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Research paper

The Cytotoxic Effects of Nyaope, a Heroin-based Street Drug, in *SH-SY5Y* Neuroblastoma Cells

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ABSTRACT

Nyaope is a local adulterated drug that contributes significantly to the psychosocial challenge of substance use in South Africa. Despite being a huge burden on society and the health care system, research into the deleterious effects of nyaope is limited. The aim of the present study was therefore to perform a chemical analysis of the drug and to assess its toxic effects on neuroblastoma cells. Gas chromatography-mass spectrometry (GC/MS) analysis showed that nyaope mainly consists of heroin and heroin-related products. *SH-SY5Y* cells were subsequently exposed to increasing concentrations of nyaope (0.625, 1.25, 2.5, 5 and 10 μg/μL) for 1, 6 or 24 h. The toxic effects of nyaope were determined by measuring lactate dehydrogenase (LDH) released into the cell culture medium as an indicator of necrosis, the mRNA expression levels of *Bax* and *Bcl-2* as markers of apoptosis, and the mRNA expression levels of *p62* and microtubule-associated protein 1 A/1B light-chain 3 (*LC3*) as indicators of autophagy. Exposing *SH-SY5Y* cells to concentrations of nyaope 5 μg/μL and greater for 24 h, resulted in a significant increase in LDH levels in the cell culture medium, unchanged mRNA expression of *Bax* and *Bcl-2* mRNA, and significantly reduced *p62* and elevated *LC3* mRNA expression levels. The chemical analysis suggests that nyaope should be considered synonymous with heroin and the toxic effects of the drug may recruit pathways involved in necrosis and autophagy.

Introduction

The prevalence of substance use and substance abuse continues to escalate at an alarming rate worldwide. Of particular concern is the escalation in polydrug use and the intake of clandestine-produced street drugs (UNODC World Drug Report, 2022). Recent data reporting opioid use in South Africa suggested a 2.6% lifetime prevalence of alcohol dependence and 0.6% for drug dependence in the general population (Herman et al., 2009), a 12.8% consumption of heroin among youth of African ancestry (South African Department of Health and South African Medical Research Council, Umthente Uhlaba Usamila: The 1st South African National Youth Risk Behaviour Survey 2002, 2003). Although there is limited research measuring the specific extent of South Africa's heroin problem, the abovementioned studies indicate a worrying increase in the availability and use of heroin in South Africa since the early 2000′s. One of the substances commonly used in South Africa is a street drug with a strong heroin base called nyaope (Mokwena and Huma, 2014; Khine et al., 2015).

We recently conducted a longitudinal study characterising 300 nyaope consumers in the Gauteng province of South Africa (Morgan et al., 2019). The majority of these participants were male, between the ages of 23 to 30 years, unemployed, and engaged in high levels of criminal activity. In addition, these participants were diagnosed with other mental illnesses that included major depressive episodes, generalised anxiety disorder and suicidality (Morgan et al., 2019; Morgan et al., 2022). These mental disorders were associated with atrophy in brain areas related to impulse control, decision-making, social-and self-perception and working memory (Mokwena and Makuwerere, 2021). Despite these alarming findings on the impact of nyaope on brain structure and functioning, research that focuses on the mechanism(s) by which this drug mediates its detrimental effects is limited.

To address this shortcoming, the present study firstly determined the chemical composition of nyaope. Thereafter, a molecular basis for the toxic effects of nyaope were investigated using an in vitro model. In this regard, the present study focussed on nyaope-induced cell death in *SH-SY5Y* cells, a human cell line derived from *SK-N-SH* neuroblastoma cells.

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The aim of our study was therefore, to determine the mode of cell death following exposure to nyaope.

Methods

Chemical analysis of nyaope

The chemical analysis of nyaope was performed in conjunction with the Department of Chemical Pathology in the Faculty of Health Sciences at the University of the Witwatersrand. Nyaope samples, obtained from the South African Police Services, were extracted using three different solvents (dichloromethane, ethanol and methanol) and subsequently subjected to gas chromatography-mass spectrometry (GC/MS) analysis in full scan mode (Agilent Technologies 5975 C with Triple Access Detection, Santa Clara, CA, USA). The National Institute of Standards and Technology (NIST) database was used to identify compounds according to their CAS registry number. The CAS registry number is a unique numerical identifier assigned by the Chemical Abstracts Service to every chemical substance described in the open scientific literature. The identifications obtained were verified against another database known as the Mass Spectral Library of Drugs, Poisons, Pesticides, Pollutants developed by Pfleger et al. (1992).

In vitro experiments

SH-SY5Y cells (Cellonex™, South Africa) were cultured in T75 cell culture flasks in a 1:1 composition of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 nutrient mixture, supplemented with 10% foetal bovine serum (FBS) (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.

