DOCTOR OF HEALTH (DHEALTH)

Effect of ethinylestradiol and levonorgestrel on nucleoside reverse transcriptase inhibitor-induced apoptosis in human cervical epithelial cancer cells

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Award date:
2019

Awarding institution:
University of Bath

EFFECT OF ETHINYLESTRADIOL AND LEVONORGESTREL ON NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITOR-INDUCED APOPTOSIS IN HUMAN CERVICAL EPITHELIAL CANCER CELLS

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A thesis submitted in partial fulfilment of the requirements of the

Department for Health, University of Bath

Doctor of Health Degree

August 2019

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Declaration of authorship

I am the author of this thesis, and the work described therein was carried out by me personally, with the exception of appended conference presentations that I co-authored in collaboration with my supervisors who supported with editing of the manuscripts as shown in the list of authors.

ABSTRACT

Preliminary findings suggest an increase in cervical cancer among sub-Sahara African women on highly active antiretroviral treatment (HAART). There has been a sharp rise in serious non-AIDS events among HAART recipients. This study explored the effect of co-administering combined contraceptive hormones with antiretroviral drugs on promoting human cervical epithelial cancer cell proliferation, DNA mutation and cell transformation. Combinations of abacavir (ABC), zidovudine (ZDV), lamivudine (3TC), stavudine (d4T) and nevirapine (NVP) were co-administered with either ethinylestradiol or levonorgestrel.

The combinations ZDV+3TC, ABC+ZDV+3TC, ZDV+3TC+NVP and d4T+3TC+NVP induced a time-dependent antiproliferative effect on HeLa cells. Co-treatment with levonorgestrel demonstrated antiproliferative effects on combination antiretroviral drug-treated cells while co-administering 0.5µg/ml ethinylestradiol increases HeLa cell proliferation ($P \leq 0.5$). Single drugs (ABC, ZDV, 3TC, d4T and NVP) showed a time-dependent DNA double strand breakage in both HeLa and Chinese hamster ovary cells. Both levonorgestrel and ethinylestradiol drastically increased apoptosis in ABC+3TC-, ZDV+3TC-, ABC+ZDV+3TC- and d4T+3TC+NVP-treated cells. Highly active antiretroviral treatment transformed pre-monocyte lymphoma (U937) cells to adherent cells with macrophage-like morphology and increased superoxide production.

Increased HeLa cell death by apoptosis following co-administration of triple combination antiretroviral drugs with levonorgestrel suggests a protective effect against human cervical cancer cell proliferation among HAART users. Despite the drastic induction of apoptosis by 0.5µg/ml ethinylestradiol, ABC+3T-, ZDV+3TC-, ABC+ZDV+3TC- and ZDV+3TC+NVP-treated cells did not significantly differ from the untreated cells in their proliferation assayed by MTT ($P \leq 0.05$), thus suggesting a proliferative effect on the human cervical epithelial cancer cells. Transformation to macrophage-like morphology and increased superoxide anion production in U937 cells suggest precipitation of serious non-AIDs events among HAART users.

The antiproliferative effect of HAART on cervical cancer cells suggests a protective role against cell proliferation. The antiproliferative effect of levonorgestrel suggests its potential as a progestogen-only contraceptive alternative for women on HAART treatment. The present results also suggest the downside effect of HAART through precipitation of serious non-AIDS events. Further *in vivo* clinical studies maybe of interest.

ACKNOWLEDGEMENTS

This research was jointly supervised by the University of Bath and the University of East London. The experimental work was carried at the University of East London under the supervision of Dr Winston A Morgan. The research design, and writing-up was guided by Dr Giordano Pula and Professor Michael D Threadgill (University of Bath). My sincere gratitude goes to my research supervisors for their outstanding support and reassurance. Special thank you to the University of East London's Medicine Research Group for welcoming me and providing resources in their tissue culture laboratory. I enjoyed the beauty of your diversity which I found invaluable in accademic cross-pollination within this beutiful universal subject of Health Sciences. My gratitude also goes to the University of East London School of Health and Bioscience technical staff (past and present) for the support rendered. To my fellow research students in the University of East London tissue culture laboratory, most especially Shazma Bashir, thank you for your kindness and patience in orientating me into the tissue culture laboratory. Thank you to Dawei Chen for the support with flow cytometric techniques. I wish to express my gratitude to Dr David Wainwright and Dr Alan Buckingham from the Department for Health (University of Bath) for the initial induction and mentoring into the Doctor of Health programme. The tolerance and support of the Department for Health administrative staff also deserves the mention. To Gillian O'Carroll, Andrea Baker, Emily Austin and Jennifer Philips, I would like to say; "What a fascinating learning experience I had working with you on administrative aspects of my journey." To Professor Hilda Marima-Matarira, Dr Agatha Benyera-Mararike, Dr Aaron Maramba and Dr Mandla Nyathi, all from Zimbabwe, thank you for your emotional support, reassurance and sharing experiences on challenges of studying abroad especially in the United Kingdom. Last but not least I would like to thank my family for enduring and coping with my ocassional absence due to this commitment.

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ABBREVIATIONS

ABC-Abacavir

ATCC-American Type Culture Collection

ATV-Atazavir

AZT-Zidovudine

AIDS-Acquired immunodeficiency syndrome

APOBEC3G-apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like-3G

APV-Amprenavir

ART-Antiretroviral therapy

ARV-antiretroviral

BHIVA-British HIV Association

BPH-Benign prostatic hypertrophy

cDNA-copy deoxyribonucleic acid

CHO-Chinese hamster ovary

CIN-Cervical intraepithelial neoplasia

CLP-Common lymphoid progenitor

CMP-Common myeloid progenitor

COC-Combined oral contraceptive

COCs-Combined oral contraceptives

CP-Compensatory proliferation

Ddc-Zalcitabine

ddi-Didanisine

DLV-Delavirdine

DPMA-depot medroxyprogesterone acetate (DPMA)

DRV-Deranavir

DMEM-Dulbecco's Modified Eagle Medium

DMSO-Dimethylsulfoxide

DSB-Double strand break

d4T-Stavudine

EDTA-Ethylenediaminetetraacetic acid

EFV-Efivarenz

EGCG-Epigallocatechin galate

EE-Ethinylestradiol

ERα-Estrogen receptor-α

ERβ-Estrogen receptor-β

ETR-Etravirine

EVs- Extracellular vesicles

E2-17β-oestradiol

FADU-Fluorometric analysis of DNA unwinding

FBS-Foetal bovine serum

FPV-Fosamprenavir

FTC-Emtricine

GPEP-G protein-coupled estrogen receptor

GPR30-G-protein-coupled estrogen receptor

GnRH-Gonadotrophin releasing hormone

HAART- Highly active antiretroviral therapy

HC-Hormonal contraceptive

hECEs-human cervical epithelial cells

HESC-Human endometrial stromal cell

HEGC-Human endometrial glandular cell

HPV-Human papillomavirus

HSC-Hematopoietic stem cell

HSIL-High-grade squamous intraepithelial lesion

IDV-Indinavir

IIs-integrase inhibitors

LNG-levonorgestrel

LNG-IUS-Levonorgestrel intra-uterine system

LPV/r-Lopinavir+ritonavir

LSIL-Low-grade squamous intraepithelial lesion

MEP-megakaryocyte-erythroid progenitor

MI-myocardial infaction

mL-Millilitre

MPP-Multipotent progenitor

mRNA- messenger RNA

MTT- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MVC-Muraviroc

NAs-Nucleoside analogues

NCE-Normochromatic rythrocyte

NFV-Nelfinavir

NHMEC-Normal human mammary epithelial cell

NK-Natural Killer

NNRTIs-Non-nucleotide/nucleoside reverse transcriptase inhibitors

NRTIs-nucleotide reverse transcriptase inhibitors

NVP-Nevirapine

OCs-Oral contraceptives

Pap-Papanicolaou

PCE-Polychromatic erythrocytes

Phr-Phenol red

PIs-Protease inhibitors

PMA- Phorbol 12-myristate 13-acetate

PR-Progesterone receptor

PRA-Progesterone receptor-A

PRB-Progesterone receptor-B

Pre-HSC-pre-hematopoietic stem cell

PS-Phosphatidylserine

SILs-Squamous intraepithelial lesions

RGV-Raltegravir

RIPK-Receptor-interacting protein kinase

ROS-Reactive oxygen species

RTV-trapinavir

SNAEs-Serious non-AIDS events

SARUA-Southern African Regional Universities Association

SQV-Saqunavir

TPV-Trapinavir

TNF-Tumour necrosis factor

TDF-tonofovir

T-20-Enfivirte

µg-Microgram

WHO-World Health Organisation

ZDV-Zidovudine

3TC-Lamivudine

CHAPTER 1

GENERAL INTRODUCTION AND BACKGROUND

1.0. Introduction

Epidemiological studies show contradictory findings on whether or not initiation of highly active antiretroviral therapy (HAART) has an effect on cervical intraepithelial neoplasia (CIN) onset and progression. Some studies have suggested a positive correlation between initiation of HAART and development of CIN among HIV positive women (Dorrucci *et al.*, 2001; Heard *et al.*, 2006; Moodley *et al.*, 2009). On the contrary, Minkoff *et al.* (2010) and Omar *et al.* (2011) maintain that HAART use seems to be associated with a significant reduction in squamous intraepithelial lesions (SILs). Sirera *et al.* (2008) and Shrestha (2010) concur that HAART treatment has no effect on initiating development of CIN but they both suggest the need to carry out related studies in larger populations to evaluate HAART's impact over longer periods of time. Due to the possibility of other predisposing factors linked to individual lifestyles, the cause-and-effect relationship is difficult to establish in most of these studies. There is limited information on whether or not co-administration of HAART with hormonal contraceptives (HCs) affects cervical cancer progression. The present study seeks to elucidate the effect of ethinylestradiol (EE) and levonorgestrel (LNG) on antiretroviral drug-induced apoptosis in human cervical epithelial cancer cells. HeLa cells are cancerous cells which are important tools for studying the pathology of human cervical epithelium *in vitro.* They characteristically exhibit cellular morphology for the cancerous cervix, hence their choice in this study.

1.1.0. The human immunodeficiency virus

The human immunodeficiency virus (HIV) is a retrovirus that causes acquired immunodeficiency syndrome (AIDS) (Costin, 2007). It is predominantly transmitted through blood and other bodily fluids such as semen, pre-ejaculation or vaginal fluids (Waheed and Freed, 2010; Watts *et al.*, 2009). HIV-1 is a potent spheroidal virus which belongs to the class of retroviruses. These retroviruses are particulate in structure with a diameter of 80-120 nm and they are encapsulated by a protein coat known as the viral envelope (Costin, 2007). In addition to AIDS, these single-stranded RNA retroviruses also cause cancer and other life-threatening diseases (Watts *et al.*, 2009; Waheed and Freed, 2010). Figure 1:1 outlines the anatomy of the HIV-1 virus.

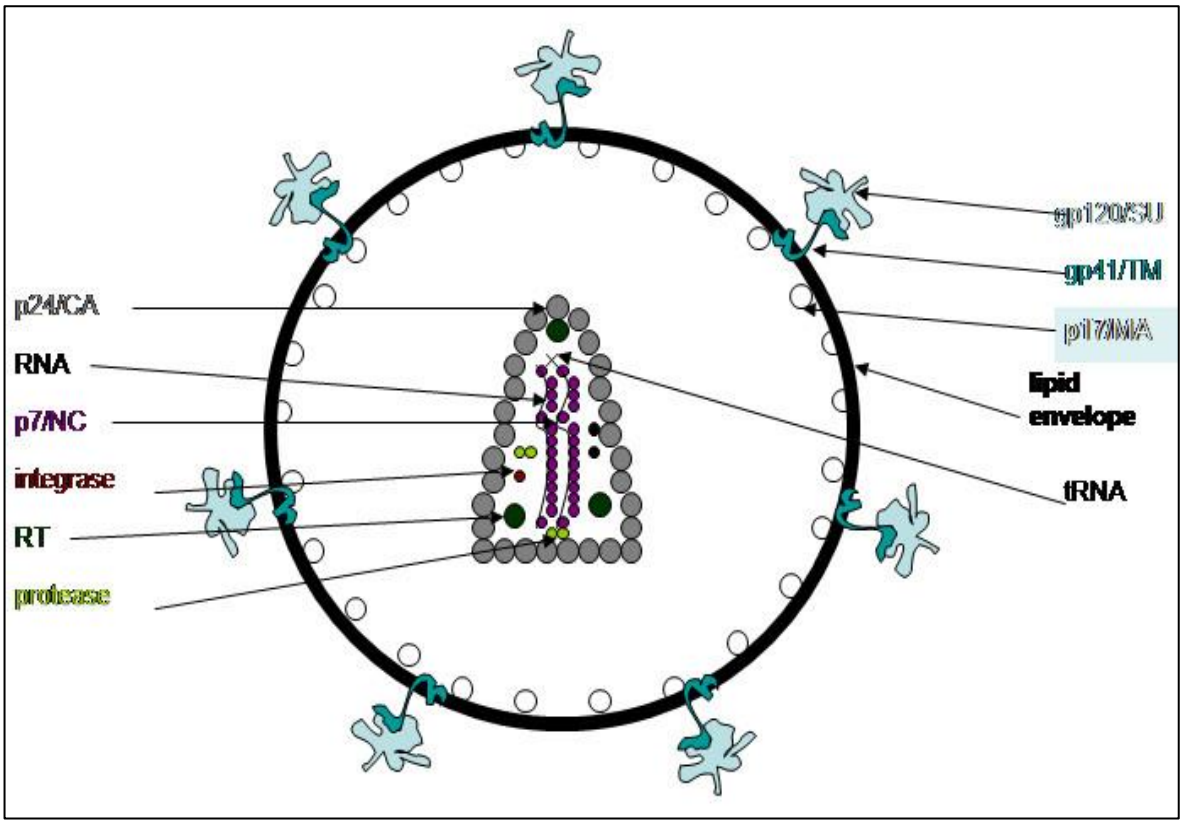

Fig 1.1: Cross-sectional structure of the HIV-1 virus. Structures represented by abbreviations are described by the full name in brackets just after the abbreviation as follows: NC (Nucleocapsid), CA (Capsid), RT (Reverse transcriptase), IN (Integrase), gp (glycoprotein) and MA represents the Matrix protein (Diagram adapted from Costin, 2007).

1.1.1. The HIV genome

The genome of the HIV consists of two RNA molecules which resemble the eukaryotic messenger ribonucleic acid (mRNA) because they have a 5´-capped end and a poly A tail at the end 3'-end (Costin, 2007; Nisole and Saïb, 2004). Figure 1.2 outlines the structure of the HIV-1 genome. Four genes are necessary for HIV replication. Firstly, the *gag* gene encodes for viral core proteins. Secondly, the *pro* gene (not shown in the diagram) codes for a protease which cleaves the Gag and Pol proteins into functionally active forms (Costin, 2007). Thirdly, the *pol* gene encodes for the reverse transcriptase enzyme (RNA-dependant DNA polymerase: Pol) responsible for DNA synthesis and formation of integrase, which is required for making a DNA copy of the genome. It also encodes the protease (PR) and integrase enzymes (Waheed and Freed, 2010). Finally, the *env* gene encodes for the HIV envelope (Env) structural proteins (Azevedo-Pereira *et al.*, 2015; Costin, 2007). After synthesis, Env (gp160) is glycosylated, followed by proteolysis into surface glycoproteins (SU or gp120) and trans-membrane glycoproteins (TM or gp41) (Waheed and Freed, 2010). While *gag*, *pol* and *env* are structural genes, *Vpu*, *Vpr, Vif* and *Nef* comprise accessory genes which may be latent and are activated at the relevant stage of the replication cycle (Costin, 2007; Waheed and Freed, 2010).

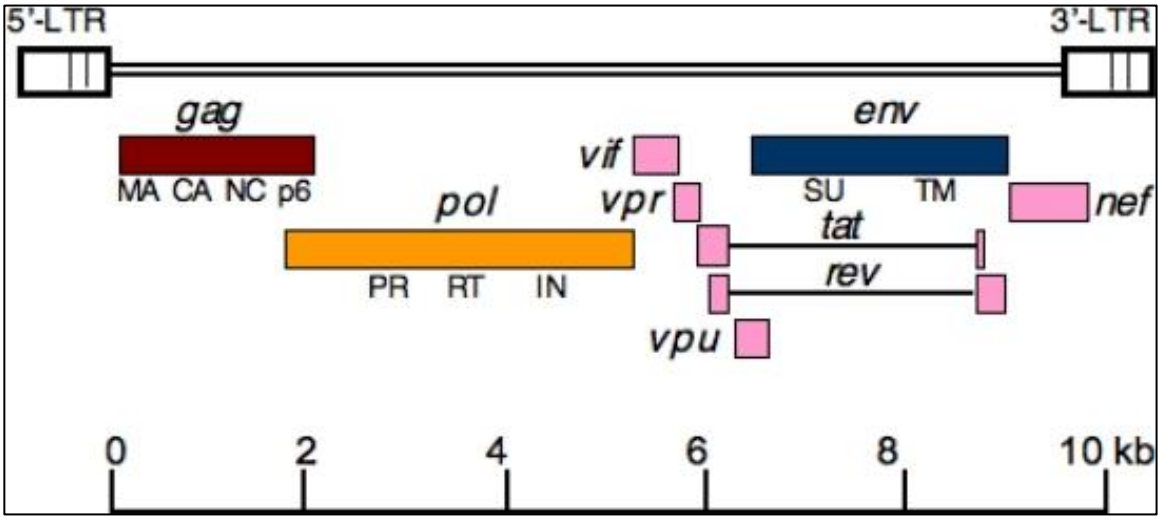

Fig1.2: Arrangement of the HIV-1 genome. The Gag polyprotein precursor for viral matrix (MA), capsid (CA) and nucleocapsid is encoded by the *gag* gene. The *pol* gene encodes the protease (PR), reverse transcriptase (RT) and integrase (IN). The *env* gene encodes the surface membrane glycoprotein (gp120) and the transmembrane glycoprotein (gp41). The starting point of the gene indicates the position of the (A) in the ATG start codon for a named gene. In the pol gene, the start implies the first T in the sequence TTTTTTAG (Adapted from: Waheed and Freed, 2010).

1.1.2. The HIV replication cycle

The retroviral life cycle can be defined by two phases. The early stage refers to the sequential steps from attachment of the virus up to integration of the viral copy deoxyribonucleic acid (cDNA) into the host cell (Röling *et al.,* 2015). The late stage begins with the expression of the viral genome up to the release and maturation of the viral progeny (Nisole and Saïb, 2004). Figure 1.3 depicts the main stages of DNA replication. Briefly, in the presence of a T-lymphocyte, HIV attaches to the CD4-receptor through the gp120 glycoprotein (Röling *et al.,* 2015; Nisole and Saïb, 2004). The hydrophobic gp40 glycoprotein is then brought into proximity with the virion (Azevedo-Pereira *et al.,* 2015). The attachment of the virus requires hydrophobic interactions between the viral envelope and the host cell membrane to allow fusion of the virion (Nisole and Saïb, 2004). The virion then releases its RNA and reverse transcriptase enzyme molecules into the host cell (Nisole and Saïb, 2004).

While in the host cell, the reverse transcriptase synthesises complementary DNA from the viral RNA strand (Röling *et al.,* 2015; Waheed and Freed, 2010). Integration is the process through which the newly synthesised double-strand DNA is transported into the nucleus of the host cell (Costin, 2007). This is aided by the enzyme integrase which binds the long terminal repeat (LTR) at each end of the viral DNA. The host DNA-dependent RNA-polymerase transcribes the virion DNA (Waheed and Freed, 2010). Some of the transcribed viral mRNA molecules synthesise the viral proteins while others are incorporated into the virus particles as the genome (Nisole and Saïb, 2004; Waheed and Freed, 2010). At the surface of the cell, the structural proteins encapsulate the viral genome and the progeny buds out through the membrane. Viral protein precursors (Gag and Gag-Pol) are then cleaved by proteases (Nisole and Saïb, 2004; Waheed and Freed, 2010). There are some cellular factors that interfere with the early stages of the viral reproduction cycle and these are perceived as defensive mechanisms against viral infections (Nisole and Saïb, 2004; Costin, 2007). The CEM 15 also known as the human apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like-3G (APOBEC3G) inhibits HIV replication. Ref1 and Lv1 block viral particles before reverse transcription, while Fv1 and Fv2 interfere with reverse transcription (Nisole and Saïb, 2004).

