaope and that observed in patients who suffer from heroin disorder (Ndlovu *et al*., 2021). As such the toxicity of nyaope is comparable to that of heroin.

Nyaope is a mixture of various drugs ranging from CNS depressants (opioids and opiates), CNS stimulants (amphetamine and meth-amphetamine) and minor

constituents such as caffeine, ARVs, acetaminophen, duracaine and calcium oxide for binding and stabilisation of the drug (Khine and Mokwena, 2016). These drugs can act synergistically, for example opioids and opiates and benzodiapines share metabolic pathways. This leads to interactions between the various drugs, resulting in greater intensity of euphoria and addiction (Dean, 2006)). Furthermore, nyaope is commonly consumed in combination with cannabis (Morgan *et al*., 2019) and the psychoactive form of cannabis, THC, upregulates opiate receptors. This form of nyaope intake may potentiate its effect as a CNS depressant, hence better blunting of pain while offering stronger addiction properties (Cichewicz, 2004; Chimalakonda *et al*., 2012). Sufferers of cocaine, morphine and/or heroin abuse frequently complain about unbearable and severe abdominal cramps. This may explain the stomach cramps experienced by nyaope users (Khine and Mokwena, 2016). It has also been proven that ARVs interact with illicit drugs (Mokwena and Huma, 2014). It is therefore clear that the complex make-up of nyaope creates the ability to recruit a variety of molecular strategies to exert its toxic effect. To understand the full picture of nyaope functioning undoubtly requires much more experimentation.

Chapter 6: Concluding remarks

The present study provides data demonstrating the toxic effects of nyaope. Exposure to Nyaope induced a decrease in cell viability in both cell lines in a concentration dependent manner. Necrosis in SH-SY5Y cells was the main mechanism of cell death, this was evidenced by the increased LDH levels after exposure to nyaope (≥5 µg/µl). The evidence for an apoptotic form of cell death caused by nyaope was less convincing with mixed findings observed for the markers (Bax/Bcl-2) studied. Finally, our data suggests that the autophagy process could have been partially initiated after nyaope exposure as increased p62 and reduced LC3 at the 24-hour time point were recorded. To our knowledge, this is the first study to determine the effect of nyaope on cell viability and death, making the comparison of our data to existing literature difficult. Thus future studies are encouraged to confirm our nyaope effects.

6.1 Limitations and future studies

A review of the literature shows that the some of the components contained in nyaope share physiological and pharmacological aspects, as well as metabolic pathways. This is suggested to lead to longer lasting plasma levels and synergy of action, resulting in prolonged effects of euphoria and hallucination. Future studies should therefore consider determining the relative contribution of each compound to the toxic effects induced by nyaope. Further, understanding the metabolic pathways of nyaope-mediated effects is important for the identification and design of intervention strategies.

The mechanisms of cell death induced by nyaope were partially addressed in the present study. Despite these promising results, some limitations are also recognised. For example, few markers were used in assessing cell death mechanisms. For instance, LDH release was used as a marker for necrosis. Assessing membrane integrity and identifying necrotic bodies as additional markers for necrosis would have strengthened the data. Measurement of cytochrome c and specific caspases as additional markers for apoptosis, could have clarified the complex results pertaining to this mode of cell death. Determining the conversion of LC3-I to LC3-II would also have made the argument for nyaope-induced autophagy more convincing. Nevertheless, the current study does provide some insights into the harmful effects of nyaope, thereby making a contribution to the limited body of knowledge about this drug that is devastating our society.