Cell viability detection by 3-(4, 5-dimethylthiazole-2-yl)−2, 5-diphenyl-tetrazolium bromide (MTT) assay

The MTT assay (Roche, South Africa) was used to assess the viability of cells. *SH-SY5Y* cells were seeded in 96 well plates 24 h 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 or plain media (negative controls) for various time intervals (1 h, 6 h and 24 h) at 37 °C with 5% CO_2. At the end of the exposure period, 0.5 mg/mL MTT solution was added to each well and the plate was incubated for 4 h 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 h. 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 (GraphPad Software Inc., San Diego, CA). Blanks were included and contained media only without cells to correct for background readings from the microplate reader. Data were plotted as the percentage of cell viability versus concentration. All drug concentrations and times were tested in triplicate and experiments were repeated at least twice. Percentage cell viability was calculated as follows:

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

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

Necrotic cell death detection by lactate dehydrogenase (LDH) assay

The LDH assay (Roche, South Africa) quantifies cytotoxicity by measuring lactate dehydrogenase enzyme (LDH) activity in the growth medium as released by damaged cells (Luhr et al., 2018). 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 experimental readings per assay. Secondly, a low control was used to determine the LDH activity released from untreated control cells (spontaneous LDH leakage) and finally, a high control was used to determine the maximum LDH released from the cells.

Cells were seeded in 96 well plates 24 h prior to treatment with nyaope. Culture medium was aspirated and replaced with colourless RPMI-1640 (Gibco, Thermo Fischer Scientific, U.K.) containing plain media (negative controls) or 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). The cells were exposed to nyaope at time intervals of 1 h, 6 h and 24 h at 37 °C with 5% CO_2. Untreated cells represented 'low controls', while cells exposed to lysis buffer alone represented 'high controls'. Following exposure, 5 μL 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 min. 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).

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

Relative gene expression analysis was used to assess apoptotic and/or autophagic modes of cell death as induced by exposure to nyaope.

Extraction and purification of RNA were carried out using an 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 h and 24 h). Following incubation, cells were detached from culture plates using Trypsin/EDTA solution (Gibco™, 0.025% trypsin and 0.01% EDTA in phosphate buffered saline (PBS)). Cells in suspension were transferred to 15 mL centrifuge tubes and pelleted by centrifugation at 800 rpm for 5 min (Hettich benchtop centrifuge, UNIVERSAL 320, Berlin) prior to commencement of the extraction process. For RNA extraction from the cells, 350 μL lysis solution followed by 3.5 μL β-mercaptoethanol was added to each cell pellet. Eluted RNA from cells was subsequently utilized for first-strand cDNA synthesis. RNA was reverse transcribed using Superscript IV VILO reverse transcription kit (Catalogue #11756050, Thermo Scientific, U.K.), as per manufacturer's instructions.

The PCR reactions were performed using TaqMan® fast advanced PCR master mix (Thermo Scientific, U.K.). Expression of mRNA for apoptotic (Bax, Bcl-2) and autophagic (LC3, p62) markers and a reference gene (18 S ribosomal RNA, TaqMan® assays ID; Hs99999901_s1), was detected in duplex reactions by comparative gene expression 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.). The primer sequences of the primer-probe pairs are propriety information of the supplier (Thermo Scientific, U.K.), however, the precise primer-probe pairs used in this investigation can be purchased using the corresponding unique 'TaqMan® assay IDs' provided above. 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 $>$ 1000 ng/μL), 5 μL TaqMan® fast advanced PCR master mix, 0.5 μL target probe, 0.25 μL reference probe, and 3.25 μL dH_2O in 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 cycling conditions: 95 °C for 3 min for initial denaturation followed by 40 cycles of 95 °C for 15 s 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). Ct values of target genes were first normalized relative to Ct readings of 18 S rRNA within the same sample and then expressed as fold changes with respect to control samples. The Ct corresponds to the number of cycles required to detect a fluorescence signal above the baseline threshold of the instrument. 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).

Statistical analysis

The data are expressed as mean ± SD of n samples. Statistical analysis was performed using GraphPad Prism software (version 9.02). The Shapiro-Wilk test was used to establish the distribution of data. Two-way analysis of variance (ANOVA), for comparison of various concentrations of nyaope, was used to identify significant differences between the different groups over time. This test was followed by Tukey's multiple comparison test. A probability value of $p < 0.05$ was considered statistically significant.