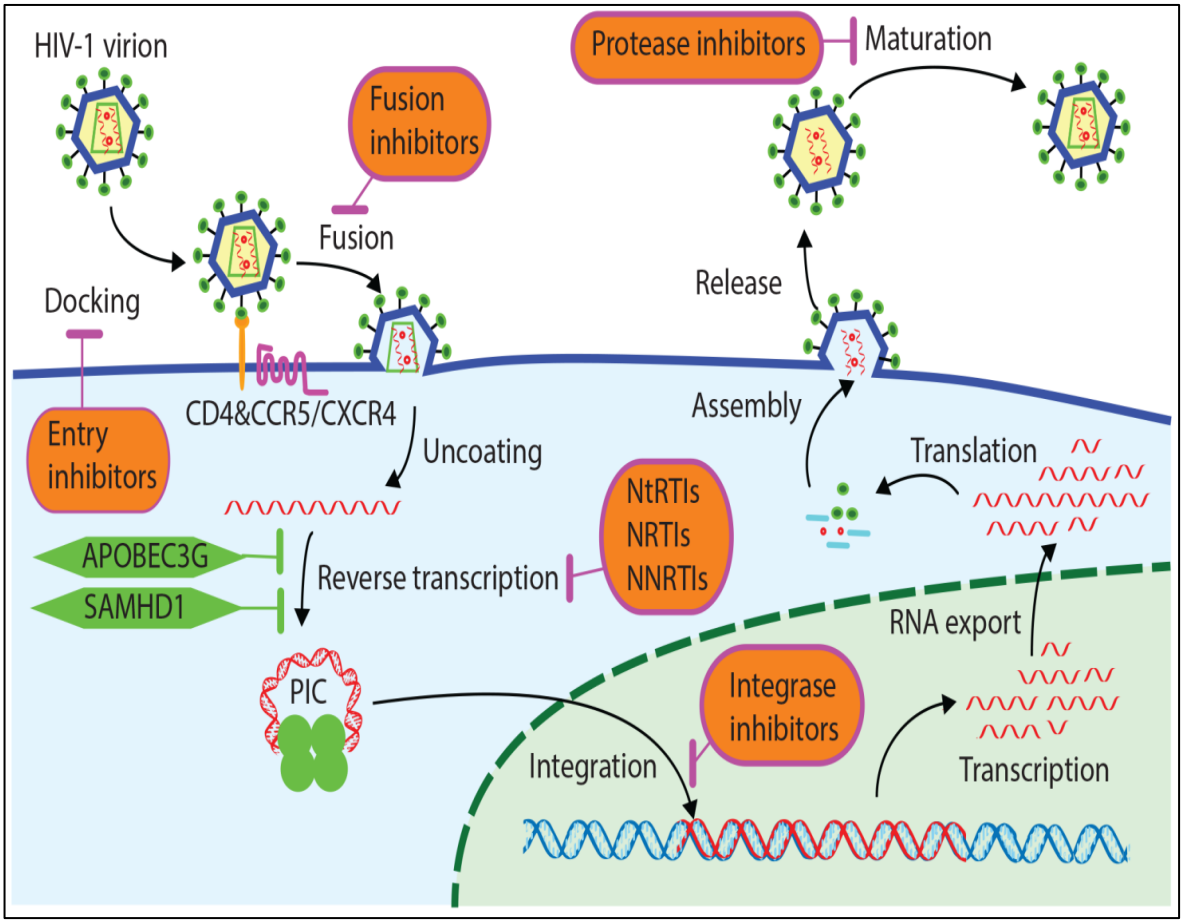

Fig 1.3: Schematic representation of the HIV replication cycle. The diagram demonstrates the sequential stages of the virus replication from the time of fusion of the single virion up to release and maturation of the newly synthesised replicates. The surface membrane protein gp120 comes into contact with membrane receptors CCR5 and CXCR4 which serve as co-receptors (Röling *et al.*, 2015). Receptor-binding results in conformational changes that expose the transmembrane protein, leading to its insertion into the target $CD4^+$ cell membrane. This results in fusion of the viral and $CD4^+$ cell membranes. The viral core is then released into the host cell. The viral core uncoats and the RNA is transcribed by the reverse transcriptase (RT). The protein inhibitor complex (PIC) protects the newly synthesised DNA while it inserts into the host DNA. The enzyme integrase (IN) facilitates integration of viral DNA into the host chromosome. The viral DNA is then transcribed and transported to the cytoplasm. Env glycoproteins incorporate into the budding viral particles. The budding viruses attach to the membrane $CD4^+$ receptors of the host cell and exude into circulatory system (Waheed and Freed, 2010; Röling *et al.*., 2015). Classes of drugs that inhibit each stage of the reproductive cycle are shown in orange. The abbreviations for inhibitor drugs depicted in the diagram are as follows: NtRTIs-nucleotide reverse transcriptase inhibitors, NRTIs-nucleoside reverse transcriptase inhibitors and NNRTIs-non-nucleoside reverse transcriptase inhibitors (Diagram adapted from Röling *et al.*, 2015).

1.2.0. Antiretroviral therapy

Antiretroviral (ARV) drugs treat infections caused by retroviruses, primarily HIV. Most have a research chemical name, generic name and brand name. It is common practice to use an abbreviation of the generic name. While combination antiretroviral therapy has its advantages, there are inherent undesired side effects associated with these drugs, thus limiting the number of useful combinations (Arts and Hazuda, 2012; Flepp *et al.,* 2001). As described earlier, when antiretroviral drugs are taken in combinations of three or more, such an approach is known as highly active antiretroviral therapy (Arts and Hazuda, 2012).

1.2.1. Classes of antiretroviral drugs

Antiretroviral drugs are classified mainly dependent on their mechanism of action in interfering with viral replication. Each class of ARV interferes with the viral reproductive cycle differently. Major classes of antiretroviral drugs include entry inhibitors (EIs), nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), integrase inhibitors (IIs) and protease inhibitors (PIs) (AIDSinfo, 2017; Flepp *et al*, 2001; Barry *et al.,* 1999).

1.2.2. Entry/Fusion inhibitors

The genesis of viral infection is the attachment of the viral gp120 protein to the $CD4^+$ receptor on cell surfaces (Flepp *et al.*, 2001). The process is facilitated by van der Waals forces and electrostatic forces between the positively charged $CD4^+$ molecules and the negatively charged gp120 socket (Arts and Hazuda, 2012). Entry inhibitors interfere with the binding, fusion and entry of an HIV virion to a T-lymphocyte cell (Barry *et al*., 1999). These drugs are used in combinatorial HIV treatment. Examples of these drugs are enfvirtide (T-20) and muraviroc (MVC) (Flepp *et al.*, 2001; Arts and Hazuda, 2012).

1.2.3. Nucleoside reverse transcriptase inhibitors

NRTIs inhibit reverse transcriptase, a DNA polymerase enzyme required for DNA synthesis. NRTIs act by competitive inhibition, hence acting as terminators by blocking natural substances needed for DNA replication (Flepp *et al.*, 2001; Olivero *et al.,* 2013). These drugs have been generally associated with host toxicity (Flepp *et al.*, 2001). Some examples of NRTIs include abacavir (ABC), didanisine(ddi), emtricitabine (FTC), lamivudine (3TC), stavudine (d4T), tenofovir (TDF), Zalcitabine (Ddc) and Zidovudine (AZT/ZDV) (Flepp *et al.,* 2001; Barry *et al.*, 1999). In this research, ABC, 3TC, d4T and ZDV are the drugs of interest, as they have been predominantly used in HAART.

1.2.4. Abacavir

Abacavir is a structural analogue of guanine that is commonly used in antiretroviral regimens (Fig 1.4). ABC metabolism is not affected by renal insufficiency, as shown by about 1% of the drug that is unchanged in urine (Akanbi *et al.*, 2012; Jackson *et al.,* 2012). The intracellular half-life of ABC ranges up to about 40 hours (Yuen *et al.,* 2008; Jackson *et al.,* 2012). While there are a number of recommended permutations, ABC+3TC (administered together with a protease inhibitor) and ABC+3TC+ZDV have found widespread use in HIV treatment (WHO, 2010). The triple combination ABC+3TC+ZDV does not constitute a HAART regimen since it consists of NRTIs only. Rather, it is known as triple NRTIs (WHO, 2010). In highly active antiretroviral combination approaches, ABC+3TC may be administered concurrently with a protease inhibitor or a NNRTI (WHO, 2016).

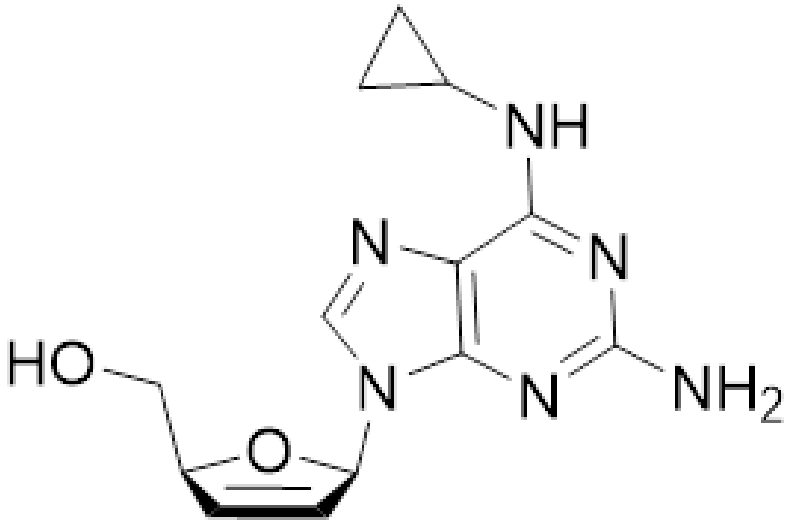

Fig 1.4: Chemical structure of abacavir (ABC).

1.2.5. Stavudine

Stavudine is a thymidine analogue whose administration was approved in 1994 (Akanbi *et al.,* 2012). There is no evidence of the effect of co-administration with food on stavudine metabolism. Due to its weak binding to proteins, d4T is distributed easily through the body, including the placenta (Akanbi *et al.,* 2012; Rana and Dudley, 1997). It efficiently clears by both renal and non-renal processes (Rana and Dudley, 1997). Stavudine use has significantly reduced in developed countries due to its perceived cumulative toxicity (WHO, 2016). Although World Health Organisation guidelines of 2010 emphasised the need to eliminate the use of d4T gradually, it is still administered in some low-resource settings (Mouton *et al.,* 2016). Figure 1.5 below outlines the chemical structure of stavudine.

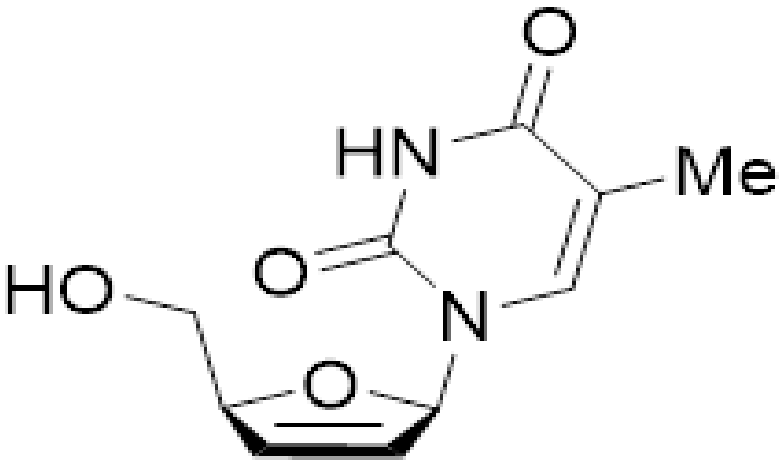

Fig 1.5: Chemical structure of stavudine/zerit (d4T).

1.2.6. Lamivudine

Lamivudine is a cytidine analogue (Figure 1.6), which was approved in 1995. 3TC weakly binds to proteins, hence it distributes well in the body and between the maternal and foetal circulation. Its bioavailability is around 82-84% in adults (Yuen *et al.*,2004; Johnson *et al.*, 1999). Due to its prolonged half-life of up to 39 h, once or twice daily administration of 3TC is desirable. 3TC is predominantly excreted through the kidneys, hence the need for dose adjustment during renal insufficiency (Else *et al.*, 2012; Johnson *et al.*, 1999; Yuen *et al.*, 2004). 3TC is phosphorylated to lamivudine 5´-triphosphate (3TC-TP), which inhibits reverse transcriptase in hepatitis B virus (HBV) and HIV-1 (Else *et al.*, 2012).

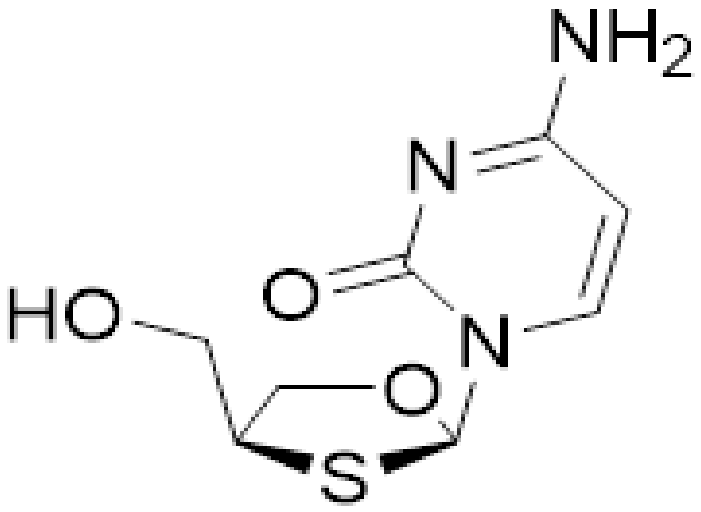

Fig 1.6: Chemical structure of Lamivudine (3TC).

1.2.7. Zidovudine

ZDV is a thymidine analogue (Figure 1.7) and was the first ARV used for monotherapy in 1987 (Akanbi *et al.,* 2012). Due to its short half-life, in practise, daily multiple dosing is common (Akanbi *et al.,* 2012; Piliero, 2004). Glucoronidation in the liver, kidneys, and intestinal mucosa constitute the major pathway for its metabolism. ZDV binds weakly to proteins and its administration with food does not affect its plasma exposure. ZDV excretion is mainly renal (Piliero, 2004; Mu *et al.,* 2016). In the host cell, ZDV/AZT is metabolised to AZT-5´-triphosphate (AZT-TP), which competitively inhibits the HIV reverse transcriptase (Mu *et al.,* 2016). Compared to monotherapy, combination therapy has resulted in reduced adverse effects of ZDV. However, haematological toxicities have been observed in combination therapy (Flepp *et al*., 2001).

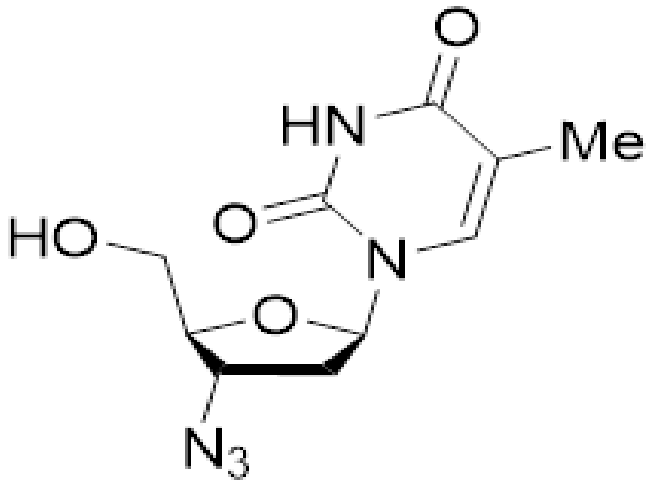

Fig 1.7: Chemical structure of zidovudine (ZDV or AZT).

1.2.8. Non-nucleoside reverse transcriptase inhibitors

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are allosteric inhibitors that bind directly to the reverse transcriptase (Röling *et al* 2015; Barry *et al.*, 1999). This group comprises a number of drugs that differ structurally from each other. These drugs share the same characteristic and mechanism of action, through binding to a site on the reverse transcriptase, thereby causing a conformational change that blocks DNA polymerase activity (Arts and Hazuda, 2012; Flepp *et. al.*, 2001). While nucleoside analogues require phosphorylation to be activated, NNRTIs do not require phosphorylation as they bind directly and noncompetitively to reverse transcriptase (Barry *et al.*, 1999). Non-nucleoside reverse transcriptase induce some host cell toxicity (Arts and Hazuda, 2012; Flepp *et al.*, 2001). Examples of NNRTIs include delavirdine (DLV), efivarenz (EFV), Etravirine (ETR) and nevirapine (NVP) (AIDSinfo, 2017; Barry *et al.*, 1999; Madigan *et al.*, 2009). Figure 1.8 presents the structure of nevirapine, a non-nucleoside reverse transcriptase inhibitor of interest in the present research on HAART combinations.

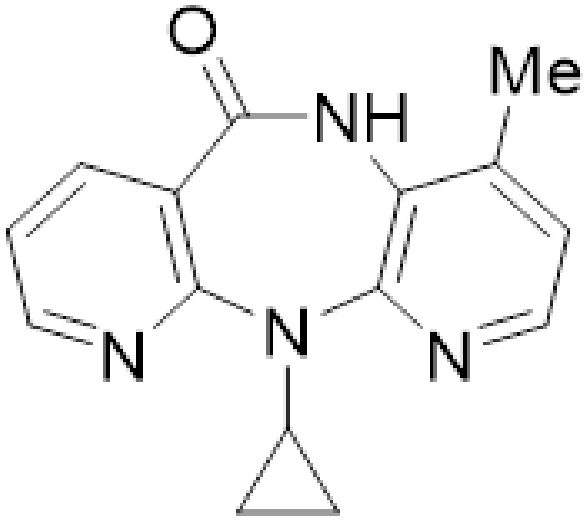

Fig 1.8: Chemical structure of nevirapine (NVP).

For some time, NVP was the NNRTI of choice in low-resource settings, where it is used in combination with other antiretroviral drugs to treat HIV-1 infected individuals (Chou *et al.*, 2010, Dong *et al.,* 2012). It was also used in monotherapy to prevent mother-to-child infection (WHO, 2010). According to the British HIV Association (BHIVA) (2008) and Dong *et al.* (2012), while single-dose NVP has been associated with rash and/or hepatotoxicity, the aetiology of its toxicity remains obscure. Although single-dose NVP was previously used to prevent mother-to-child transmission, combinations with tinofovir (TDF) and emtricitabine (FTC) have been shown to reduce resistance mutations associated with NVP (Benaboud *et al.*, 2011; WHO 2010). The combinations d4T+3TC+NVP and ZDV+3TC+NVP have found wide use for HIV-positive patients in low-resource settings although the current trend is to withdraw d4T gradually due to its reported toxicity (WHO, 2010).

1.2.9. Integrase inhibitors

Integrase inhibitors block the action of integrase, an enzyme that facilitates insertion of viral genome into the host cell (Flepp *et al.,* 2001; Barry *et al.,* 1999). Blocking the integrase therefore stops further viral replication (Arts and Hazuda, 2012; Flepp *et al.*, 2001). In practice, integrase inhibitors are administered concurrently with other anti-HIV medication. Raltegravir (RGV) is an example of a widely-used integrase inhibitor (WHO, 2010).

1.2.10. Protease inhibitors

Protease inhibitors act by competitively blocking the action of the protease enzyme, which is responsible for cleaving the HIV precursor proteins into smaller units that can be integrated into the HIV structure (Röling *et al.,* 2015; Flepp *et al.,* 2001; Arts and Hazuda, 2012). Resultantly, protease inhibitors lead to the production of defective viral particles that cannot infect $CD4^+$ cells, thus averting viral replication (Barry *et al.,* 1999; Flepp *et al*., 2001). While protease inhibitors can be effective upon lone administration, prolonged use leads to viral resistance (Arts and Hazuda, 2012). In practice, protease inhibitors are administered concurrently with other anti-HIV medication (WHO, 2010). Examples of PIs include atazanavir (ATV), duranavir (DRV), fosamprenavir (FPV), indinavir (IDV), lopinavir + ritonavir (LPV/r), nelfinavir (NFV), ritonavir (RTV), saqunavir (SQV) and trapinavir (TPV) (Arts and Hazuda, 2012; Flepp *et al.,* 2001).

1.3.0. ARV combination therapy

Combination therapy currently provides a promising approach in pharmacy with the aim of improving the efficacy of different drugs (Bulusu *et al.*, 2016; Murugan *et al*., 2016). While synergistic effects of combination drugs may be beneficial, toxic effects may also result from the combination mixtures, as exemplified by carbamazepine toxicity if co-administered with several drugs and inhibitors (Bulusu *et al.*, 2016). Combination therapy has been shown to exhibit high therapeutic effects in other diseases such as cancer (Murugan *et al.*, 2016). The large number of possible combinations and permutations makes combination research less feasible. This section discusses antiretroviral drug regimens that are widely used in low-resource settings.

Combination antiretroviral drugs act by obstructing HIV replication, thereby keeping the number of the viral progeny very low (Schutz and Wendrow, 2001; Barry *et al.*, 1999). Combination antiretroviral treatment ensures that, should resistance to one drug arise, continued suppression results from other drugs (Arts and Hazuda, 2012). Combinatorial antiretroviral treatment creates multiple obstacles to HIV replication to keep the number of budding offspring low and reduce the possibility of a superior mutation (Schutz and Wendrow 2001; Flepp *et al.*, 2001). It has been shown that mono-antiretroviral therapy ultimately leads to superior mutations over time (Barry *et al.*, 1999; Flepp *et al.*, 2001; Arts and Hazuda, 2012), hence the combinatorial use of antiretroviral drugs ensures longer-lasting effects (WHO, 2016; AIDSinfo, 2017). As a result, the standard of care is to use combinations of antiretroviral drugs (BHIVA, 2008; WHO, 2010). Table 1.1 and Table 1.2 depict some of NRTIs based combination ARVs currently used in low-resource settings, although the trend is to exclude d4T gradually.