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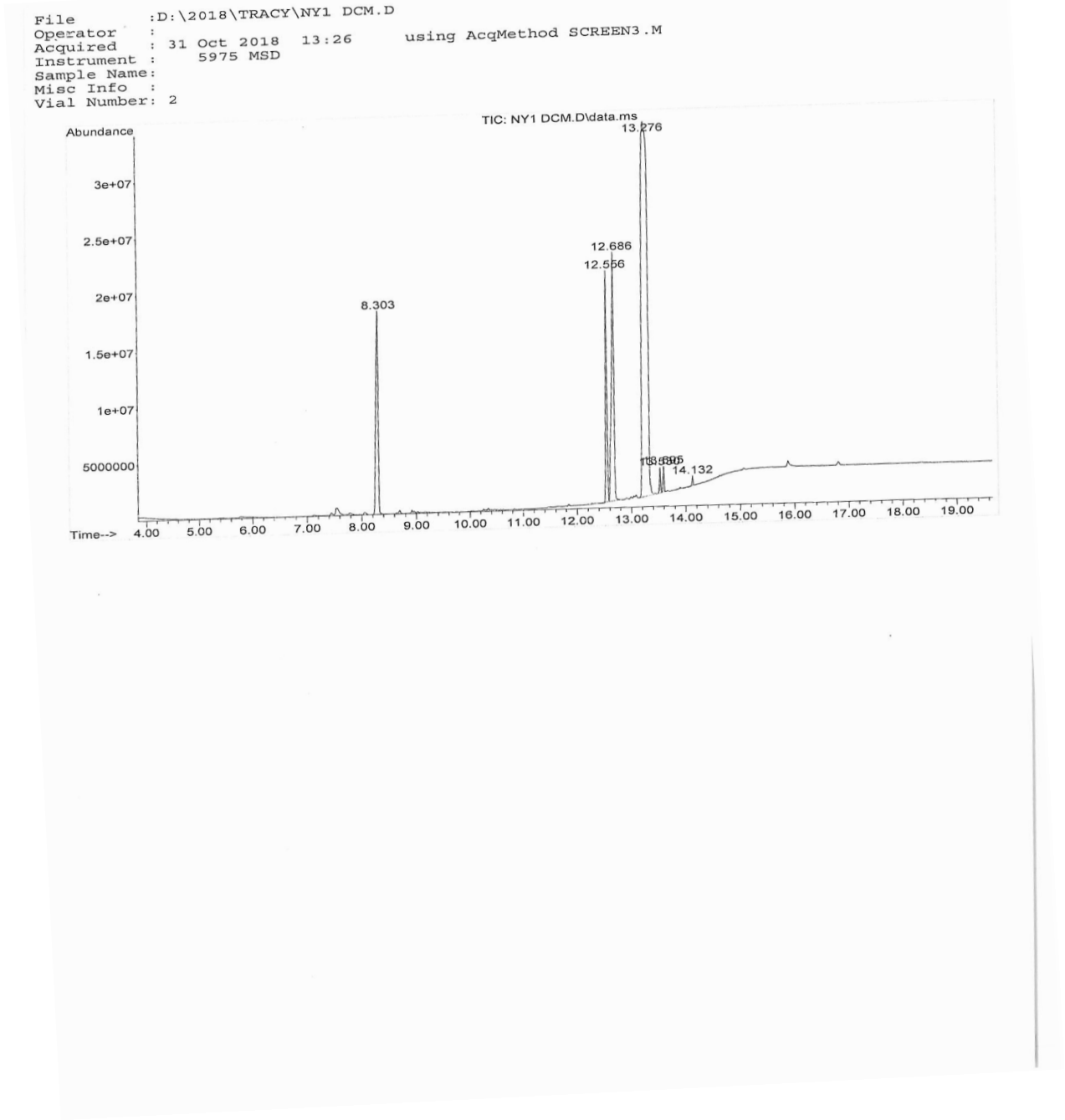
Appendix I. Nyaope components



Library Search Report

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ALS Vial : 2 Sample Multiplier: 1

Search Libraries: C:\Database\MPW2007.L Minimum Quality: 50

Unknown Spectrum: Apex
Integration Events: ChemStation Integrator - autoint1.e

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			P204				
			Amineptine artifact (ring)		6036	999806-03-6	1
			P205				
2	12.556	8.61	C:\Database\MPW2007.L				
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			P812				
			Bumetanide -SO2NH MEAC		2782	999802-78-2	38
			P812				
			Naloxone ME		565	999800-56-5	37
			P813				
3	12.688	16.50	C:\Database\MPW2007.L				
			Heroin-M (6-acetyl-morphine)		525	059833-14-6	95
			P744				
			Fenbendazole 2ME		7409	999807-40-9	38
			P743				
			Nisoldipine-M -H2O		5090	999805-09-0	22
			P919				
4	13.274	60.81	C:\Database\MPW2007.L				
			Morphin 2AC	@	225	000561-27-3	96
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			Heroin-M (6-acetyl-morphine)		525	059833-14-6	87
			P744				
			Naloxone AC		361	999800-36-1	60
			P916				
5	13.528	0.75	C:\Database\MPW2007.L				
			Lauroscholtzine artifact AC		6743	999806-74-3	55
			P956				
			GC stationary phase		2627	000000-00-0	22
			P1076				
			Protriptyline-M (HO-) 2AC		393	999800-39-3	11
			P895				
6	13.595	0.75	C:\Database\MPW2007.L				
			Pyritinol-M		949	000000-00-0	18
			P232				
			GC stationary phase (UCC-W-982)		1018	000000-00-0	17
			P1077				
			Decamethyltetrasiloxane		5429	000141-62-8	10
			P666				
7	14.132	0.17	C:\Database\MPW2007.L				
			GC stationary phase		2627	000000-00-0	43
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