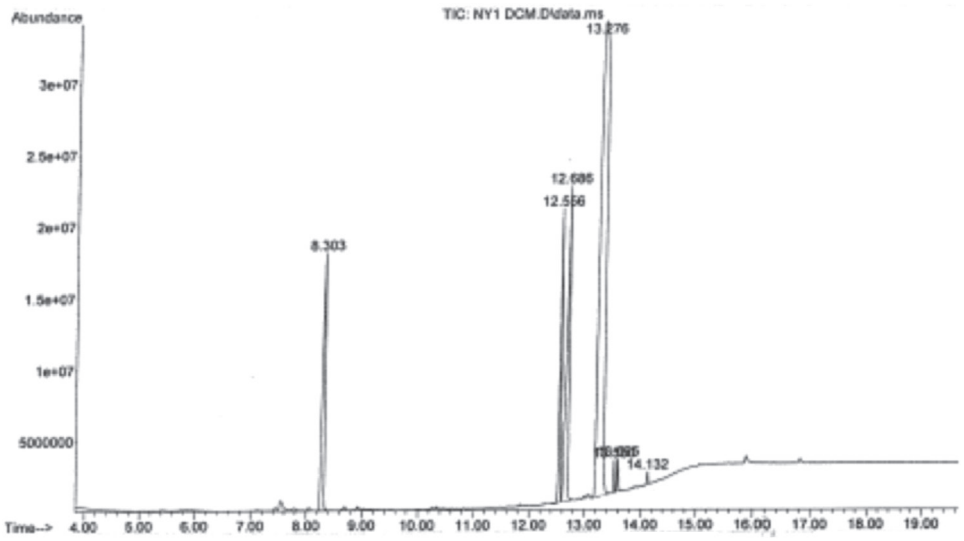
Results

Chemical analysis of nyaope

When nyaope samples were extracted using dichloromethane, significant peaks were identified for morphine, heroin, codeine and caffeine (Fig. 1). A similar pattern of peaks was observed when nyaope samples were extracted using ethanol (Fig. 2). Extraction of nyaope using methanol yielded a more comprehensive list of compounds (Fig. 3). Here significant peaks were identified as 1(3 H)-isobenzofuranone, 1 H-purine-2, 6-dione 3,7-dihydro-1,3,7-trimethyl-acetamide, acetylcodeine, 6-MAM and diacetylmorphine. Despite slight differences, all three solvents showed high concentrations of heroin and heroin-related products.

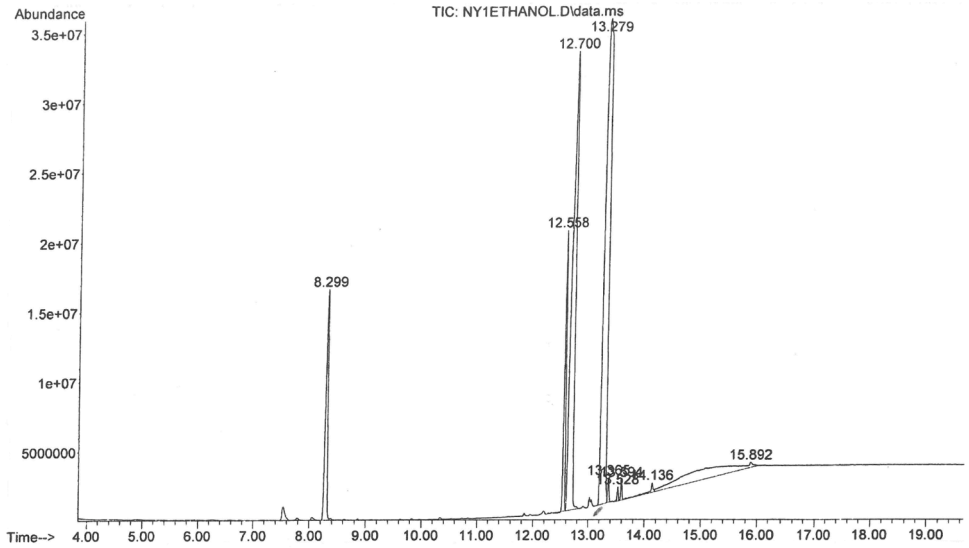
Viability of SH-SY5Y cells following nyaope exposure

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 h. At all-time points tested, exposure to nyaope induced a



Retention time (min)	Library / ID	Reference no	CAS #	Quality (%)
8.303	Caffeine	191	000058-08-2	95
12.556	Codeine	224	006703-27-1	93
12.685	Heroin-M (6-acetyl-morphine)	525	059833-14-6	95
13.276	Morphine 2AC	225	000561-27-3	96

Fig. 1. Chromatograph and compounds identified of nyaope samples extracted with dichloromethane.



Retention time (min)	Library / ID	Reference no	CAS #	Quality (%)
8.299	Caffeine	191	000058-08-2	94
12.558	Codeine	224	006703-27-1	93
12.700	Heroin-M (6-acetyl-morphine)	525	059833-14-6	95
13.279	Morphine 2AC	225	000561-27-3	95

Fig. 2. Chromatograph and compounds identified of nyaope samples extracted with ethanol.

significant decrease in cell viability of *SH-SY5Y* cells (Fig. 4). 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 h 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 controls). Following 6 h of nyaope exposure, significant decreases in the number of metabolically active cells were 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 μg/μL 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 h. At this time interval all concentrations of nyaope caused a significant decrease in the number of viable cells (Fig. 4). Of note, the data showed that at concentrations of 5.0 μg/μL and 10.0 μg/μL, nyaope seemed to have achieved its maximum effect with respect to cell viability at all time periods.