Table 1.1: Some multiple class combination ARVs used in low-resource countries

Combination	Generic Name
ZDV+3TC +EFV	Zidovudine/Lamivudine/Efavirenz
d4T +3TC +NVP	Lamivudine/Stavudine/Nevirapine
ZDV +3TC +NVP	Lamivudine/Nevirapine/Zidovudine

(After: Barry *et al.*, 1999; Flepp *et al.*, 2001)

Table 1.2: Combined Nucleoside/Nucleotide analogues

Combination	Brand name
ABC +3TC	Epzicom (US) and Kivexa (Europe)
ABC + ZDV +3TC	Trizivir
ZDV +3TC	Combivir
TDF +FTC	Truvada

(After: Barry *et al.*, 1999; Flepp *et al,* 2001)

The present research investigates the effect of single nucleoside reverse transcriptase inhibitors and nevirapine on cervical epithelial cancer cell proliferation and apoptosis. This is coupled with an analysis of the effect of hormonal contraceptives on combination antiretroviral drug-induced apoptosis in human cervical epithelial cancer cells. From Tables 1.1 and 1.2, the following combinations are of interest: d4T+3TC+NVP, ZDV+3TC+NVP, ABC+ZDV+3TC, ABC+3TC and ZDV+3TC. These regimens are reasonably accessible in low-resource settings. Of these, ZDV+3TC+NVP is a frequently used HAART regimen. The combination d4T+3TC+NVP still finds its use in sub-Saharan Africa, although the World Health Organisation is advocating for its gradual withdrawal due to long-term toxicity (WHO, 2010). Recent epidemiological contradictions of CIN development following initiation of HAART necessitate cell-culture experiments that would determine the role of HAART treatment and use of HCs as genotoxic agents. This serves to clarify the cause-and-effect relationship of exposure to highly active antiretroviral therapy in combination with levonorgestrel and ethinylestradiol. The British HIV Association and World Health Guidelines will be used to outline the clinical applications of these drug combinations.

1.3.1. Abacavir + lamivudine (Kixeva)

This drug is well tolerated but patients may experience hypersensitivity in the first six weeks, hence the need for counselling prior to initiating treatment (BHIVA, 2008). Kixeva is usually administered with a protease inhibitor. This regimen poses an increased risk of myocardial infarction in patients with a risk of cardiovascular events (CVD) (WHO, 2016; BHIVA, 2008). Due to higher virological failure rate associated with baseline viral loads higher than 100 000 copies/ml, the BHIVA Treatments Guidelines suggest that the use of Kixeva be reserved for first-line treatments in which Truvada has shown contraindications. It is therefore interesting to assess the *in vitro* association of this double combination with EE and LNG.

1.3.2. Zidovudine + lamivudine (Combivir)

Combivir is poorly tolerated, hence the BHIVA Treatments Guidelines (2008) recommend that this medicine be used in specific circumstances. Such circumstances include women with an intention to get pregnant and those travelling to low-resource settings where there are limited nucleotide backbone alternatives. There remains a paucity of information on whether the contraceptive hormones EE and LNG have an effect on human cervical epithelial cancer proliferation upon co-administration with Combivir.

1.3.3. Triple combinations

AZT+3TC+NVP and d4T+3TC+NVP are HAART regimens that are administered as first-line treatments in some countries. The WHO guidelines of 2016 recommend limited use or, if feasible complete withdrawal of d4T due to observed toxicities. The combination ABC+ZDV+3TC is triple NRTIs. As is the case for3'-end double combinations described earlier, little is known about the effect of co-administration of hormonal contraceptives with triple antiretroviral combinations.

1.3.4. Antiretroviral treatment and myocardial infarction

While HAART has significantly increased the life expectancy among HIV-infected individuals, documentation of myocardial infarction (MI) has depicted major challenges with choices of ARVs. In a study involving thirteen antiretroviral drugs, indinavir, ABC lopinavir-retinovir and didanosine showed high risk of MI with cumulative use (Worm *et al.*, 2010). However the research group was wary of confounding factors that could have influenced the results. In follow-up studies carried out in the UK, increased exposure to PIs was shown to be associated with MI. Interestingly, in the same study, no such evidence was found for NNRTIs-exposed patients although the sample size was smaller in this group (Worm *et al.*, 2010). The NNRTI nevirapine in combination with NRTIs has therefore found widespread use in low-resource countries (WHO, 2010). Bedimo *et al*. (2011) did not observe an effect of current or cumulative use of ABC on MI. To this effect, the regimen Kixeva (ABC+3TC) is considered among first-line drugs that are administered with PIs (BHIVA, 2008).

1.4.0. Human papillomavirus and cervical cancer

It has since been suggested that classes of high risk papillomaviruses such as HPV16 and HPV18 are associated with development of high-grade cervical intraepithelial neoplasia and carcinoma (Mougin *et al.*, 2001; Ponten and Guo, 1998). While most women are likely to be infected by HPV during their lifetime, only a small proportion has been shown to be at a risk of developing cancer (Mougin *et al.*, 2001; Cox, 1995). This suggests the role of other factors in promoting genotoxic transformation in human cervical epithelial cancer cells. Oestrogen and progesterone have been implicated as cofactors (Webster *et al*., 2001). The long latency period between infection by HPV and cancer emergence suggests that there should be other predisposing factors responsible for cancer initiation (Mougin *et al*., 2001).

Viral oncoproteins E6 and E7 are associated with malignant conversion of cervical cells. On their own, oncoproteins are not sufficient for conversion of cells to malignant phenotype (zur Hausen, 2009). The oncoproteins from high-risk HPV stimulate pleiotropic cell growth and at the same time induce mutations and aneuploidy in host DNA (Thierry, 2009; zur Hausen, 1999). Interaction of E6 with p53 (a checkpoint protein for G1/S-phase transition) results in degradation of p53. This has been suggested to cause mutagenic events and aneuploidy karyotype formation (Fei and de Villiers, 2008; zur Hausen, 2009). E7 is also able to induce mutations in host DNA (Thierry, 2009). Expression of both E6 and E7 proteins has been shown to be necessary for proliferation of cervical carcinoma cells. DeFillipis *et al*. (2003) demonstrated that repression of E7 proteins activates the Rb pathway and triggers senescence, while repression of E6 proteins activates the p53 pathway, hence triggering both senescence and apoptosis.

1.4.1. The HeLa cell

HeLa cells were obtained from a tissue biopsy of Henrietta Lacks (hence the abbreviation HeLa) who died in 1951. Henrietta Lacks had developed a 2-3cm cervical tumour. HeLa cells provided the first successful culture growth of cells from adenocarcinoma of the cervix (Lucey *et al.,* 2009). HeLa cells have since been shown to contain HPV18 and this has been associated with aggressive carcinoma (Peran *et al.*, 2010; Mougin *et al.* 2001; Ponten and Guo, 1998). The HeLa cell is widely used as a model of a human cervical epithelial cancer cell line, hence the choice of this cell in the present study.

1.5.0 Cytotoxicity and Genotoxicity

Cytotoxicity refers to the degree to which any compound can be toxic to the cells. Cytotoxic compounds thus have the potential of inhibiting or preventing the normal physiological functions of the cell. These substances can induce necrosis by disrupting the cell membrane, hence leading to cell lysis. Alternatively cytotoxic substances can initiate a process of programmed cell death through apoptosis (Sadeghi-Aliabadi *et al.,* 2010). Where some of these compounds result in non-pathophysiological rates of apoptosis, these can be candidates for treatment of cancer, since they can inhibit proliferation and growth of the cancer cell (Kang *et al.,* 2012; Sadeghi-Aliabadi *et al.,* 2010). On the contrary, apart from preventing normal physiological processes, cytotoxic substances may damage the DNA of a cell. The extent to which a substance damages DNA is known as genotoxicity (Moreno-Villanueva *et al.,* 2011). Human cells have well-developed DNA-repair mechanisms which deal with DNA alterations and damage. Alternatively, some damaged

cells are destroyed *via* apoptosis. When the genotoxicity of a substance is high, DNA damage-repair mechanisms are impaired, hence resulting in excessive apoptosis and possibly cancer (Christmann *et al.,* 2003; Matt and Hofmann, 2016).

There is limited information on the effect of hormonal contraception use on cancer cell proliferation and apoptosis upon co-administration with antiretroviral drugs. The present study seeks to investigate the proliferative activity of antiretroviral drugs (with and without contraceptive hormones) on HeLa cells and the genotoxic effect of each single antiretroviral drug on these cells.

1.5.1. Apoptosis and cancer

Apoptosis refers to programmed cell death. It is triggered by either exogenous stimuli, such as ultraviolet radiation, or endogenous stimuli such as genotoxic chemicals and intracellular oxidative stress (Elmore, 2007). Apoptosis and the genes that control it have an effect on malignancy. DNA damage activates and stabilises p53 in the nucleus and cytoplasm, thereby stimulating other proteins that stimulate apoptotic pathways (Lowe and Lin, 2000). Malfunctioning of apoptotic pathways may result in diseases such as cancer, neurodegenerative diseases and autoimmune disorders (Rastogi and Sinha, 2009). It is therefore intriguing to investigate whether exposure to levonorgestrel and ethinylestradiol simultaneously with highly active antiretroviral therapy affects apoptosis in cervical epithelial cancer cells. Caspases are enzymes that facilitate apoptosis by cleaving and inactivating PARP-1. This stops PARP-1 triggering repair of DNA which is being cut (Kang *et al.,* 2012). The process leads to digestion of DNA and degradation of cytoskeleton (Matt and Hofmann, 2016; Kang *et al.,* 2012). Morphological changes associated with apoptosis include membrane blebbing, cell shrinkage and condensation of nuclear chromatin, nuclear fragmentation and DNA cleavage (Matt and Hofmann, 2016; Lowe and Lin, 2000). While cells can trigger both apoptosis and necroptosis in response to DNA damage, apoptosis is perceived to be the prevalent form of cell death (Matt and Hofmann, 2016). The two major pathways of apoptosis are the extrinsic and intrinsic pathways as summarised in Figure 1.9.

1.5.2. The extrinsic apoptosis pathway

This pathway is signalled through proteins such as Fas and tumour necrotic factor (TNF) receptor *via* their intracellular domain (Kang *et al*., 2012). When TRAIL binds to death receptors DR4/and or DR5, this leads to trimerisation of the receptors and subsequent

recruitment of the Fas-associated death domain (FAAD) protein (Fulda *et al.*, 2010). FAAD attracts initiator procaspases -8 or -10 which are then activated through proteolysis to respective caspases (Matt and Hofmann, 2016). Cellular FLICE-inhibitory protein (c-FLIP) and ubiquitination of caspases-8 regulate procaspases activation (Matt and Hoffman, 2016; Kang *et al.*, 2012). Caspases -8 and/or -10 activate the executor caspases -3, -6 and -7 thus up-regulating apoptosis. Alternatively, caspase-8 cleaves BID signalling expression of BAX/BAK, thereby playing a role in up-regulation of mitochondrial-dependent apoptosis (Kang *et al.*, 2012; Fulda *et al.*, 2010).

1.5.3. The intrinsic apoptosis pathway

The intrinsic pathway is activated by members of the BCL-2 family. Loss of growth factor signals and severe cellular stress activate this pathway (Kang *et al.*, 2012). In the case of DNA damage, p53 is activated and it in turn activates PUMA and NOXA. Activated PUMA/NOXA stimulates BAX and BAK, thereby activating cytochrome c release from mitochondria (Matt and Hofmann, 2016; Fulda *et al.*, 2010). Binding of cytochrome c to Apaf-1 leads to activation of caspase-9, which in turn, up-regulates the expression of caspases -3, -6 and -7, leading to apoptosis (Matt and Hofmann, 2016). Inhibitors of apoptosis are targeted by smac/DIABLO, which blocks their activity (Matt and Hofmann, 2016; Fulda *et al.*, 2010).

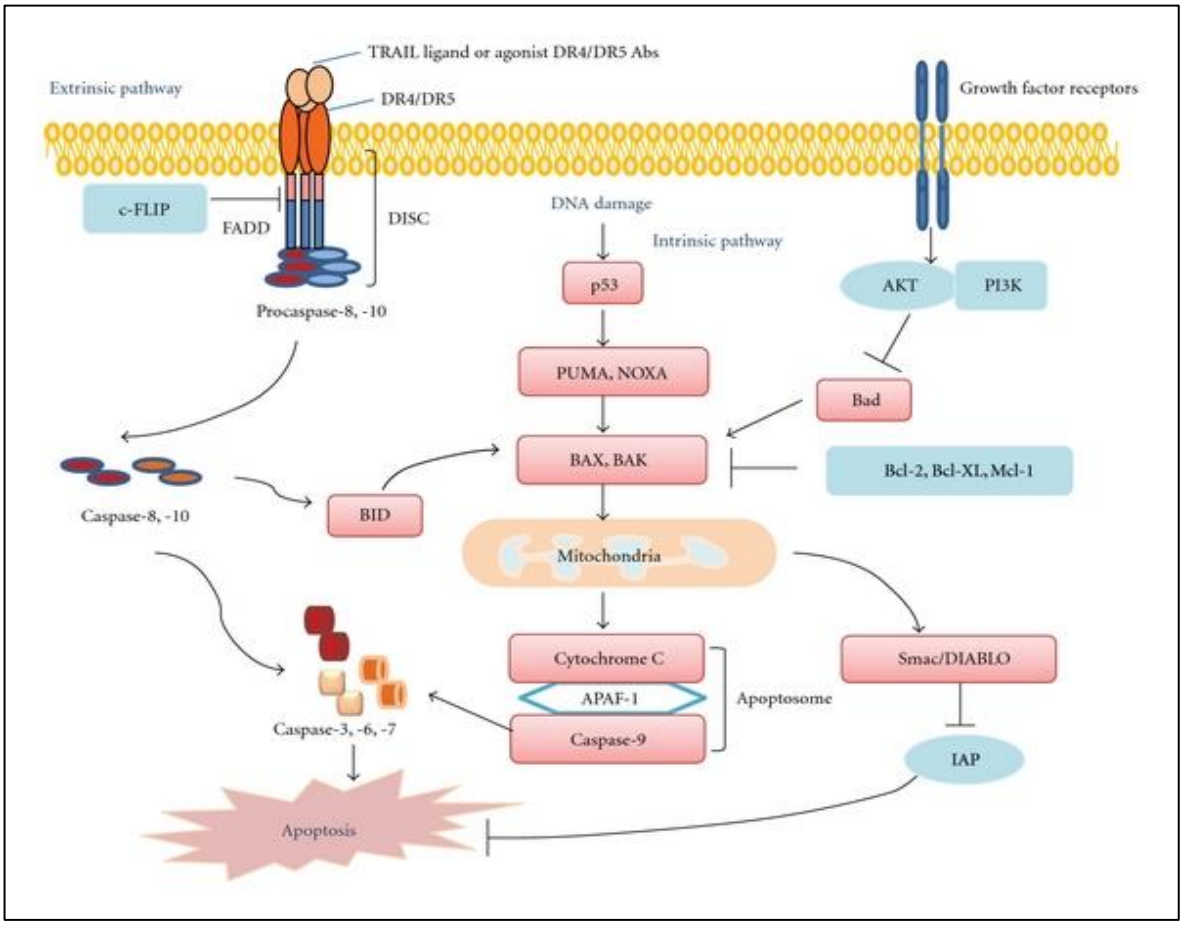

Fig 1.9: Schematic representation of the intrinsic and extrinsic apoptotic pathways. The extrinsic pathway is signalled through cell membrane receptors by death factors such as FAS and TNF receptors. The binding of TRAIL to death receptors results in a cascade leading to activation of caspases -3, -6 and -7, which are executors of apoptosis. In the intrinsic pathway for apoptosis, membrane receptors signal apoptosis *via* an intracellular domain that results in DNA digestion by caspases. The protein Apaf-1 is activated by cytochrome c, thus activating caspase-9 which in turn activates executioner caspases. Inhibitors of apoptosis are inactivated by smac/Diablo (After: Kang *et al.*, 2012).

1.5.4. Apoptosis and compensatory proliferation

Optimal apoptosis is beneficial for eliminating damaged and potentially cancerous cells (Matt and Hofmann, 2016). Apoptosis that is too drastic may result in pathophysiology, leading to undesirable conditions such as neurodegenerative disease, tissue dysfunction and cancer (Ryoo and Bergmann, 2012; Matt and Hofmann, 2016). Studies in *Drosophila* wings have demonstrated evidence that some proapoptotic proteins, especially caspases, can induce proliferation in adjacent cells as a compensatory mechanism to replace lost cells (Ryoo *et al.,* 2004). This phenomenon is referred to as compensatory proliferation (CP) or apoptosis-induced proliferation (Ryoo and Bergmann, 2012; Ryoo *et al*., 2004). One well-known mechanism in which CP occurs has been studied in *Drosophila*, in which apoptosis is initiated but the effector caspases are inhibited thereby maintaining the 'undead cells'. These cells emit mitogens which signal overgrowth (Fan and Bergmann, 2008a; Ryoo and Bergmann, 2012). The second mechanism of CP observed in *Drosophila* eyes is dependent on effector caspases, which activate Hedgehog signalling for CP (Fan and Bergmann, 2008b). Ryoo and Bergmann (2012) maintain that such conditions of persistent mitogen signalling and overgrowth may contribute to certain kinds of cancer.

1.5.5. Necroptosis and autophagy

Although apoptosis is the predominant mechanism of programmed cell death, necroptosis and autophagy are other mechanisms of controlled cell death (Su *et al.,* 2015). Necroptosis is a form of programmed necrosis. Morphological characteristics of the necroptic cell resemble those of a necrotic cell (Su *et al*., 2015; Matt and Hofmann, 2016). Necroptosis is characterised by lack of activation by caspases but instead receptor-interacting protein kinases -1 and -3 (RIPK1 and RIPK3) activate the process. Activation of necroptosis can be induced by death ligands such as TNFα and Apo2/TRAIL (Matt and Hofmann, 2016). Autophagy entails 'self-death' in which intracellular membrane structures, such as Golgi apparatus, package protein complexes so as to degrade and renew these proteins. This is one mechanism that cells have evolved in order to survive under stressful conditions (Su *et al.,* 2015).

1.6.0. Haemopoietic cells and macrophages

Blood is formed through the haemopoietic system, in which the pluripotential stem cell gives rise to separate cell lineages (Lim *et al.,* 2013). Red blood cells carry oxygen while platelets prevent bleeding. Hematopoietic stem cells (HSC) differentiate into common myeloid progenitors (CMP) and common lymphoid progenitors (CLP) (Lim *et al*., 2013;

Shaikh and Bhartiya, 2012). The CMP mature into megakaryocyte-erythroid progenitor (MEP) and granulocyte monocyte progenitors (GMP). The GMP, in turn, commit to mature erythrocytes, megakaryocytes, platelets, monocytes, macrophages and granulocytes (Lim *et al*., 2013; Shaikh and Bhartiya, 2012; Domen *et al*., 2006). Fig 1.10 shows the pluripotent stem cell and the progeny cells that arise from the stem cell.

Although monocytes and macrophages originate from a common progenitor, monocytes are short-lived, while macrophages have a longer lifespan (Shaikh and Bhartiya, 2012; Parihar *et al.,* 2010). In the presence of differentiation factors, monocytes escape the apoptotic route and instead undergo differentiation into macrophages (Gordon and Taylor, 2005; Parihar *et al.*, 2010). While monocytes are vital for the defence system, their accumulation may lead to undesired medical conditions, such as atherosclerosis, multiple sclerosis and arthritis (Lim *et al*., 2013; Parihar *et al*., 2010). The present research will also explore the potential of single and combination antiretroviral drugs to differentiate the U937 cells. These U937 cells are myeloid-derived cells that have found widespread use in evaluating the effect of various substances on their responses to different stimuli (Chanput *et al.,* 2015).

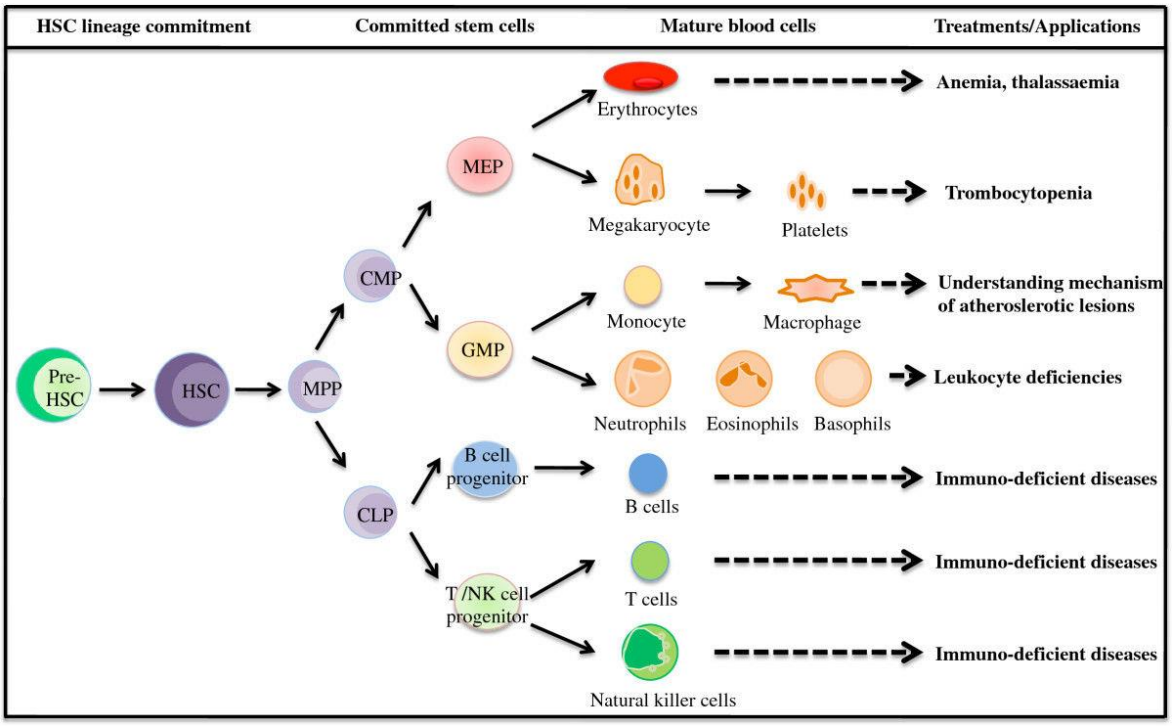

Fig 1.10: Diagrammatic representation of the differentiation of haematopoietic pluripotent stem cells. Pre-hematopoietic stem cells (Pre-HSC) mature into haematopoietic stem cells (HSCs). The HSC matures into a multipotent progenitor (MPP) which then further differentiates into common myeloid progenitor (CMP) and common lymphoid progenitor (CLP). CMP mature into megakaryocyte-erythroid progenitor (MEP) and granulocyte monocyte progenitors (GMP). The GMP in turn commits to mature erythrocytes, megakaryocytes, platelets, monocytes, macrophages and granulocytes. CLP differentiates into B cell, T cell and natural killer (NK) cell progenitors, followed by their differentiation into the corresponding mature cells. Each cell lineage may serve as a treatment tool for anaemia, thalassemia, leukocyte deficiencies and immune-deficiency related conditions. Macrophage differentiation may serve to clarify molecular mechanisms behind certain disorders, as exemplified by atherosclerotic lesions (Adopted with modifications from Lim *et al.*, 2013).