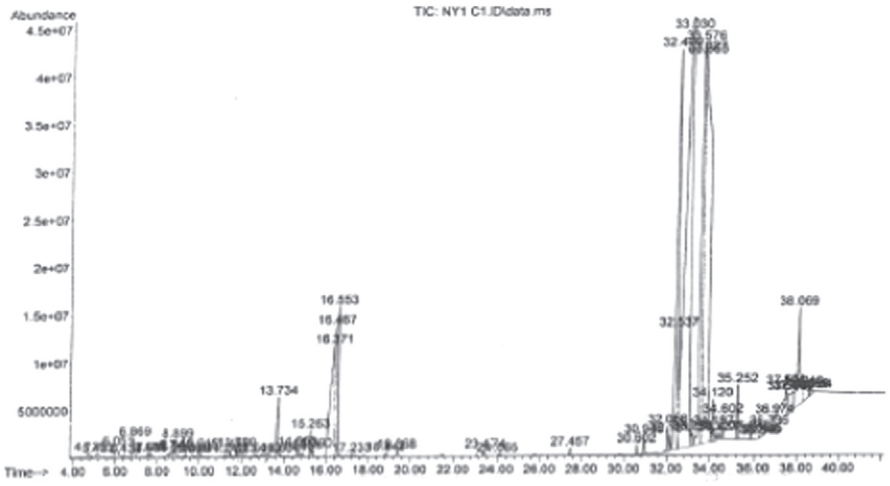
Necrotic cell death

In order to assess the mechanism of nyaope toxicity and cell death, the concentration of lactate dehydrogenase (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 (Fig. 5). 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 h of 5 μg/μL nyaope exposure, when compared to control (Fig. 5). 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 h to 6 h to 24 h (Fig. 5).

Apoptosis

Cell death by apoptosis was assessed by RT-PCR analysis of the relative mRNA expression of the gene that codes for the pro–apoptotic protein, *Bax*, and the gene that codes for the anti-apoptotic protein, *Bcl-2*, at two time periods of exposure (1 h and 24 h), with 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 (Fig. 6A) and 24-hour (Fig. 6B) 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 $p < 0.001$ (Fig. 6A) and at 24 h it was $p < 0.05$ (Fig. 6B) when compared to control.

Similar to *Bax* expression, exposing *SH-SY5Y* cells to the lower



Retention time (min)	Library / ID	Reference no	CAS #	Quality (%)
13.734	1(3H)-isobenzofuranone	52288	000569-31-3	99
16.371	1H-purine-2,6-dione 3,7dihydro-1,3,7-trimethyl-acetamide	53168	000058-080-2	97
16.457 (same as 16.371)	1H-purine-2,6-dione 3,7dihydro-1,3,7-trimethyl-acetamide	53168	000058-080-2	97
16.553 (same as 16.371)	1H-purine-2,6-dione 3,7dihydro-1,3,7-trimethyl-acetamide	53170	000058-080-2	97
32.479	acetylcodeine	148310	1000153-00-2	98
32.537	6-monoacetylmorphine	140705	002784-73-8	99
33.033 (same as 32.537)	6-monoacetylmorphine	140705	002784-73-8	99
33.575	diacetylmorphine	160913	000561-27-3	99
33.627 (same as 33.575)	diacetylmorphine	160913	000561-27-3	99

Fig. 3. Chromatograph and compounds identified of nyaope samples extracted with methanol.

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 h and 24 h. At 1 h 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; Fig. 6C), while at 24 h this stimulation was even further enhanced (Fig. 6D). 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 h (Fig. 6E) and 24 h (Fig. 6F).

Autophagy

LC3 and *p62* are death receptor markers used to monitor the autophagy process. At the 1-hour time point, mRNA expression levels of *p62* were significantly decreased when *SH-SY5Y* cells were exposed to nyaope at all concentrations tested ($p < 0.05$; Fig. 7A), except when exposed to 10 μg/μL. There was no significant differences in mRNA expression levels of *p62* between cells exposed to 10 μg/μL nyaope and controls ($p > 0.05$, Fig. 7A). This pattern of *p62* expression levels was different at the 24 h′ 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 controls, 5 μg/μL of nyaope reducing expression level of *p62* compared to controls but was not found

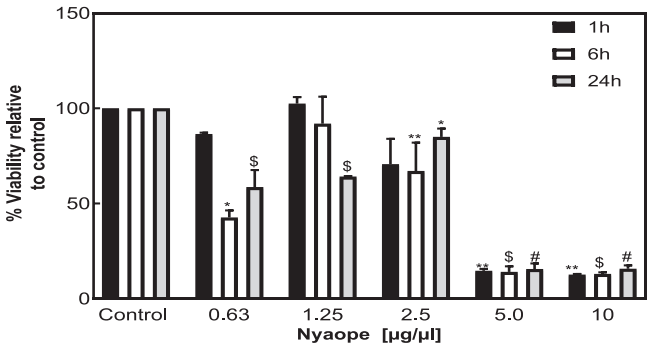


Fig. 4. The effect of nyaope on cell viability. *SH-SY5Y* cells were exposed to increasing concentrations of nyaope for 1, 6 and 24 h. Cell viability was measured by MTT assay. The results were normalised against control (untreated group), which was set at 100%. Results are expressed as mean ± SD from 3 independent experiments performed in triplicate. * ** *$p < 0.0001$ (#), * **$p < 0.001$ ($), * *$p < 0.01$ and*$p < 0.05$ compared to control (Two-way ANOVA followed by Tukey's multiple comparison *post-hoc* test).

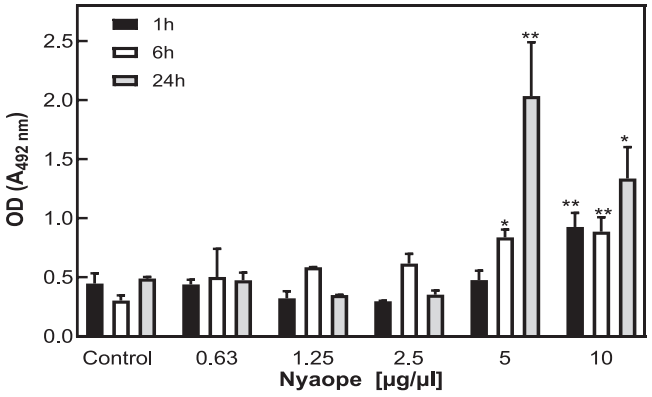


Fig. 5. Cell death by necrosis as measured by LDH detection in the cell culture medium. *SH-SY5Y* cells were exposed to increasing concentrations of nyaope for 1, 6 and 24 h. Results are expressed as mean ± SD from 2 independent experiments done in triplicate. * *$p < 0.01$ and *$p < 0.05$ when compared to control (Two-way ANOVA followed by Tukey's multiple comparison *post-hoc* test).

to be significant ($p < 0.303$), while 10 µg/µL of nyaope caused an increased expression of *p62* when compared to control ($p < 0.05$; Fig. 7B).

The overall assessment of *LC3* mRNA expression levels showed that nyaope induced a reduction in mRNA expression levels of *LC3*, as the concentration of nyaope increased. This pattern of decreasing *LC3* expression levels was comparable at the two time points studied (Figs. 7C and 7D). After 1-hour exposure, there were no significant differences between untreated control cells and cells exposed to nyaope at concentrations of 0.63 µg/µL, 1.25 µg/µL and 2.5 µg/µL ($p > 0.05$, Fig. 7C). This result was accompanied by a significant decrease in *LC3* expression levels as the concentration of nyaope increased to 5 µg/µL and 10 µg/µL when compared to control ($p < 0.05$; Fig. 7C). After 24 h of nyaope exposure *SH-SY5Y* cells displayed a dose-dependent decrease in mRNA expression of LC3 (Fig. 7D) 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 (Fig. 7D). This data showed that higher concentrations of nyaope lead to greater *LC3* degradation when compared with lower concentrations.

Discussion

Chemical analysis of nyaope

Characterization or profiling of street drugs is important to gain insights into the active ingredients that mediate its effects. Therefore, drug profiling provides the chemical basis that underpins the pathophysiological impact of the drug. The profiling exercise simultaneously identifies additional substances that are added during the preparation or production of the drug by the dealer. Identifying the composition of a street drug is therefore vital to understanding its mechanism of action and to informing remedial strategies to address harmful effects.

Nyaope has previously been described as a low-grade heroin to which various other compounds have been added as bulking agents (Khine et al., 2015; Mthembi et al., 2018). In the current study, nyaope samples were subjected to three different extraction solvents, namely dichloromethane, ethanol and methanol. The three methodologies provided comparable data that unequivocally showed that the nyaope samples contained significantly high levels of 6-acetyl-morphine (6-AM) and morphine. In contrast to previous reports (Khine et al., 2015; Mthembi et al., 2018), we found nyaope to consist mainly of heroin and heroin intermediates of exceptionally high quality and purity. Our data, therefore, do not support the description of nyaope as a low-grade version of heroin. Indeed, our study highlights this misconception and encourages scientists and healthcare professionals to refer to nyaope in the same vein as heroin.

A study from Austria compared the purity of 415 samples of street heroin with drug-related hospital emergencies. All 415 samples contained adulterants. The study did not find a correlation between heroin purity and heroin-related deaths (Risser et al., 2007). An older study from 1984 analysed over 12000 samples of white heroin, brown heroin and cocaine (Cunningham et al., 1984). Eleven adulterants were consistently found in the samples. The antimalarial agent, Quinine, had a greater than 5% frequency of occurrence in white and brown heroin. A 2018 study that chemically analysed over 500 samples of seized drugs in the US found that Quinine was present in 42.5% of heroin samples (Fiorentin et al., 2019). Academic, media and policy reports on heroin in most other parts of the world do not classify heroin by their grade, diluents or adulterants. In the South African context, there are a dearth of studies analysing mass samples of street heroin. Consequently, the frequent emphasis on 'low-grade', 'cocktail' and the focus on adulterants such as ARVs may lack sufficient scientific evidence and diverts attention from a serious growing heroin problem in South Africa.