1.6.1. The U937 cell and differentiation studies

In cases of chronic inflammatory diseases and in the tumour microenvironment, inhibition of apoptosis promotes monocyte survival and hence their conversion to macrophages (Lin *et al.,* 2014; Parihar *et al.,* 2010). The accumulation of macrophages and the continued expression of inflammatory milieux has since found widespread use for *in vitro* studies involving molecular mechanisms behind disorders such as cancer and atherosclerotic lesions (Lim *et al.,* 2013; Heusinkveld and van der Burg, 2011). Two distinct states of macrophages have been categorised as polarisation of the classical type I (M1) macrophages and type II (M2) macrophages (Heusinkveld and van der Burg, 2011; Martinez and Gordon, 2014). M1 macrophages attract cells of the adaptive immunity in response to signals from bacteria and IFNγ **(**Heusinkveld and van der Burg, 2011). These cells are associated with expression of reactive oxygen species (ROS), inducible nitric oxide synthatase (iNOS) and activation of natural killer cells; hence these are associated with inflammatory response (Martinez and Gordon, 2014). M2 macrophages are associated with promotion of tumour growth and polarisation of T cells, hence dampening the immune response (Housinkveld and van der Burg, 2011; Martinez and Gordon, 2014).

1.6.2. Reactive oxygen species and apoptosis

Reactive oxygen species are metabolic end-products of oxygen metabolism. Ideally, in a balanced physiological process, the rate of formation of reactive oxygen species is balanced by the rate of their elimination (Elmore, 2007). Overproduction of reactive oxygen species results in intracellular oxidative stress (Elmore, 2007). This may result in an increase in endogenous stimulation of apoptosis. The U937, a histolytic lymphoma cell, has been widely used to evaluate the role of chemical stimuli in cellular transformation as well as ROS production (Matés and Sánchez-Jiménez, 2000; Meshkini *et al*., 2009). The present study also seeks to evaluate the effect of single and combination antiretroviral treatment on morphological changes in U937 cells, including the potential to transform pre-monocyte U937 cells into adherent cells. It is also of interest to determine the amount of superoxide anion produced following treatment of U937 cells with antiretroviral drugs.

1.7.0 Overview of the problem

HAART entails combining two nucleoside reverse transcriptase inhibitors (NRTIs) with one non-nucleoside reverse transcriptase inhibitor (NNRTI), in the present study, nevirapine (NVP). Alternatively a combination of two NRTIs may be administered together with a protease inhibitor. The advent of HAART has resulted in increased survival

of HIV-infected individuals. However, acquired immunodeficiency syndrome-associated cancers present a challenge in modern day cancer epidemiology (Hsu *et al.*, 2013; Engels *et al.,* 2006). AIDS-defining malignancies include Kaposi's sarcoma, non-Hodgkin lymphoma and cervical cancer (Engels *et al.,* 2006). HIV patients are also at an increased risk of non-AIDS-defining cancers (NADCs), such as anal, lung and liver cancer and Hodgkin lymphoma (Hsu *et al*., 2013; Engels *et al.,* 2006).

The introduction of HAART from the 1990s led to an improved life expectancy among HIV-positive patients (Shiels *et al*., 2011). Incidences of AIDS-defining malignancies such as Carposi's sarcoma and non-Hodgkin lymphoma have seen an up to four-fold decrease (Deeken *et al.*, 2012; Shiels *et al.,* 2011). While AIDS-defining cancers have been associated with chronic immune activation, the observed decrease in AIDS defining cancers with HAART has been attributed to the progress in CD4+ restoration, hence the efficiency of immune system (Hsu *et al.*, 2013; Deeken *et al.*, 2012). On the contrary, there has been a sharp rise in non-AIDS defining cancers, such as cancers of the lung, kidney, anus, skin, and Hodgkin's lymphoma by around three-fold (Deekeen *et al.,* 2012; Shiels *et al.*, 2011). There is limited information on whether HAART affects cervical cancer progression among users of hormonal contraceptives. Globally, hormonal contraceptive (HC) formulations with levonorgestrel and ethinylestradiol have since been most commonly used (Blackburn *et al.*, 2000).

1.7.1. HAART use and progression of cervical intraepithelial neoplasia

A study in USA indicated that progression of neoplasia in high-risk human papillomavirus infected women was inversely proportional to $CD4^+$ count. However the results suggested that, even in the era of HAART, some women developed high-grade cervical intraepithelial neoplasia (Lillo *et al.,* 2001). McKenzie *et al.* (2011) demonstrated that cervical squamous intraepithelial lesion (SIL) is more prevalent among women on non-nucleoside reverse transcriptase inhibitor (NNRTI) regimen than those not on this treatment. Similarly, a cross-sectional study among women in Cape Town (South Africa) linked high prevalence of abnormal Pap smears and high-risk human papillomavirus (HR-HPV) to HAART initiation (Moodley *et al.*, 2009).

1.7.2. HAART use and cervical intraepithelial neoplasia regression studies

A seven-year cohort study carried out by Adler *et al.* (2012) in Soweto (South Africa) identified an increased likelihood of SIL regression among HAART users. A related study

carried out in Johannesburg (South Africa) concurs with these findings, as HAART use was associated with reduction of both incidence and progression to cervical neoplasia (Firnhaber *et al.*, 2012). Omar *et al.* (2011) demonstrated higher rates of high-grade squamous intraepithelial lesion (HSIL) and low-grade squamous intraepithelial lesion (LSIL) among HIV-positive women from a cohort derived from Soweto. In this cohort, HAART was shown to reduce risk of progression. In their eight-year cohort study in Taiwan, Chen *et al.* (2014) found a four-times higher incidence of cervical neoplasia among HIV-positive women compared to HIV-negative women. The study, however demonstrated the protective effect of HAART against cervical neoplasia in HAART-adherent women and they postulate that prolonged HAART use for more than three years may reduce the risk of cervical neoplasia. Minkoff *et al.* (2010) maintain that initiation of HAART is associated with reduction in HPV infection although the protective effect is reduced among non-adherent women.

1.7.3. Studies demonstrating no effect of HAART on CIN

In a study carried out in South Western Nigeria, Ezechi *et al.* (2014) demonstrated no effect of antiretroviral therapy against development of squamous intraepithelial lesion (SIL). However, their study was not clear on whether the participating women were on HAART (combination of three or more drugs), single or double drug combinations. It would appear the authors present observations on generalised antiretroviral treatment. According to Shrestha *et al.* (2010), HAART does not have an effect on HPV infection and neoplasia progression but they suggest the need for further research. Similarly, from their cohort study in Germany, Sirera *et al.* (2008) maintain that use of HAART presents a similar CIN incidence to women not on HAART.

Such observed variations in CIN progression among different epidemiological studies necessitate *in vitro* studies in which confounding factors are precluded. Whether HAART affects cervical intraepithelial neoplasia risk progression into cervical cancer among users of hormonal contraceptives needs further elucidation. The present study thus seeks to evaluate the cause-and-effect relationship between exposure of human cervical epithelial cancer cells to antiretroviral treatments (both before and following exposure to levonorgestrel and ethinylestradiol that are common constituents of combined hormonal contraceptives) and apoptotic cellular transformations. To this effect, HeLa cells are an important tool for studying the pathology of the human cervical epithelium cancer cells *in vitro*.

1.8.0. Combined oral contraception and cervical cancer

Hormonal contraception is widely used globally, including in sub-Saharan Africa, where apparently a high rate of HIV infection has been observed (Blackburn *et al.*, 2000). The major benefit of combined oral contraceptives is prevention of unwanted pregnancies (Practice Committee of American Society for Reproductive Health, 2008; Messiou *et al.*, 2009). In addition, reduction in mortality, improved bone health and prevention of ovarian, endometrial and colorectal cancer have been associated with combined oral contraceptive (COC) use (Messiou *et al.*, 2009). On the contrary, the Practice Committee of American Society for Reproductive Health (2008) concurs that combined oral contraceptives possibly act as co-factors for HPV-induced development of CIN. Such variation in observed results on cause-and-effect makes it necessary to investigate the effect of continued hormonal contraceptive (HC) use by HAART patients. Whether or not HAART affects cervical cancer progression among hormonal contraceptive users is an area which needs further elucidation. It may thus be important to design *in vitro* experiments to evaluate the effect of exposure to levonorgestrel or ethinylestradiol and HAART on genotoxicity and apoptosis in human cervical epithelial cells.

1.8.1. Constituents of combined oral contraceptives

Progestins provide the dominant contraceptive benefits in combined oral contraceptives. These include suppression of follicle-stimulating hormone (FSH) and luteinising hormone (LH) associated with ovulation (Practice Committee of American Society for Reproductive Health, 2008). Progestins also increase cervical mucus secretion and result in decreased tubular motility, hence lowering sperm mobility (Blackburn *et al.*, 2000). In addition to progestin, combined oral contraceptives contain oestrogens, which stabilise the endometrium, thereby minimising bleeding (Practice Committee of American Society for Reproductive Health, 2008). Monophasic COCs have consistent dosages of oestrogen, while multiphasic OCs have varying doses (Blackburn *et al.*, 2000).

The present study considered the oestrogen ethinylestradiol (EE) and the progestin ß-levonorgestrel (LNG) because these are constituents of COCs predominantly used in sub-Saharan Africa and other low-resource settings where the prevalence of HIV infection is high (Blackburn *et al.*, 2000). EE is the most commonly used oestrogen in COCs. It is a semisynthetic female hormone derived from oestradiol with an ethinyl substitution at C17 position (Figure 1.11A). EE is often present in low doses in combination with relatively higher doses of progestin (Practice Committee of American Society for Reproductive

Health, 2008). Levonorgestrel is a synthetic progestogen (Figure 1.11B). Whether or not hormonal contraceptives affect cell viability and apoptosis of human cervical cancer cells upon co-administration with antiretroviral drugs remains obscure, hence warranting the need for further exploration.

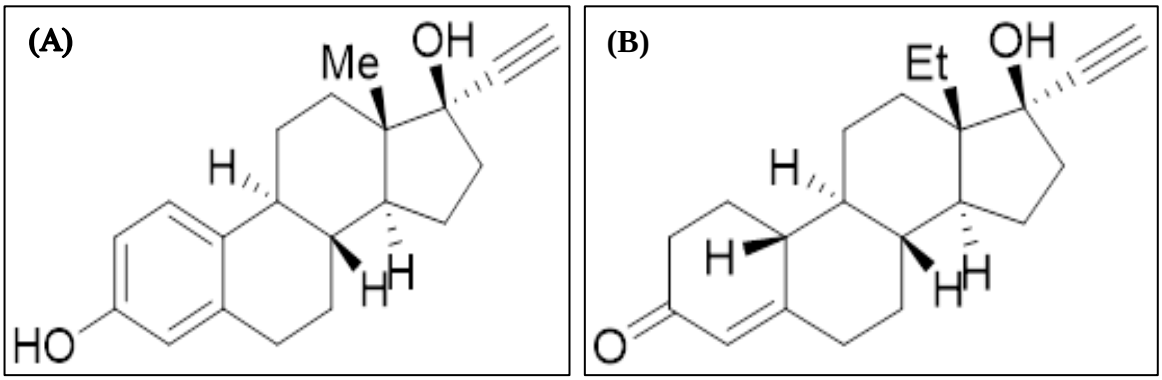

Fig 1.11: Chemical structures of ethinylestradiol and levonorgestrel. (A) Ethinylestradiol (B) Levonorgestrel.

1.8.2. Physiological concentrations of ethinylestradiol and levonorgestrel

Different clinical studies have demonstrated plasma mean maximum concentrations (Cmax) for ethinylestradiol (EE) ranging from around 33pg/ml to about 85pg/ml. Steady-state concentration of EE have been reported to be in the range 70±30pg/ml (mean±SD) (Stanczyk *et al.,* 2013; Archer *et al.,* 2012; Junior *et al.,* 2011). Plasma C_{max} for levonorgestrel (LNG) has been reported to be in the range around 2400±930pg/ml with steady-state concentrations of 1847±930pg/ml (Archer *et al*., 2012; Stanczyk *et al*., 2013).

18.3. Physiological concentrations of antiretroviral drugs

Related ranges of serum C_{max} values have been reported for ZDV, 4T, 3TC, ABC and NVP (Donnerer *et al.,* 2003, Donnerer *et al.,* 2008 and van Praag *et al.,* 2002) with absolute mean ranges of C_{max} for these drugs as summarised as follows: 635ng/ml for zidovudine, 930ng/ml for stavudine, 1195ng/ml for lamivudine, 1380ng/ml for abacavir and 6199ng/ml for nevirapine (Donnerer *et al.,* 2003, Donnerer *et al.,* 2008; van Praag *et al.,* 2002).

1.9.0. Philosophical basis of the research

The present research utilises a deductive approach of initiating the research from what is generally known (assumed knowledge). Deduction is an aspect of positivism (Herbert and Higgs, 2004). In a positivist approach, experimental designs will be informed by the gaps in knowledge (Herbert and Higgs, 2004). The knowledge gaps on the correlation between co-administration of antiretroviral drugs with contraceptive hormones and cervical cancer progression, inform the present experimental design. The observed scientific results will inform the knowledge gaps, hence making the last stage inductive.

1.9.1. Research hypothesis and questions

The present research seeks to establish whether interaction of antiretroviral drugs with levonorgestrel or ethinylestradiol have an effect on human cervical epithelial cancer cell proliferation and apoptosis. The underlying research hypothesis is thus:

H_0: Exposure to combined oral contraceptive hormones (ethinylestradiol and levonorgestrel) and antiretroviral drugs does not promote DNA mutation, cell transformation and human cervical epithelial cancer cell proliferation.

H_1: Exposure to combined oral contraceptive hormones (ethinylestradiol and levonorgestrel) and antiretroviral drugs promotes DNA mutation, cell transformation and human cervical epithelial cancer cell proliferation.

The following research questions are therefore of interest:

(a) Does exposure to antiretroviral drugs and levonorgestrel or ethinylestradiol alter the proliferation of human cervical epithelial cancer cells?

(b) Do nucleoside reverse transcriptase inhibitors or nevirapine cause DNA double strand breaks in their supposed carcinogenic effect?

(c) Does exposure to levonorgestrel or ethinylestradiol have an apoptotic effect on antiretroviral drug-induced apoptosis in human cervical epithelial cancer cells?

(d) Do antiretroviral drugs have the potential of transforming U937 cells to adherent cells with macrophage-like characteristics?

1.9.2. Specific objectives of research

a. To evaluate the effect of single and combination antiretroviral treatment (with and without contraceptive hormones) on the viability of human cervical cancer cells.

b. To determine the effect of antiretroviral drugs on inducing DNA double-strand breaks on Chinese hamster ovary (CHO) and human cervical epithelial cancer cells (HeLa).

c. To evaluate the effect of contraceptive hormones (levonorgestrel and ethinylestradiol) on antiretroviral drug-induced apoptosis in human cervical epithelial cancer cells.

d. To explore the effect of single and combination antiretroviral drugs on transformation of the U937 cells into 'macrophage-like' adherent cells.

e. To measure the amount of intracellular superoxide anion produced by transformed adherent cells following 72 h treatment of U937 cells with single and combination antiretroviral treatment.

1.9.3. General approach to the problem

The following section summarises the general approach and methodology used to explore the effect of ethinylestradiol and levonorgestrel on combination antiretroviral drug-induced apoptosis. In a bid to explore the precipitation of serious non-AIDS events (SNEs), the effect of highly active antiretroviral therapy on transforming premonocyte U937 cells to cells with macrophage-like characteristics is also outlined.

1.9.3.a. Cell viability assay

24 h effective concentrations (EC) were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT). Determination of antiretroviral drug

concentrations lying within EC_{15-25} was established, as these are the desired concentrations for toxicological assays because they do not induce toxicity that is too drastic. The MTT assay was then used to evaluate the effect of administering single and combination antiretroviral treatment on HeLa cell proliferation over 24, 48 and 72 h. The effect of ethinylestradiol and levonorgestrel co-administration on Hela cell proliferation was also evaluated using the MTT assay over a period of 72 h.

1.9.3.b. Fluorimetric analysis of DNA unwinding

Using the fluorimetric analysis of DNA unwinding (FADU), concentrations of 50µg/ml of each antiretroviral drug were then assayed for their potential to cause double strand breaks in CHO and HeLa cells. The efficiency of reparing DNA strand breaks was evaluated by treating HeLa cells with each antretroviral drug for 72 h followed by continued culture in drug-free medium under microscopic observation over a period of two weeks.

1.9.3.c. Determination of apoptosis

Annexin-V/PI staining quantified normal, early apoptotic, late apoptotic and necrotic cells, following treatment of HeLa cells with either single or combination antiretroviral drugs over a period of 48 h.

1.9.3.d. Transformation of U937 cells

U937 cells were seeded in 24-well plates and treated with single and combination antiretroviral drugs. The cells were then incubated for 72 h and the number of adherent and non-adherent cells counted. Morphological transformation was assessed using an EVOS inverted microscope. Concurrently, the superoxide anion production potential of these transformed cells was evaluated cognisant of the association betweem MI macrophages and superoxide anion production (Matés and Sánchez-Jiménez, 2000; Meshkini *et al.*, 2009).

1.10. Dissemination of research findings and ideas

Open Access (OA) publication has been shown to accelerate the timely dissemination of information, especially in low-resource settings (Eysenbach, 2006). This is mainly due to limited financial resources to access some subscription based databases (Eysenbach, 2006). The Southern African Regional Universities Association (SARUA) supports the notion of OA publication in order to encourage visibility in these mostly low and medium countries. During the taught phase of my doctoral programe (first and second year) prior to the

research phase, I was fascinated by writing skills, evidence-based practice and health policy studies courses delivered. Consequently we wrote a short opinion article with Dr Hilda Matarira of the University of Zimbabwe (Chemical Pathology Department) on hormonal contraception and HIV from a Zimbabwean perspective and disseminated it in the OA Journal of Public Health in Africa. The vision in this short review also informed some introductory concepts to this thesis. During the research phase, I presented ordinary and electronic posters at a number of international conferences. Cognisant of some unanswered questions in the present research, feeedback from these conferences lead to introspection into future directions as discussed in Chapter 6. The following is a summary of dissemination articles:

1.10.1 Short communication/opinion article

- Mafuva, C., and Marima-Matarira, H.T., 2013. Hormonal Contraception and HIV/AIDS Transmission: Challenges for Zimbabwe's Reproductive Health Service Providers in Promoting Informed Contraceptive Choices. Journal of Public Health in Africa. 2013; 4(e16):73-75. Available from: *https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5345434/*

1.10.2 Conference proceedings

- Mafuva, C., and Morgan, W.A., 2016. Transformation of monocytes to adherent cells by combination antiretroviral treatment. *Proceedings of the British 'Pharmacology 2016' annual conference*, 13-15 December 2016, Queen Elizabeth II Hotel, London, United Kingdom. Available from: *http://roar.uel.ac.uk/6457/1/Pharmacology%202016%20abstract-%20Christopher%20Mafuva%20Winston%20Morgan%202.pdf*
- Mafuva, C., and Morgan, W.A., 2017. Effect of contraceptive hormones on a nevirapine based antiretroviral combination induced apoptosis in human cervical epithelial cancer cells. *Proceedings of the 32nd Annual Conference on Genes and Cancer*, 11-13 April 2017; pp72, Robinson College, University of Cambridge, United Kingdom.
- Mafuva, C., Pula, G., and Morgan, W.A., 2017. Role of contraceptive hormones on combination antiretroviral treatment induced apoptosis in human cervical cancer cells. Proceedings of the 13th World Congress on Inflammation; 8-2 July 2017, London, United Kingdom. Available from: *http://static.springer.com/sgw/documents/1614767/application/pdf/13.+WCI+London+2017+Abstract+Book+-+Springer+Portal.pdf* pp101-102.