Heroin, or diamorphine, is a diacetyl derivative of morphine. In the body, it is rapidly converted to 6-AM or monoacetylmorphine (MAM). In our analysis of nyaope, this major heroin derivative appeared in significant amounts together with 3-acetyl morphine (3-AM). 3-AM is a less active metabolite of heroin compared to the more active 6-AM. A study by Avvisati et al. (2019) showed that 6-AM displays effects similar to that of pure heroin. For instance, it exhibits robust reinforcing properties in a rat self-administration setting akin to heroin, and evokes drug-seeking behaviour equal to heroin (Avvisati et al., 2019). 6-AM is further metabolised to morphine (morphine 2AC) and hence the presence of high concentrations of this compound was not surprising.

Besides heroin, notable amounts of codeine and caffeine were also identified in the nyaope samples, yet in much smaller quantities. It is possible that these substances identified in the samples may be cutting agents, or alternatively by-products of the fabrication process. The presence of codeine and caffeine in adulterated heroin samples have previously been reported (Florea et al., 2019) Nevertheless since these compounds by themselves have addictive properties (Addicott, 2014), their presence may add to the potential of nyaope to induce substance use disorder. Codeine, a strong psychostimulant, has been suggested to be a gateway substance to other opiates including morphine and heroin (Nkansah-Amankra and Minelli, 2016). It is well known that the administration of codeine relieves pain and, when ingested in higher