CHAPTER 2

EFFECT OF ANTIRETROVIRAL DRUG CO-ADMINISTRATION WITH CONTRACEPTIVE HORMONES ON HUMAN CERVICAL CANCER CELL VIABILITY

2.1.0. Introduction

Ethinylestradiol and levonorgestrel elicit their effect through interaction with hormone receptors. Different responses to oestrogen exposure are elicited through binding to estrogen receptor-α (ERα) and estrogen receptor-β (ERβ). The receptors have different subtypes and several isoforms (Yu *et al.*, 2010; Mazzucco *et al.*, 2006). Oestrogen modulation of gene transcription is termed genomic action (Felty and Roy, 2005). In addition to the genomic pathway, there is evidence that a non-genomic signalling pathway *via* ERs contributes to cell proliferation (Mosli *et al*., 2013; Mazzucco *et al.*, 2006). It has since been established that 17β-oestradiol (E2)-dependent cellular proliferation is mediated through ERα control of gene transcription and kinase activation (Pesiri *et al*., 2015; Scaling *et al*., 2014; Atawia *et al*., (2013). Leucine (L^{429}) and alanine (A^{430}) are located within the ERα ligand-binding domain and contribute to E2-induced proliferation although the mechanism of their action has not been fully elucidated (Pesiri *et al*., 2015).

Mosli *et al.* (2013) demonstrated that *in vitro* exposure of non-transformed benign prostatic hyperplasia (BPH-1) epithelial cells to catechol oestrogens (CEs) have a neoplastic effect. They further demonstrated that the CE 4-hydroxyestradiol ($4\text{-}OHE_2$) showed more carcinogenicity, relative to the parent hormone 17β-oestradiol (E2). It has also been shown that oestrogen has proliferative effects in normal and MCF-7 breast cancer cells (Scaling *et al*., 2014; Pesiri *et al*., 2015). G protein-coupled estrogen receptor (GPER), previously known as GPR30, modulates breast cancer proliferation downwards. In MC7 10A cells, stimulation by oestrogen, in the presence of GPER-selective agonist G-1, was shown to increase the mitogenic index (Scaling *et al*., 2014). Mazzucco *et al.* (2006) demonstrated that dual activation of ERα and ERβ showed reduced cell proliferation thus suggesting that ERα and ERβ modulate each other's activity. This research explores the effect of ethinylestradiol (EE), a derivative of 17β-oestradiol, on antiretroviral drug-induced proliferative effect in human cervical epithelial cancer cells. It utilises the HeLa cell as the cancer cell model. The cells have continued to have widespread use as a model for human cervical cancer research.

Two isoforms of progesterone receptor (PR), namely progesterone receptor-A (PRA) and progesterone receptor-B (PRB), are associated with endometrial differentiation (Vereide *et al.,* 2006). Deregulation of either of the genes has been associated with carcinogenesis of the endometrium and failure of progestin therapy (Gunderson *et al*., 2014; Kudesia *et al*., 2014, Vereide *et al*., 2006). Levonorgestrel inhibits human endometrial stromal cell (HESC) and human endometrial glandular cell (HEGC) proliferation, while inducing apoptosis in both cell lines (Zhao *et al.,* 2015). This is attenuated through an increase in permeability of gap junctions and upregulation of Cx43 (Zhao *et al*., 2015). The levonorgestrel intra-uterine system (LNG-IUS) has since been shown to induce regression of complex endometrial hyperplasia (Wildemeersch *et al*., 2015; Haoula et *al*., 2011; Zhao *et al.,* 2015). LNG-IUS down-regulates expression of PRA, PRB, ER-α and ER-β (Gomes *et al.,* 2009. Vereide *et al.,* 2014). Complex atypical hyperplasia (CAH) was also shown to decrease after progestin therapy (Gunderson *et al*., 2014; Kudesia *et al*., 2014). Women with CAH and endometrial carcinoma were shown to have an overall response of about 65% upon receiving progestin-only treatment (Gunderson *et al*., 2014). Progestin-based treatment of CAH and endometrial cancer was shown to be effective and associated with an increase in desired pregnancy rates (Kudesia *et al.,* 2014). Here, the effect of co-administration of levonorgestrel (a synthetic progestogen) with ARVs on HeLa cell proliferation is investigated.

While androgens and oestrogen have a pathological role in benign prostatic hyperplasia (BPH), Atawia *et al.* (2013) demonstrated the protective effect of silymarin in preventing testosterone-induced BPH in rats by protection against decline in caspase-3 activity. Baker and Bauer (2015) also demonstrated that epigallocatechin gallate (EGCG) modifies oestrogenic activity in MFC-7 breast cancer cells, hence making it a suitable chemo-preventive agent. Wang *et al.* (2004) demonstrated the antiapoptotic effect of oestrogen in normal cervical and human epithelial cancer cells (HT3 and CaSki), where baseline apoptosis was mediated by $P2X_7$-receptor-induced activation of the pathway. It was shown that the antiapoptotic effect was dependent on attenuation of caspase-3 and caspase-9. Interestingly, oestradiol did not affect apoptosis in SiHa and HeLa cells (Wang *et al.,* 2004). This demonstrates that some cells acquire apoptosis-eversion characteristics hence enabling growth and proliferation of the tumour. As outlined in the previous chapter, HPV alone does not necessarily induce cervical cancer (zur Hausen 2009; Thiery, 2008). While HPV-6, E2 and E7 proteins are required for neoplastic transformation of normal human cervical cancer cells, it has since been shown that oestrogen and progesterone upregulate

the level of HPV-E2 and E7 protein-induced apoptosis in HeLa cells (Webster *et al.*, 2001; Wang, 2004). To date there is paucity of information regarding the effect of co-administration of either LNG or EE with HAART on human cervical epithelial cancer cell proliferation.

While HAART reduces HIV-1 transmission, a Burkina Faso follow-up study isolated genital HIV-1 in patients on HAART (Low *et al.*, 2014). NVP (nevirapine: a first-generation non-nucleoside reverse transcriptase) and ZDV (zidovudine: a nucleoside reverse transcriptase inhibitor) regimens were shown to exhibit higher rates of cervacovaginal shedding relative to d4T (stavudine: a first-generation nucleotide reverse transcriptase inhibitor) and efavirenz (EFV)-based regimens (Low *et al.*, 2014). McGuigan *et al.* (2013) evaluated the anticancer activity of the phosphorodiamidate phosphate prodrugs for nucleoside analogues (NAs), among which were ABC (abacavir), 3TC (lamivudine) and d4T. These studies were devoid of co-administration of contraceptive hormones. Stavudine did not show proliferative activity against HeLa cells, while its phosphorodiamidates showed anticancer activity. This could be due to the greater concentration of active metabolites achieved. In the same study, d4T showed cytostatic activity against murine L1210 cells. 3TC also displayed cytostatic activity for HeLa cells, while its prodrug displayed some antiproliferative activity. ABC and its prodrugs did not exhibit proliferative activity. It is interesting to speculate whether or not co-administration with either levonorgestrel and/or ethinylestradiol affects the observed proliferative trend.

There is paucity of information regarding whether pre-exposure to combined oral contraceptives followed by co-administration with HAART has an effect on cervical intraepithelial neoplastic progression. Using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, this section seeks to evaluate the effect of pre-exposure to contraceptive hormones (levonorgestrel and ethinylestradiol) on proliferation of human cervical epithelial cancer cells. The effect of subsequent co-administration of antiretroviral drugs is also investigated. In this section, proliferative assays were performed using low effective concentrations (EC_{15-25}) of each antiretroviral drug estimated from the 24 h proliferation assay. Drug sensitivity is usually defined as IC_{50} which is that concentration resulting in 50% growth inhibition (Sadegh-Aliabadi *et al.*, 2010; Trivedi *et al.*, 1990). The half-maximum effective concentration (EC_{50}) is that concentration of a drug eliciting half its maximum effect after a specific exposure time. Most toxicological

studies use minimal stimulating concentrations, resulting in relative decreases in absorbance while IC_{50} or EC_{50} equivalents may be used as high-dose exposures.

In the present section, the proliferative effect of single and combination antiretroviral treatment (with or without contraceptive hormones) on HeLa cells over a treatment period of 72 h is elucidated. Moodley *et al* (2009) and Sirera *et al* (2008) agree that there is need to evaluate the role of other factors, such as use of hormonal contraceptives and smoking on the onset of CIN among women on highly active antiretroviral therapy (HAART). In the past two decades, most single-drug treatments have since been substituted with HAART, due to their observed toxicities (BHIVA, 2008; WHO, 2010). Presently, HAART is the predominant intervention, although other double and triple combinations have been recommended (WHO, 2016). The effect of single drugs on HeLa cell proliferation is explored concurrently with antiretroviral drug combinations used in low-resource settings.

Phenol red (Phr)-containing medium used in most cell culture experiments ensures monitoring the sterility of the medium, cell culture environment and the aseptic technique. However, it has since been suggested that Phr has weak oestrogenic properties in concentrations used in most tissue culture medium and Phr was shown to stimulate proliferation of MCF-7 cancer cells (Hofland *et al.*, 1987). Ortmann *et al.* (1990) demonstrated that Phr and oestradiol have a synergistic stimulatory effect on gonadotrophin-releasing hormone (GnRH) secretion by pituitary cells. To this effect, phenol red free medium is preferred for hormone-based assays (Webster *et al.*, 2001; Wang *et al.*, 2004).

2.1.1. Objectives of the chapter

a. To evaluate the effect of single and combination antiretroviral treatment (with and without contraceptive hormones) on the viability of human cervical epithelial cancer cells over a 72 h treatment.
b. To evaluate the morphological changes on the cervical epithelial cancer cells after 24 h treatment with single antiretroviral drugs and contraceptive hormones at varying concentrations.

The mechanistic effect of single drugs on HeLa cell proliferation is explored concurrently with antiretroviral drug combinations used in low-resource settings. The nucleotide reverse transcriptase inhibitors abacavir, zidovudine, lamivudine and stavudine simultaneously with nevirapine (a first-generation widely used non-nucleotide reverse transcriptase inhibitor) are considered. These constitute the backbone of the most widely used

nucleotide plus non-nucleotide antiretroviral drug combinations namely; abacavir and lamivudine (ABC+3TC), zidovudine and lamivudine (ZDV+3TC), zidovudine, abacavir and lamivudine (ZDV+ABC+3TC) zidovudine, lamivudine and nevirapine (ZDV+3TC+NVP) and zidovudine, stavudine and nevirapine (ZDV+d4T+NVP).

2.2. Materials and Methods

2.2.1 Materials

Cell culture media, Dulbecco's Modified Eagle Medium (DMEM) with and without phenol red were purchased from Life Technologies (United Kingdom). Antiretroviral drugs (abacavir, lamivudine, nevirapine, stavudine and zidovudine), penicillin-streptomycin, sodium pyruvate, dimethylsulfoxide (DMSO), non-essential amino acids, glutamine, charcoal-treated foetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), were obtained from Sigma (United Kingdom). 96-well plates were purchased from Nunc Denmark. The HeLa cell line was obtained courtesy of the University of East London tissue culture research laboratory. Trypsin/ ethylenediaminetetraacetic acid (EDTA) was purchased from Life Technologies (UK).

2.2.2 HeLa cell culture and maintenance

HeLa cells were maintained in 75cm^2 flasks. The cells were grown in DMEM with phenol red supplemented with 10% FBS 1% non-essential amino-acid solution, 1% sodium pyruvate, 2mM glutamine and 1% (v/v) penicillin/streptomycin [10,000 U/ml penicillin, 10,000 µg/ml streptomycin]. The cells were incubated at 37^{o}C and 5% atmospheric CO_2. The cells were split every 3 to 4 days at approximately 75% confluence. For hormone assays, HeLa cells were sub-cultured in DMEM devoid of phenol red supplemented with charcoal-treated FBS under similar conditions.

2.2.3 Cryopreservation of HeLa cells

Cells were grown to about 75% confluence in T75 culture flasks. The cell medium was aspirated from the culture flask with growing cells, the cells washed with 5ml PBS and the PBS aspirated. 2ml of trypsin/EDTA was added to cover the surface of the flask and the flask returned to the incubator for about a minute. Trypsin was quenched by adding 8 ml of medium to the flask followed by gently pipetting up and down about 4 times while washing the sides and middle part of the flask in order to harvest the highest number of cells. The cells were transferred to a 15ml centrifuge tube and spun at 1000rpm for about 5 minutes. The supernatant was removed, cells re-suspended in 1ml of fresh freezing

medium (with 10% DMSO and 20% FBS) and transferred to a cryotube. The cells were placed at -80^{o}C overnight and then transferred to liquid nitrogen the next day. Upon resuscitation, up to 15 passages were used for experimental assays.

2.2.4 Standard curves for MTT assay

The MTT cell proliferation assay measures the cell proliferation rate and, conversely, when metabolic events lead to apoptosis or necrosis, the reduction in viability (Sieuwerts *et al*., 1995). Metabolic reduction of the yellow tetrazolium salt (MTT) results in a purple formazan whose concentration is directly proportional to viable cells. This resultant purple complex is quantifiable by spectroscopy (Sieuwerts *et al*., 1995, Sylvester, 2011; van Meerloo *et al*., 2011). For drug-sensitivity measurements, the absorbance value of untreated wells are compared to wells exposed to the drug and the result expressed as a percentage: [Absorbance of treated well/Absorbance of the control well] x 100. The decrease in cell absorbance is directly proportional to decrease in cell viability and is indicative of cell growth inhibition.

The optical cell count was adapted as outlined in the American Type Culture Collection (ATCC) MTT cell proliferation assay instruction guide and from Trivedi *et al.* (1990). Briefly, cells were released from the T75 flask by trypsin (Life Technologies). 1.0 x 10^6 cells per ml were re-suspended. The cells were serially diluted in cell culture medium from 1.0 x 10^6 to about 1.0 x10^3 cells per ml. The culture medium contained 10% charcoal treated FBS, 2mM glutamate, 1% (v/v) penicillin/streptomycin [10,000 U/ml penicillin, 10,000 µg/ml streptomycin] and 1% essential amino acids. At short exposure time up to 24 h, 100µl of medium were deemed sufficient as nutrients will still be present in the medium at the end of the incubation period. As the incubation period is increased to 72 h, a higher volume of medium (200µl) was used to ensure continuous supply of nutrients and limiting the accumulation of metabolic end products.

For preliminary 24 h assays, 100µl of serially diluted cells were seeded in quadruplicates onto 96-well plates. Four control wells with medium only constituted the reagent blank for absorbance readings. The cells were left overnight (24 h at 37^{o}C and 5% atmospheric CO_2) to adhere and recover from handling. The medium was aspirated, fresh medium was added and the cells incubated under same conditions for a further 24 h. 10µl of 5µg/ml MTT reagent were added to each well and the well plates further incubated for 1 h. The medium/MTT solution was removed from the wells and 100µl DMSO added. The plates were left in the dark for 2 h and the absorbance read on a microplate reader at 540nm.

Average values from the quadruplicate readings were determined and the average value for the blank subtracted. Three independent experiments were considered. Ideally, the seeding cell density should lie within the straight line of the curve (Sieuwerts *et al.,* 1995; van Meerloo *et al.,* 2011). Seeding densities for hormone treatment were estimated using 200µml of the medium over 24 h, 48 h and 72 h post adherence of cells. The procedure was repeated as described above but this time using 200µl of medium and 20µl MTT solution.

2.2.5 Preliminary 24-hour MTT assay for NRTIs

100µl of 5.0 x 10^4cells/ml were seeded in 96-well plates. The plates were incubated at 37^{o}C and 5% CO_2 for 24 h. After the 24 h incubation period, working stock solutions of 1000µg/ml of the water-soluble nucleotide reverse transcriptase inhibitors ABC, ZDV, 3TC and d4T were freshly prepared in sterile medium consisting of 1% (v/v) penicillin/streptomycin [10,000 U/ml penicillin, 10,000 µg/ml streptomycin], 10% charcoal-treated FBS, 2mM glutamate and 1% essential amino acids. The working solutions were then adjusted to final concentrations of 50µg/ml, 100µg/ml, 150µg/ml, 200µg/ml, 250µg/ml and 300µg/ml of each drug by diluting with the complete medium to a final volume of 1ml. The control solution consisted of medium only (0µg/ml). The vehicle control and all treatment medium were adjusted to a final concentration of 0.25% (v/v) DMSO. The medium was replaced with 100µl of the freshly prepared medium with varying drug concentrations for a further 24 h. Each concentration was administered in quadruplicates and the average absorbance taken. For every given drug concentration, each experiment was repeated three times. Morphological changes were observed using an EVOS® inverted microscope.

2.2.6 Preliminary 24-hour MTT assay for nevirapine

The preliminary 24 h MTT assay was carried out in order to determine the concentrations of NVP ideal for toxicity and apoptosis assays in HeLa cells. Nevirapine is a non-nucleoside reverse transcriptase inhibitor that is sparingly soluble in water. Its solubility in DMSO is ≥22mg/ml. In *in vitro* assays, it has since been established that DMSO concentrations of 1% and below do not affect the toxicity in HeLa cells (Trivedi *et al.*, 1990). 10mg of nevirapine were dissolved in 0.5 ml of DMSO, thus giving a stock solution of 20mg/ml. This solution was diluted in DMEM (consisting 10% charcoal-treated FBS, 2mM glutamate, 1% (v/v) penicillin/streptomycin [10,000 U/ml penicillin, 10,000 µg/ml streptomycin] and 1% essential amino-acids to give solutions of 40µg/ml, 80µg/ml,

120µg/ml, 160 µg/ml and 200µg/ml. The maximum concentration of 200µg/ml ensured the limit of 1% DMSO in solution. The vehicle control and all treatment media were adjusted to a final concentration of 1% DMSO. HeLa cells were then treated in quadruplicate in 96-well plates with each of the concentrations to determine single-drug viability effect as previously described for NRTIs. The experiment was repeated three times (n=3).

2.2.7. Preliminary 24-hour MTT assay for ethinylestradiol and levonorgestrel

The preliminary 24-h MTT assay was carried out in order to determine the concentrations of EE and LNG ideal for toxicity and apoptosis assays in HeLa cells. Briefly, approximately 0.5 $x10^4$ HeLa cells per well were seeded in flat bottom 96-well plates. The cells were seeded in a suspension DMEM without phenol red, supplemented with 10% FBS 10ml/L minimum essential amino-acids and 1% (v/v) penicillin/streptomycin [10,000 U/ml penicillin, 10,000 µg/ml streptomycin]. The cells were then incubated overnight (24 h) at 37^{o}C and 5% CO_2 to allow them to attach. Following cell attachment, the medium was aspirated and replaced with 4µg/ml, 8µg/ml, 12µg/ml, 16µg/ml and 20µg/ml of EE and LNG in DMEM with its constituent supplements. The cells were then incubated for a further 24 h, viewed on an inverted EVOS® microscope and photographs captured with great caution since the culture medium was colourless, hence giving a grey background.

2.2.8. 72-Hour viability assay for antiretroviral drug treated cells

About 3.0 x 10^4 cells/ml were suspended in DMEM without phenol red supplemented with 10% charcoal-stripped FBS, 10ml/L minimum essential amino-acids and 1% (v/v) penicillin/streptomycin [10,000 U/ml penicillin, 10,000 µg/ml streptomycin]. 200µl of cell suspension (6.0 x 10^3 cell per well) were seeded in flat bottom 96-well plates. A quadruplicate of wells with 200µl of medium only constituted the reagent blank. The plates were incubated at 37^{o}C and 5% atmospheric carbon dioxide for 24 h. The medium was replaced with 200µl quadruplicates of the freshly prepared single and combination antiretroviral drugs at final concentrations of 50µg/ml of each drug. The vehicle control and all treatment medium were adjusted to a final concentration of 0.25% DMSO. The control cells were treated with the vehicle medium without antiretroviral drugs. Blank wells constituted of the medium only with no cells. For each concentration, the set of quadruplicates was commensurate with the number of single drug and HAART combinations under investigation. The cells were then incubated for 72 h and the morphology of the cells captured by an EVOS® microscope over time.

2.2.9. 72-Hour viability assay for ARV co-treatment with contraceptive hormones

Approximately 6.0 x10^3 cells per well were seeded in flat-bottom 96-well plates. A quadruplicate of wells had 200µl of medium without cells and this constituted the reagent blank. The plates were incubated at 37^{o}C and 5% atmospheric carbon dioxide. After 24 h of initial incubation, the working stock solutions of ethinylestradiol and levonorgestrel were adjusted to final concentrations of 0.5µg/ml, 1.0µg/ml and 2.0µg/ml or 1.0µg/ml, 2.0µg/ml and 4.0µg/ml, by diluting with DMEM. The medium was replaced with quadruplicates of 200µl hormone solutions. Control wells were treated with 200µl of vehicle medium only. Blank wells constituted of the medium only with no cells. The wells were incubated for 24 h. A mixture of antiretroviral drugs at final concentration of 50µg/ml for each drug was freshly prepared and supplemented with hormones maintained at original concentrations. The medium was then aspirated and replaced with 200µl of the antiretroviral drug/hormone mixtures and incubated for a further 48 h. After the total exposure time (72 h), cell proliferation was assessed by MTT. The set of quadruplicates constituted of 50µg/ml of single drugs (ABC, ZDV, 3TC, d4T and NVP) or antiretroviral drug combinations (ABC+3TC, ZDV+3TC, ABC+ZDV+3TC, ZDV+3TC+NVP and d4T+3TC+NVP). At each stage, the cells were viewed on an inverted EVOS® microscope and photographs captured. Each set of experiments was repeated three times. The procedure of the proliferation assay is summarised in Figure 2.1.

2.2.10 Data Analysis

For preliminary proliferation assays, differences in proliferation rates among groups were compared by one-way ANOVA followed by Dunnett's multiple comparison. Three independent experiments were considered for each set of MTT assay.