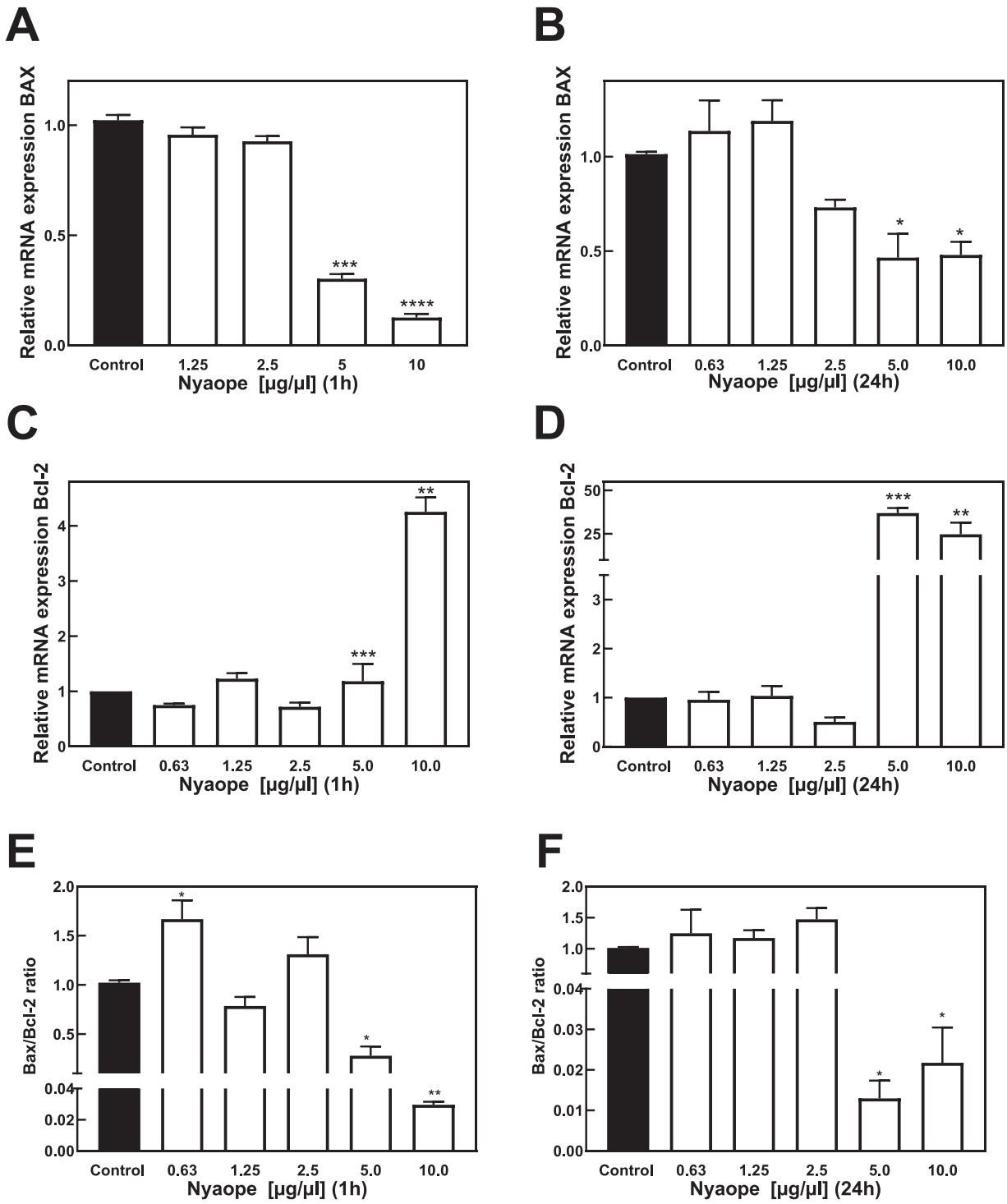


Fig. 6. mRNA expression of *Bax* (A, B), *Bcl-2* (C, D) and Bax/Bcl-2 ratio (E, F) in *SH-SY5Y* cells following exposure to increasing concentrations of nyaope for 1 h and 24 h. The mRNA expression levels were examined by RT-PCR and were normalised relative to expression of the housekeeping gene, *18 s rRNA*. Results are expressed as mean ± SD from 2 independent experiments performed in triplicate. * ** *p < 0.0001, * **p < 0.001, * *p < 0.01 and *p < 0.05 when compared to control (Two-way ANOVA followed by Tukey's multiple comparison *post-hoc* test).

doses, may lead to pleasurable, euphoric sensations. The physiological mechanisms of codeine-induced euphoria are well documented. Codeine binds to μ-opioid receptors to elicit its effects on the central nervous system (Chidambaran et al., 2017). Codeine is normally O-demethylated to form morphine via the activity of the cytochrome P450 enzyme CYP2D6 (Goldsack et al., 1996). However, studies have shown that codeine can also be converted to codeine-6-glucuronide and that this metabolite can generate opioid effects (Susce et al., 2006). The combined action of morphine and codeine-6-glucuronide may therefore lead to an escalated nyaope-mediated pleasurable experience.

The effects of caffeine on the central nervous system are varied. Clinical studies have shown an association between chronic caffeine ingestion and negative mood states such as anxiety, restlessness, insomnia, tachycardia (Herz, 1999), and impaired cognitive function (Ritchie et al., 2007; Santos et al., 2010). On the other hand, preclinical experiments reported caffeine administration to stimulate motor activity