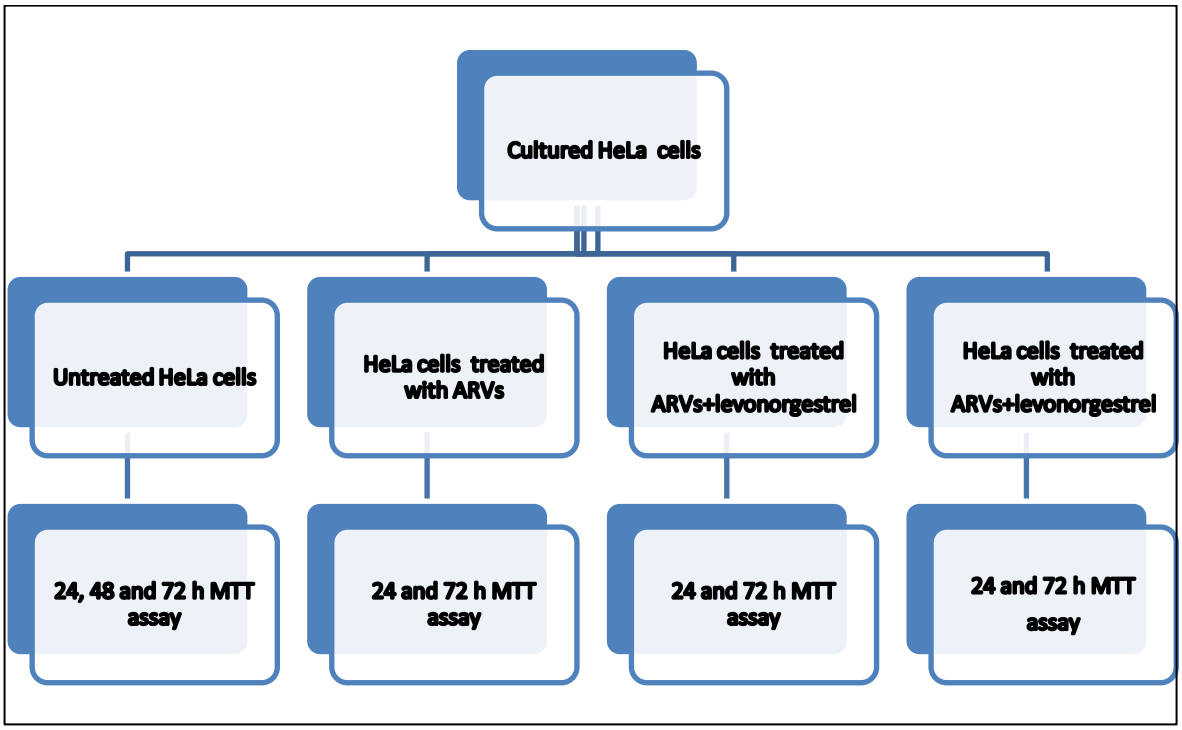

Fig 2.1: Outline of the experimental design for the effect of antiretroviral drug co-administration with contraceptive hormones on human cervical epithelial cancer cell proliferation. HeLa cells were treated with either single drugs or the combination ARVs at a final concentration of 50µg/ml of each drug. The rate of proliferation for ARV-only treated cells was compared to the untreated control after 24 h, 48 h and 72 h using the MTT assay. Simultaneously, 0.5µg/ml, 1.0µg/ml and 2.0µg/ml ethinylestradiol (EE) or 1.0µg/ml, 2.0µg/ml and 4.0µg/ml of levonorgestrel (LNG) were administered for 24 h followed by co-administration with single and combination drugs and cell proliferation evaluated against the control after further 48 h.

2.3.0 Results

The results for the effect of contraceptive hormones on HeLa cell proliferation upon co-administration with both single and combination antiretroviral drugs are presented in this section.

2.3.1 MTT Standard curves

Figure 2.2 and Figure 2.3 show the standard curves for 24 h and 72 h MTT assays respectively.

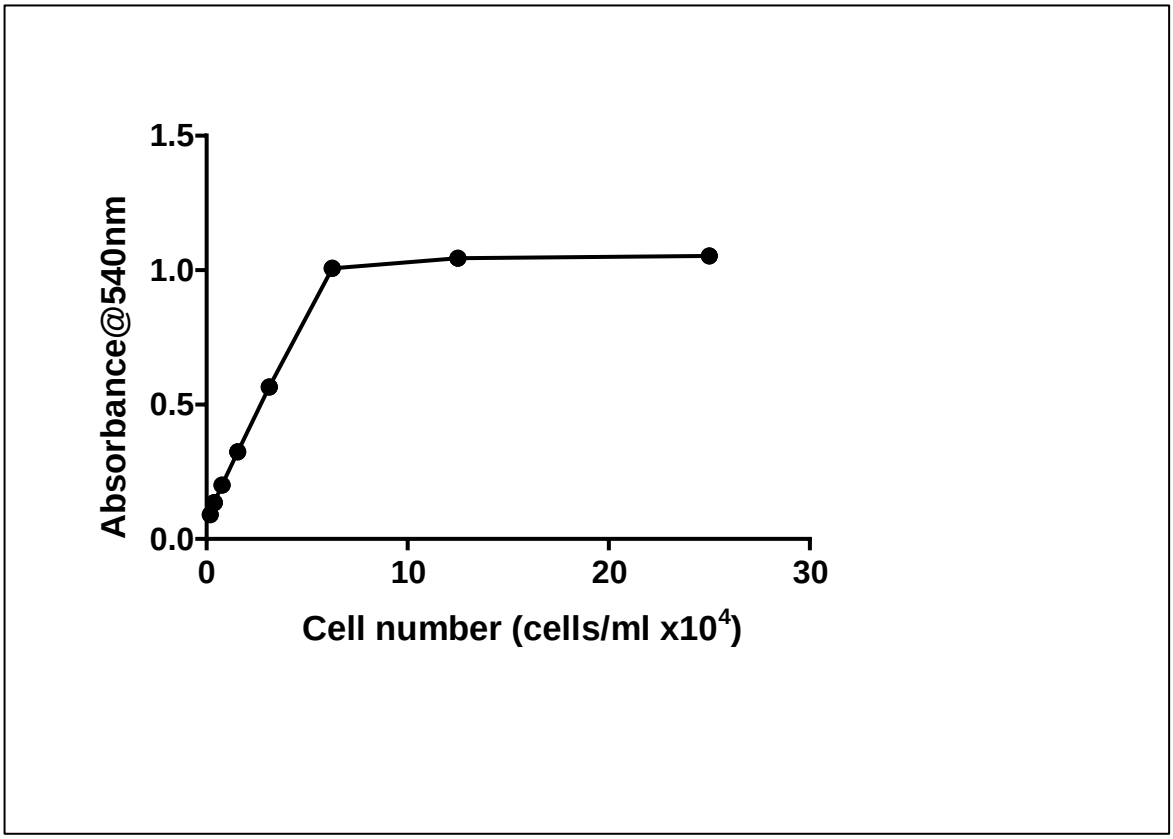

Fig. 2.2: Linearity between absorbance and HeLa cell numbers over a range of densities in 48 h. To each well was added 100µl of the given cell concentration and cells left for 24 h to allow for adherence. The medium was changed and the cells further incubated for 24 h. 10µl of MTT (5µg/ml) was added to each well and cells incubated in the dark for 2 h after which the suspension medium was discarded. The formazan (i.e. the intensely absorbent product of cellular metabolic activity in the MTT assay) in each well was extracted with 100µl of DMSO. The linearity of the MTT reduction for each cell concentration was determined using the Multiskan Spectrum® ELISA plate reader. 100µl of 5.0 x 10^4 cells per ml (5.0 x 10^3 cells per well) was preferred for further experiments as it fell within the linear portion of the plot and extrapolates to an absorbance of 0.840 on (GraphPad Prism 7.0).

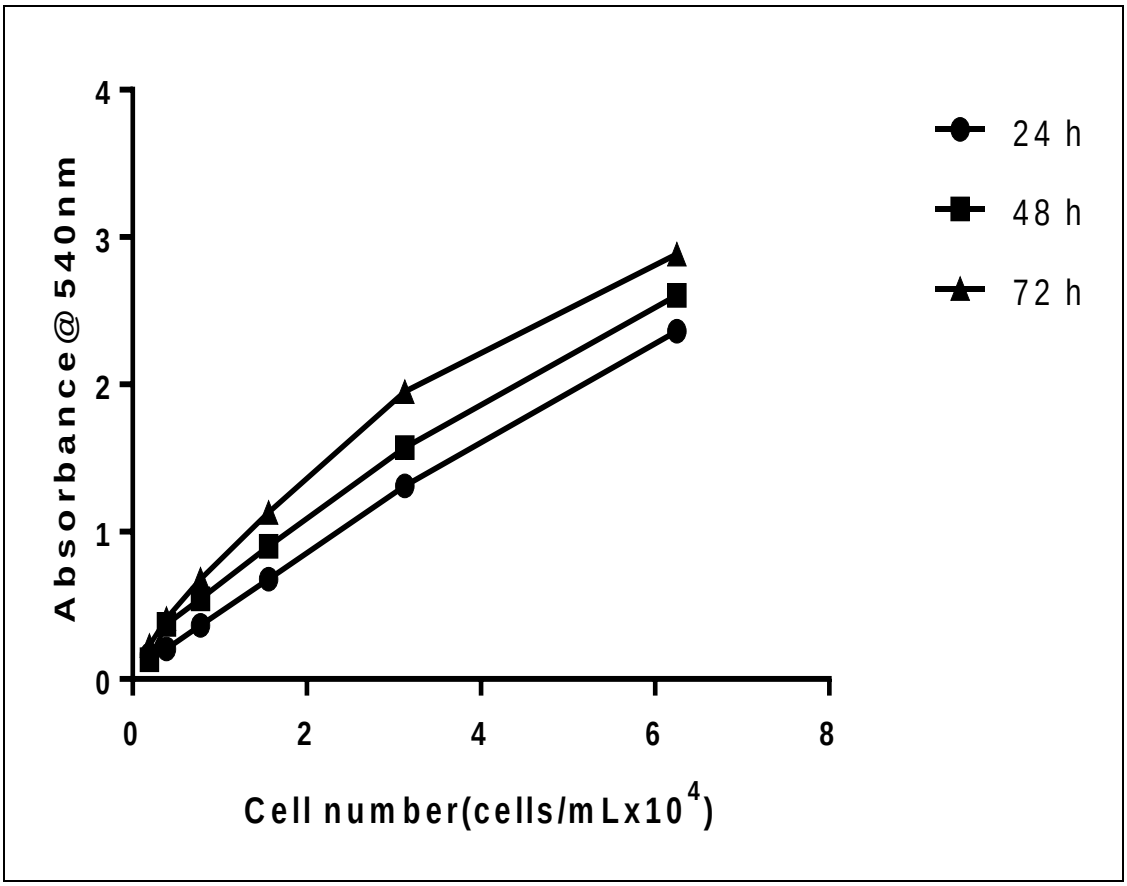

Fig. 2.3: Linearity between absorbance and HeLa cell numbers over a range of densities in 96 h. To each well was added 200µl of the given cell concentration and cells left for 24 h. The medium was changed and the cells further incubated for 24, 48 and 72 h. 20µl of MTT dye (5µg/ml) was added to each well and cells incubated in the dark for 2 h after which the suspension medium was discarded. The formazan in each well was extracted with 200µl of DMSO. The linearity of the MTT reduction for each cell concentration was determined using the Multiskan Spectrum® ELISA plate reader. 200µl of 3 x 10^4 cells per ml (6.0 x 10^3 cells per well) was preferred for further experiments as it fell within the linear portions of the plots for all time exposures.

2.3.2. 24-Hour antiproliferative effect of NRTIs and NVP on HeLa cells

The proliferative effect for each drug at given concentrations was expressed as a percentage absorbance relative to that of the untreated cells. Figure 2.4 summarises the proliferative assay results for NRTIs. With increasing antiretroviral drug concentration from 50µg/ml to 300µg/ml, the mean percentage absorbance for ABC and ZDV decreased in a concentration-dependent manner, from 82% to 37% and 84% to 47%, respectively. The absorbance for 3TC fell from an average of 80% following administration of 50µg/ml to 64% at 300µg/ml. This 3TC decrease depicts a linear concentration-dependent decrease up to 150µg/ml after which an almost static trend is observed. The trend is inconsistent for d4T-treated cells, whose absorbance decreases from 76% at 50µg/ml to 67% at 300µg/ml but in a non-linear trend. Figures 2.5(a) and 2.5(b) depict the MTT proliferation results and dose-response curves for NVP-treated HeLa cells, respectively. The computed mean relative absorbance value for formazan produced by the NVP-treated cells fell in a concentration-dependent manner, from 82% with 50µg/ml to 58% at 200µg/ml. The use of DMSO as a vehicle was shown to have no effect on HeLa cell rate of proliferation following 24 h exposure at concentrations up to 1% v/v.

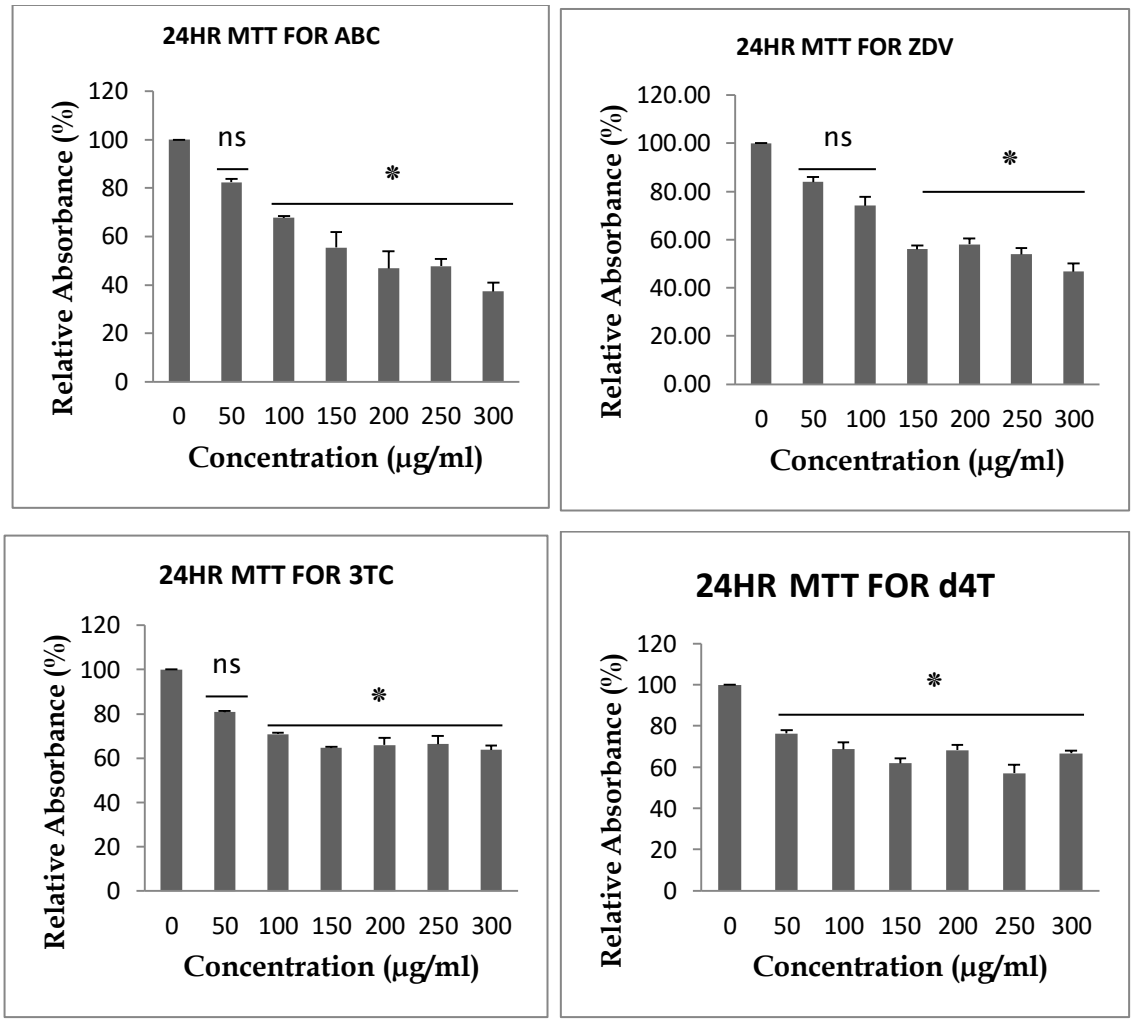

Fig 2.4: Viability of HeLa cells exposed to different concentrations of NRTIs for 24 h. Cell proliferation was determined by MTT assay. Data are expressed as the comparison of absolute absorbance values of treated cells compared to the absolute absorbance values for the untreated control whose cell survival is set to 100% (mean ± SEM, n=3 independent experiments). Data were analysed using one-way ANOVA followed by Dunnett's multi-comparison test (P≤0.05). Asterisks (*) indicate data that are significantly different compared to the control (untreated cells) while ns denotes no significant difference.

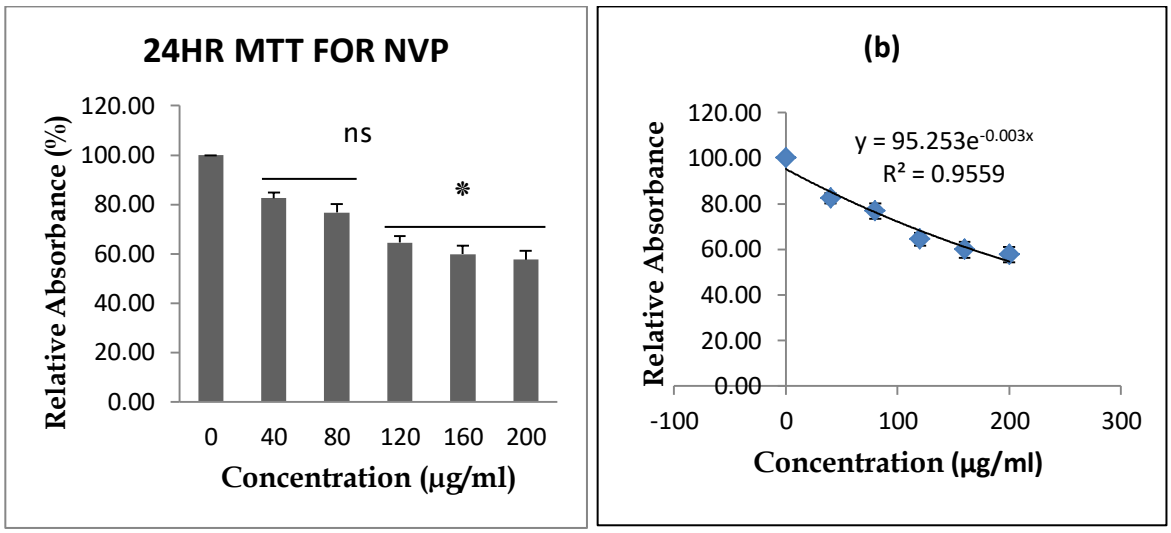

Fig 2.5: (a) Viability of HeLa treated with varying concentrations of nevirapine for 24 h. Cell proliferation was determined by MTT assay. Data are expressed as the comparison of absolute absorbance values of treated cells compared to the absolute absorbance values for the untreated control whose cell survival is set to 100% (mean ± SEM, n=3 independent experiments). Data were analysed using one-way ANOVA followed by Dunnett's multi-comparison test (P≤0.05). Asterisks (*) indicate data that are significantly different compared to the untreated control while ns denotes no significant difference. (b) Dose response curve for NVP. Drug concentration was plotted against the relative percentage of absorbance (mean ± SEM, n =3 independent experiments). The equation of this line was used to estimate the relative absorbance for 50µg/ml of NVP.

2.3.3 Determination of percentage viability at 50µg/ml drug treatment

Table 2.1 depicts percentage of absorbance relative to the control (vehicle-treated cells), following treatment with 50µg/ml of ABC, ZDV, 3TC and d4T. The relative absorbance (Abs) value for 50µg/ml NVP ([a]) was estimated from the equation of the line of best fit by calculating the value of x when y=50 (Figure 2.5b).

Table 2.1: Minimum exposure concentration data from 24-hour proliferation assays.

Treatment	ABC	ZDV	3TC	d4T	NVP
%Abs of 50µg/ml	82.4±1.5	84.0±2.1	80.8±0.6	76.4±1.5	82.0[a]

The table summarises the percentage of absorbance of cells treated with 50µg/ml of each antiretroviral drug relative to untreated control cells (% Abs of 50µg/ml ± SEM, n=3 independent experiments). ([a]) Indicates a value estimated from linear interpolation.

2.3.4 Morphological observation of HeLa cells after 24-hour ARV treatment

Photographs were captured using an EVOS® inverted microscope. Detailed microscopic pictures and change of cellular morphology with treatment at different concentrations of antiretroviral drugs is shown in Appendix 1. The major morphological changes observed with varying antiretroviral combinations are summarised in this section.

For ABC-treated cells, as the concentration increases to 50µg/ml and 100µg/ml, there is no obvious morphological change compared to untreated cells. At these concentrations, there are some dividing cells as evidenced by large nuclei. As the concentration increases to 200µg/ml and 300µg/ml, the cells become spindle- and star-shaped, respectively, while there is a decrease in cell density with increasing concentration. At 300µg/ml, some cells with protruded membrane and apoptotic bodies are observed. Cells treated with 50µg/ml and 100µg/ml of ZDV still exhibit the generic HeLa morphological characteristics. Increasing the concentration to 200µg/ml and 300µg/ml results in cells that are heterogenous in morphology. There is a gradual decrease in cell density with increasing ZDV concentration. Following treatment with 50µg/ml and 100µg/ml 3TC, the typical HeLa morphology is mantained. With 200ug/ml and 300µg/ml 3TC treatment, the cells are still viable and actively dividing with no obvious morphological changes although the cells appear less dense. With 300µg/ml 3TC treatment, some large nuclei of dividing cells are still apparent. Treatment with up to 200µg/ml d4T does not elicit obvious morphological differences relative to untreated cells regarding the nuclear/cytoplasmic ratio. Large nuclei of dividing cells are still apparent. Cells treated with 300µg/ml dT4 have large dividing

nuclei, although there seems to be a mixture of some anvil, spindle- and normal-shaped cells. At this concentration, some cells with protruded membrane and apoptotic bodies are observed. Most nevirapine-treated cells exhibit nomal morphological features up to 80µg/ml. However, from 120µg/ml to 200µg/ml the cells are increasingly becoming spindle-shaped and less dense.

HeLa cells remained able to accomplish cell division and growth. Concentrations of 50µg/ml of each antiretroviral drug were thus considered for further proliferation, apoptosis and toxicity experiments. In this 24 h treatment, all the antiretroviral drug treatments result in reduction of viability by within 20-25% compared to the untreated control. Such reduction in viability have been recommended as low-dose concentrations for toxicological assays. Although d4T significantly altered the proliferation rate in medium with phenol red, the inhibitory effect was within the 20-25% range in Phr-free medium which was used for later assays. While 50µg/ml was chosen for further assays involving all drugs, it is important to note the different molarities of each drug as shown in Table 2.2 below.

Table 2.2. Concentration of antiretroviral drugs used for further assays

Drug	MW	Conc. (µg/mL)	µmol/L
Abacavir (ABC)	286.33	50	175
Stavudine (d4T)	224.21	50	223
Lamivudine (3TC)	229.26	50	218
Zidovudine (ZDV)	267.24	50	187
Nevirapine (NVP)	266.30	50	188

2.3.5. Twenty-four hour viability effect of contraceptive hormones on HeLa cells

Figure 2.6 depicts the MTT assay results for treating HeLa cells with varying concentrations of hormones for 24 h. There is a concentration-dependent decrease in proliferation following treatment with both ethinylestradiol and levonorgestrel.

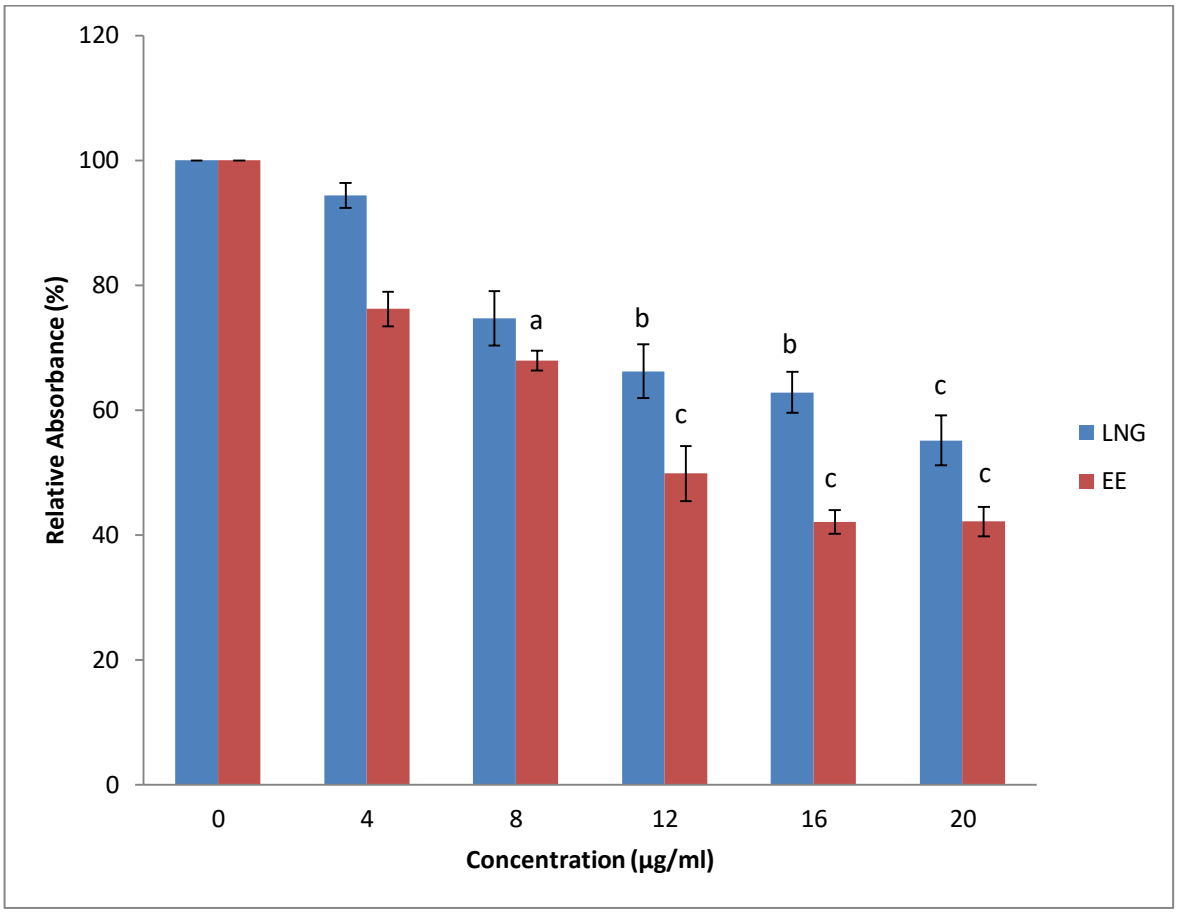

Fig 2.6: Effect of levonorgestrel (LNG) and ethinylestradiol (EE) on HeLa cell viability over 24 h. Cell viability was determined by MTT assay. Data are expressed as the percentage of absolute absorbance values of treated cells compared to the absolute absorbance values for the untreated control whose cell survival is set to 100% (mean ± SEM, n=3 independent experiments). Data were analysed using one-way ANOVA followed by Dunnett's multi-comparison test. The relative absorbance of drug-treated cells was compared to the untreated control, where a, b and c represent significant differences for P≤0.05, P≤0.01, and P≤0.001 respectively.

2.3.6 Morphology of cells treated with ethinylestradiol and levonorgestrel

Figure 2.7 and Figure 2.8 depict the appearance of HeLa cells following 24 h treatment with EE and LNG, respectively. As shown in Figure 2.7, treatment with 4.0µg/ml EE results in some cells becoming apoptotic. Further exposure to 4.0µg/ml EE for 24 h results in most of these treated cells becoming shrunk and characterised by membrane blebbing thus depicting grossly apoptotic cells (Figure 2.9). Microscopic observation of the morphology in 2.0 EE treated cells over 48 h did not show any obvious morphological transformation (Figure 2.9). Concentrations of 0.5µg/ml, 1.0µg/ml and 2.0 µg/ml EE were therefore preferably used in succeeding assays on the role of contraceptive hormones upon co-administration with antiretroviral drugs. Figure 3.7 shows no apparent morphological changes with 4.0µg/ml LNG, although further increases in concentration depict varying morphological changes. No morphological changes were observed with 4.0µg/ml LNG over 48 h of treatment hence concentrations of 1.0µg/ml, 2.0µg/ml and 4.0µg/ml LNG were used for later assays. Phenol red-free medium was preferred for these assays because other experiments involved treatment of cells in the presence of hormones (ethinylestradiol and levonorgestrol). The common practice to use phenol red-free medium, coupled with charcoal-treated foetal bovine serum in hormone-based assays, ensures controlled levels of various hormones (Wang *et al.*, 2004; Webster *et al.*, 2001).

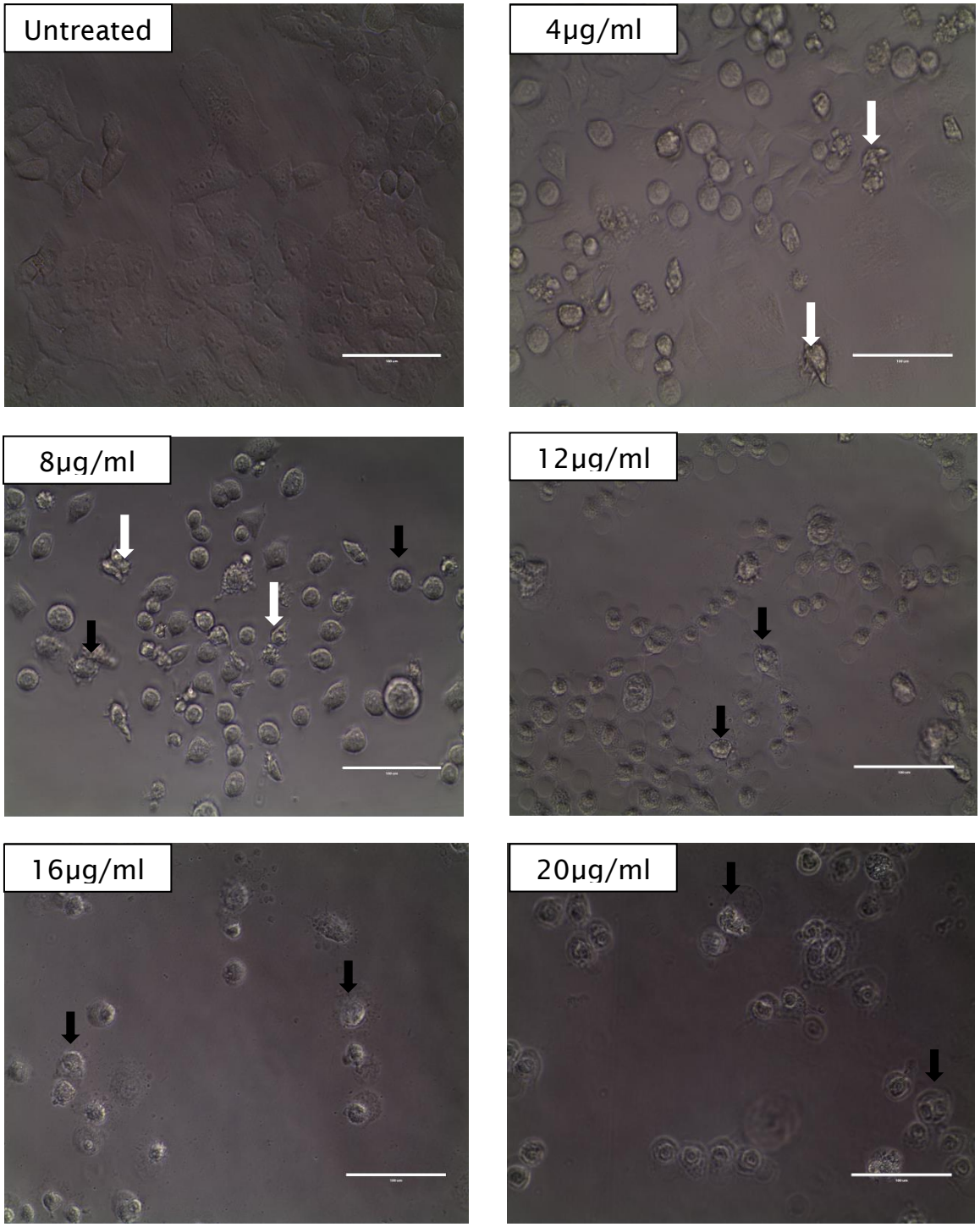

Fig 2.7: Microscopic appearance of cells after single drug treatment with ethinylestradiol (EE). At 12µg/ml, most cells have lost their polygonal structure and have an increased nuclear / cytoplasmic ratio. At 16µg/ml and 20µg/ml as cells are dying, there is increased cellular debris and most cells become detached from the cell plates. White arrows illustrate changes in cellular morphology, as typified by a spherical shape, while black arrows depict cells that have grossly lost their normal morphology and are dying. The scale bars represent 100µm.

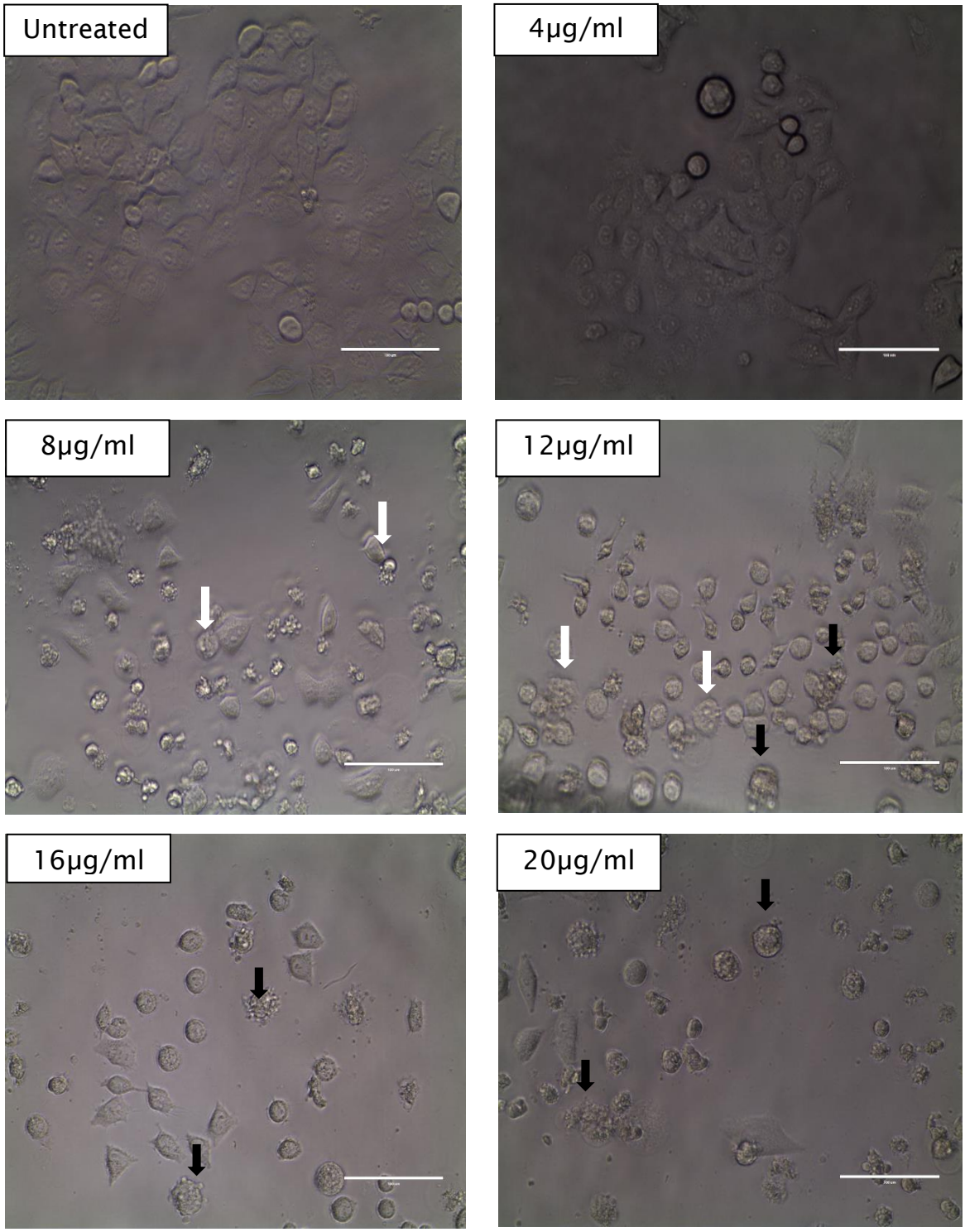

Fig 2.8: Microscopic appearance of cells after single drug treatment with levonorgestrel. At 12µg/m and 16µg/ml most cells have lost their polygonal structure and characterised by an increased nuclear cytoplasmic ratio. Although at 12µg/ml and 16µg/ml, there is increased cellular debris, there is a mixture of detached cells and some cells that are still attached to the cell plate. At 16µg/ml and 20µg/ml, most cells are dying, there is increased cellular debris and most cells become detached from the cell plates. White arrows illustrate changes in cellular morphology as the polyhedral shape is lost typified by a spherical morphology, while black arrows depict cells that have grossly lost their normal morphology and are dying. The scale bars represent 100µm.

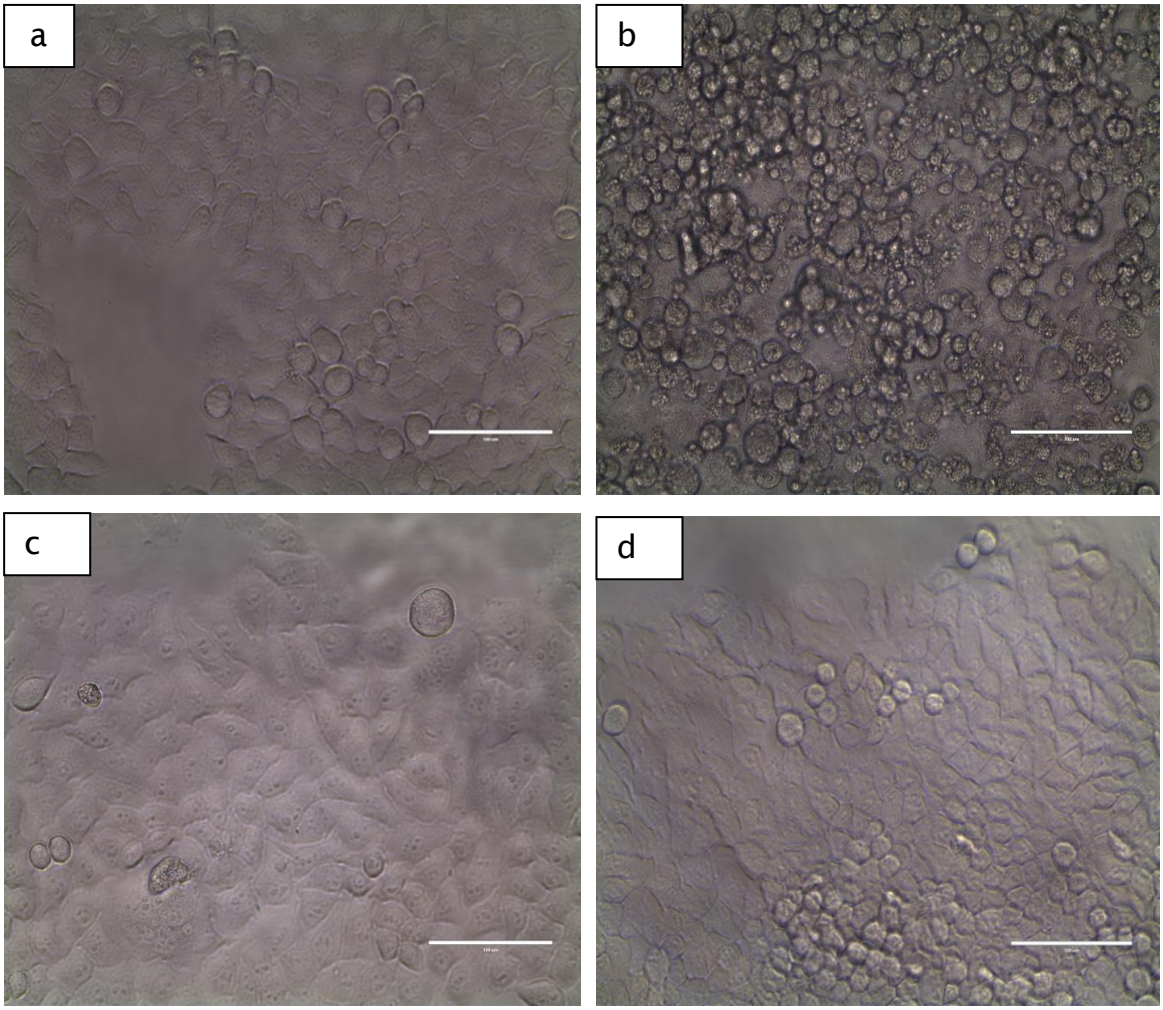

Fig 2.9: Morphological assay of 48 h hormone treatment for HeLa cells. 0.5 x 10^4 cells per well were seeded in 96-well plates and incubated overnight at 37^{o}C and 5% CO_2 for cells to adhere. The medium was removed and replaced with 100µl of untreated (control) medium (a), 4.0µg/ml ethinylestradiol (b), 2.0µg/ml ethinylestradiol (c), or 4.0µg/ml levonorgestrel (d). The cells were incubated at 37^{o}C and 5% CO_2. The plates were left for 48 h and morphological appearance captured by an EVOS® inverted microscope. Cells in a, c and d maintain their polygonal elongated morphology, while those in b showed a drastic change in morphology with most of them being spherical and characterised by loss of some visible nuclei.

2.3.7. Effect of antiretroviral drug treatment on HeLa cell viability

Figure 2.10 depicts the results from MTT viability assay for single drug treatment over 72 h. Most cells depict normal morphology for ABC-, 3TC- and d4T-treated cells, with very few cells however showing altered morphology as depicted by loss of the typical polygonal structure and membrane blebbing after 72 h of treatment. After 72 h, ZDV- and NVP-treated cells had predominantly normal morphology, although a higher proportion of atypical cells were noted compared to those treated with ABC, 3TC and d4T. Figure 2.11 shows the viability of combination antiretroviral-treated cells over 72 h. Combination antiretroviral-treated cells were predominantly polygonal although there was a general decrease in cell density with time.