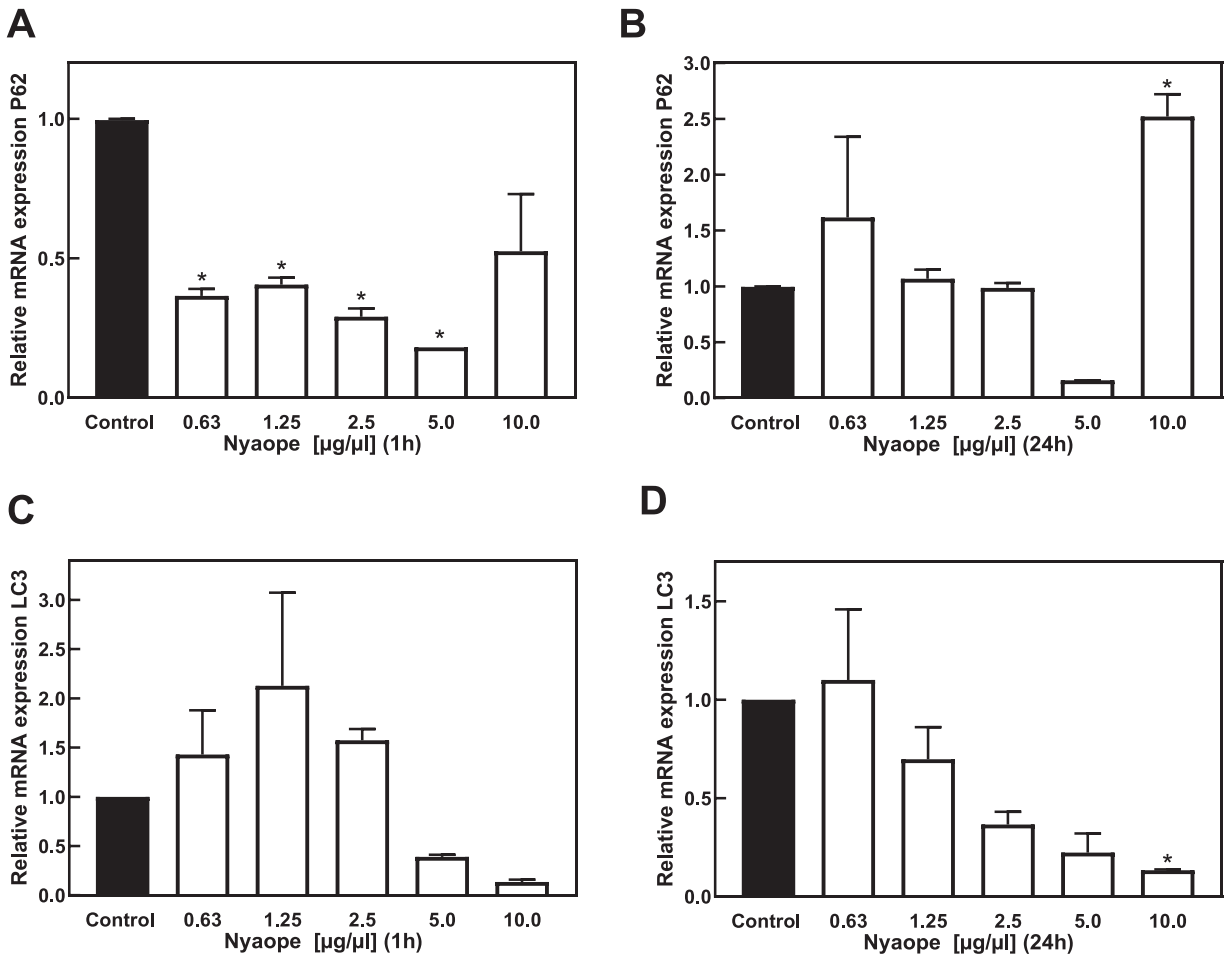


Fig. 7. mRNA expression of *p62* (A and B) and *LC3* (C and D) in *SH-SY5Y* cells was examined at 1 h and 24 h after exposure to increasing concentrations of nyaope. The mRNA expression levels of these autophagy markers were normalised relative to expression of the house keeping gene, *18 s rRNA*. Results are expressed as mean ± SD from 2 independent experiments performed in triplicate. *$p < 0.05$ compared to control (Two-way ANOVA followed by Tukey's multiple comparison *post-hoc* test).

in rodents (Acevedo et al., 2016). The effects of caffeine are proposed to be mediated via activation of adenosine A1/A2 receptors that influence dopaminergic neurotransmission and subsequent protein kinase A (PKA)-dependent mechanisms (Acevedo et al., 2016). Caffeine, in conjunction with heroin, may have synergistic effects that may result in a greater deleterious impact on the wellbeing of nyaope consumers.

Unlike those of others (Khine et al., 2015; Mthembi et al., 2018), our extractions showed no significant traces of other cutting agents. A reason for this discrepancy could be differences in methodology. Khine and co-workers used two independent methods to characterise their nyaope samples that included both gas chromatography-mass spectrometry (GC-MS) as well as time-of-flight direct sample analysis mass spectrometry (TOF DSA MS). These authors reported nyaope to contain antiretroviral drugs, antidepressants and benzodiazepines, in addition to morphine, caffeine, codeine and amphetamine (Khine et al., 2015). Despite variations in experimental procedures, the data collected shows that nyaope has a strong opiate base and is therefore an extremely unsafe substance to consume.

Viability of SH-SY5Y cells following nyaope exposure

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 chemical composition of nyaope suggests that the cell toxicity induced by nyaope is likely mediated by heroin and its related compounds, or to other drugs present in the nyaope cocktail (caffeine) or a combination thereof. Insights into the toxic mechanisms of heroin (Cunha-Oliveira et al., 2010) and caffeine (Herz, 1999) 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., 2008).

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 can lead to the release of calcium from intracellular stores and the activation of oxidative stress pathways (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. In view of the cell viability results, subsequent experiments were conducted in order to identify the mechanism of cell death following nyaope exposure, focusing on necrosis, apoptosis and/or autophagy.

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). 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, although these authors associated their findings with an apoptotic mode of cell death (Cunha-Oliveira et al., 2007). Moreover, the significant increase in LDH levels in the culture medium of cells exposed to high concentrations of nyaope, was time-dependent. LDH activity in the culture medium increased as the incubation time was lengthened from 1 to 24 h. Our results therefore suggest that nyaope-induced cell death can at least in part be ascribed to the involvement of necrosis.

Cell death by apoptosis induced by substances of abuse has been documented previously (Krasnova et al., 2005; Cunha-Oliveira et al., 2007). 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 and increase Bax/Bcl-2 ratio (Krasnova et al., 2005; Cunha-Oliveira et al., 2007). 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.

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 (Schläfli 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 (Klionsky et al., 2021). LC3 play a critical role in autophagosome biogenesis and turnover. During autophagy the pro-peptide 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 either 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. Increased levels of cytoplasmic LC3 has been shown to be essential for starvation-induced autophagy (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 h, 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 (ubiquitination 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 (Feng et al., 2013; Hayashi et al., 2014). It has been shown that morphine induces autophagy in hippocampal neurons and neuroblastoma cells akin to that used in the present study (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.

Conclusions

The present study provides data demonstrating toxic effects of nyaope. Exposure to nyaope induced a decrease in cell viability in *SH-SY5Y* cells in a concentration dependent manner. Necrosis was the main mechanism of cell death as evidenced by the increased LDH levels after exposure to nyaope (≥5 μg/μL). The data 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, the 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.

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 society.

CRediT authorship contribution statement

Willie M.U. Daniels – conceptualising, design, data analysis and manuscript writing. Matome M. Sekhotha – provision of nyaope samples and permits, chemical profiling of nyaope samples, data analysis and presentation. Nirvana Morgan – conceptualising, funding acquisition, manuscript review and editing. Ashmeetha Manilall – design of in vitro experiments, data analysis, manuscript review and editing.

Declaration of Competing Interest

The authors have no known conflicts of interest to declare.

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