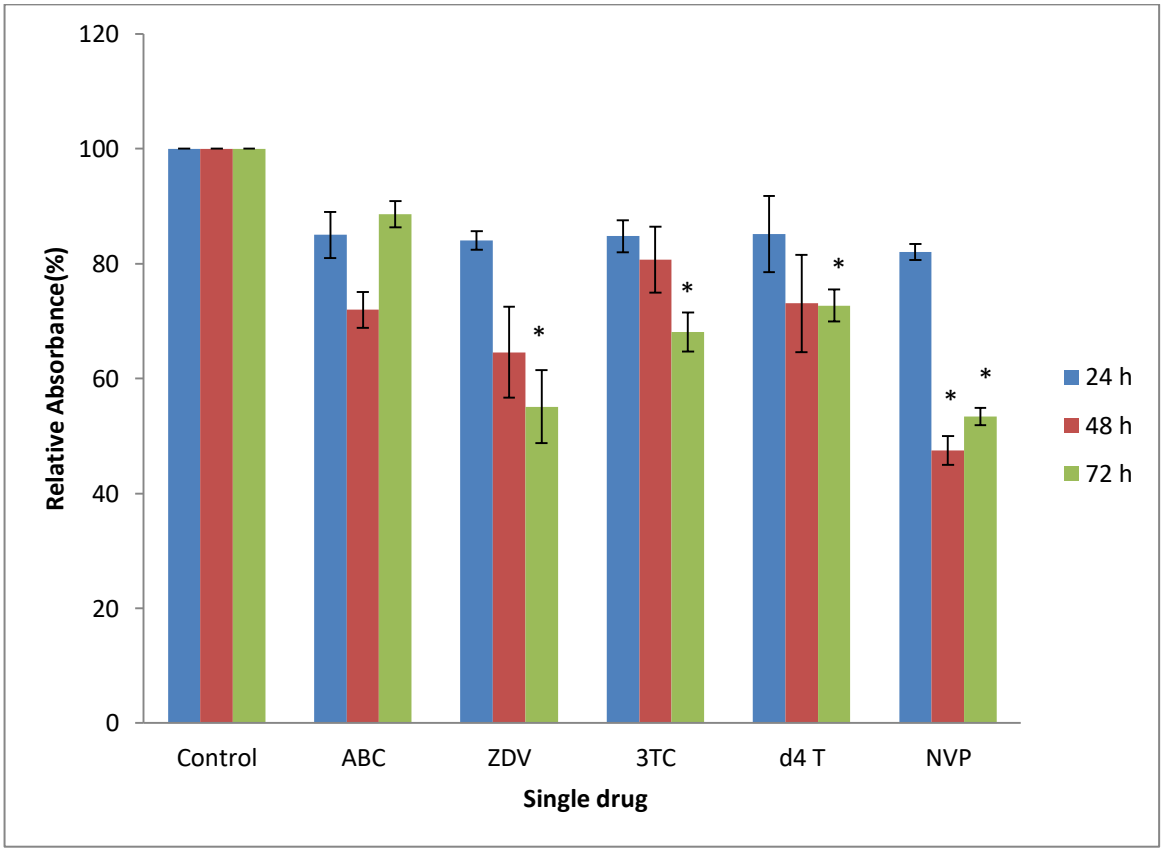

Fig 2.10: Effect of single antiretroviral treatment on HeLa cell proliferation. HeLa cells were seeded in phenol red-free DMEM for 24 h to allow attachment. The cells were treated with single antiretroviral drugs at final concentrations of 50µg/ml for 24, 48 and 72 h. Untreated cells served as the control. Significant differences among the treatments were determined by one-way ANOVA followed by Dunnett's multiple comparison test. Data are expressed as the comparison of absolute absorbance values of treated cells compared to the absolute absorbance values for the untreated control whose cell survival is set to 100% (mean ± SEM, n=3 independent experiments. Asterisks (*) indicate significant differences where $P \leq 0.05$.

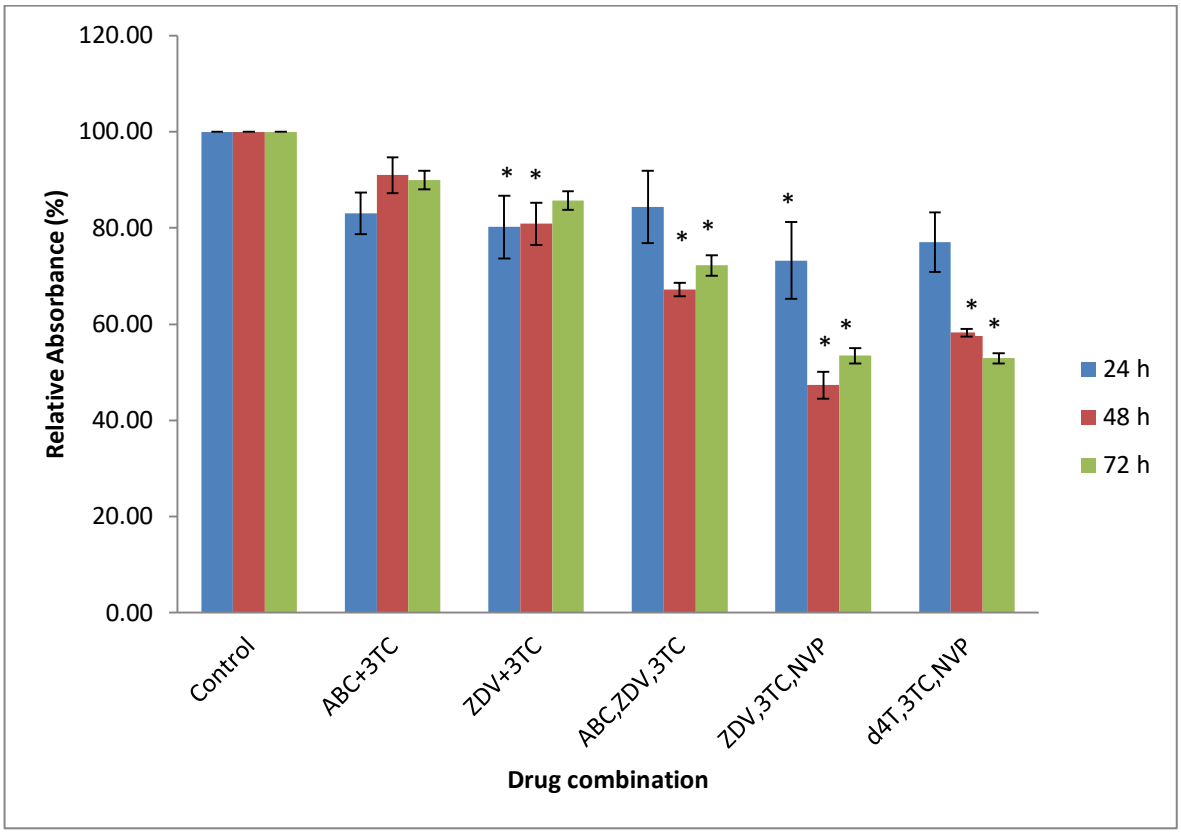

Fig 2.11: Effect of combination antiretroviral treatment on HeLa cell proliferation. HeLa cells were seeded in phenol-red free DMEM for 24 h to allow attachment. The cells were then treated for 24, 48 and 72 h with combination antiretroviral drugs at final concentrations of 50µg/ml of each drug. Untreated cells served as a control. Following each exposure time the treated cells were compared to untreated cells using one-way ANOVA followed by Dunnett's multiple-comparison test. Data are expressed as the percentage of absolute absorbance values of treated cells compared to the absolute absorbance values for the untreated control whose cell survival is set to 100% (mean ± SEM, n=3 independent experiments). Asterisks (*) represent significant differences where $P \leq 0.05$.

2.3.8 Effect of antiretroviral drug co-treatment with levonorgestrel on HeLa cell viability

Figure 2.12 depicts the viability of HeLa cells following co-administration of single antiretroviral drugs with levonorgestrel (LNG). Untreated cells (control) and those treated with 1.0µg/ml, 2.0µg/ml and 4.0µg/ml grew well with typical polygonal morphology, colour and loose chromatin over the whole 72 h treatment time. Co-treatment of ABC, ZDV and NVP with 1.0µg/ml, 2.0µg/ml and 4.0µg/ml LNG appeared normal. Co-treatment of ABC with 4.0µg/ml over 72 h results in normal morphology but darker than the control and dense at the plate periphery. ABC-, ZDV- and NVP-treated cells appeared less dense compared to the control. 3TC and d4T showed increased numbers of spherical clumped morphology upon co-administration with 4.0µg/ml LNG. Figure 2.13 demonstrates the effect of co-administering LNG with combination antiretroviral drugs on HeLa cell proliferation. While treatment with 1.0µg/ml, 2.0µg/ml and 4.0µg/ml depicted an increase cellular clumping with increasing concentration, most of the cells had polyhedral morphology.

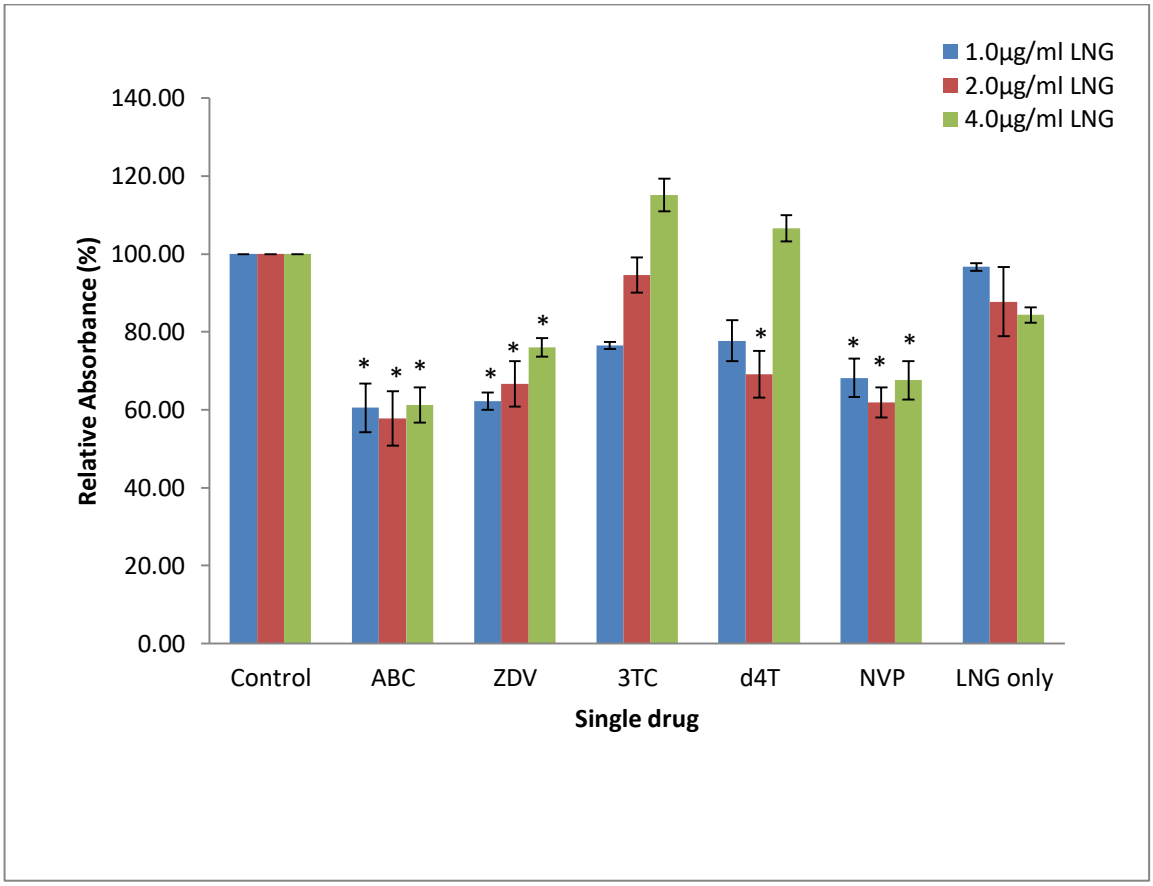

Fig 2.12: Effect of single antiretroviral drug co-treatment with levonorgestrel (LNG) on HeLa cell proliferation. HeLa cells were seeded in phenol red-free DMEM for 24 h to allow attachment. The medium was replaced with solutions of 1.0µg/ml, 2.0µg/ml and 4.0µg/ml levonorgestrel which were then incubated for 24 h. The medium was then replaced with each single drug at a final concentration of 50µg/ml co-administered with 1.0µg/ml, 2.0µg/ml and 4.0µg/ml levonorgestrel. The cells were incubated for a further 48 h. After the total exposure time (72 h), the viability of drug-treated cells with each hormone concentration co-treatment were compared to untreated cells using one-way ANOVA followed by Dunnett's multi-comparison test. Data are expressed as the percentage of absolute absorbance values of treated cells compared with the absolute absorbance values for the untreated control whose cell survival is set to 100% (mean ± SEM, n=3 independent experiments). Asterisks (*) indicate data significantly different compared to the control (P<0.05).

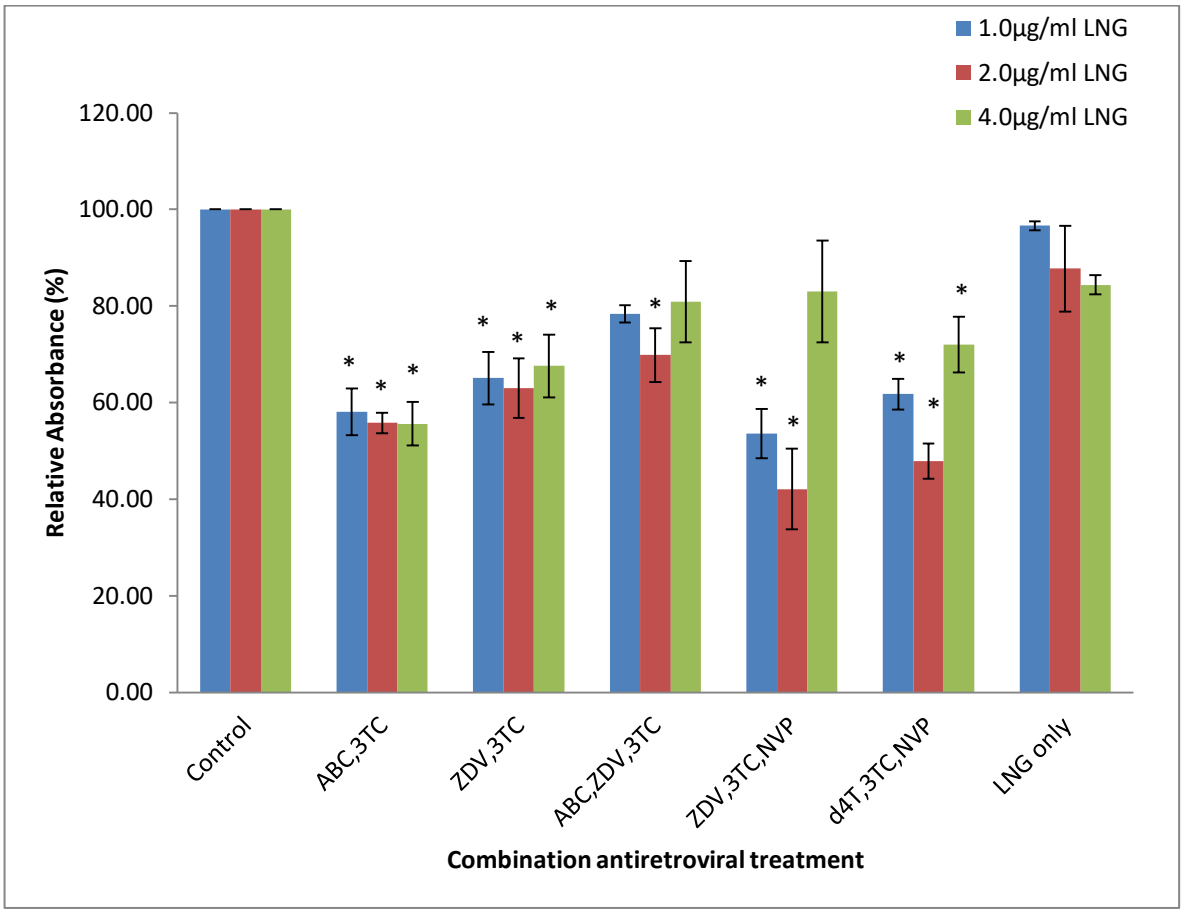

Fig 2.13: Effect of combination antiretroviral co-treatment with levonorgestrel (LNG) on HeLa cell proliferation. HeLa cells were seeded in phenol red-free DMEM for 24 h to allow attachment. The cells were pre-treated with levonorgestrel at concentrations of 1.0µg/ml, 2.0µg/ml and 4.0µg/ml for 24 h. The cells were then co-treated with combination antiretroviral drugs for a further 48 h, thus making the total exposure time 72 h. For each concentration of levonorgestrel, the relative absorbance for drug-treated cells was compared to untreated cells by one-way ANOVA followed by Dunnett's multiple comparison test. Data are expressed as the percentage of absolute absorbance values of treated cells compared to the absolute absorbance values for the untreated control whose cell survival is set to 100% (mean ± SEM, n=3 independent experiments). Asterisk (*) indicate significant difference compared to untreated cells ($P<0.05$).

2.3.9 Effect of antiretroviral co-treatment with ethinylestradiol on HeLa cell viability

The effect of single antiretroviral co-treatment with ethinylestradiol on the viability of HeLa cells over a 72 h treatment period is shown in Figure 2.14. All single drugs co-treatments with 0.5µg/ml EE showed normal morphology. With increasing EE concentration, the cell density appears to be decreasing for 3TC, ZDV and NVP. Figure 2.15 depicts the effect of combination antiretroviral co-treatment with ethinylestradiol on HeLa cell viability over a 72 h treatment period. 0.5µg/ml EE co-treatment with d4T+3TC+NVP decreases the cell density.

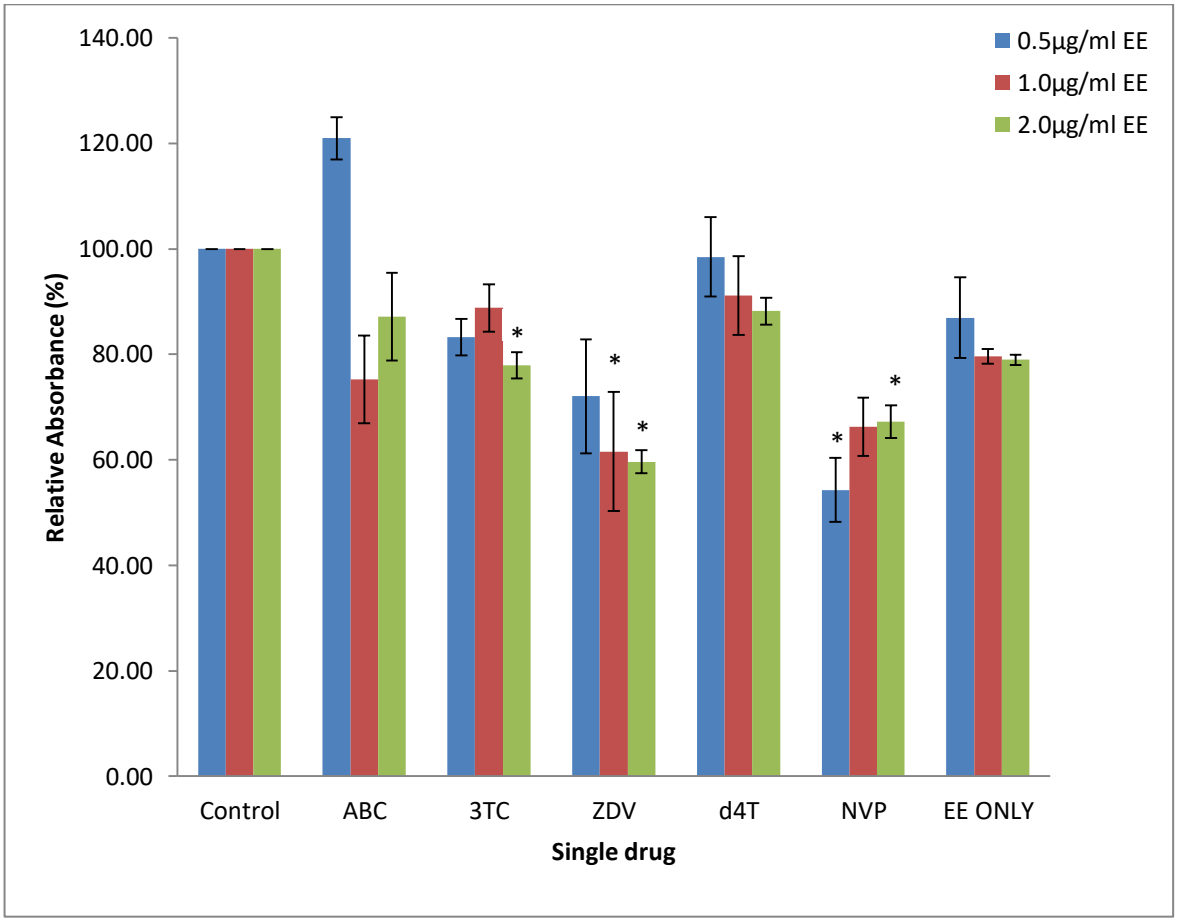

Fig 2.14: Effect of single antiretroviral co-treatment with ethinylestradiol (EE) on HeLa cell proliferation. HeLa cells were seeded in phenol red-free DMEM for 24hrs to allow attachment. The medium was replaced with 0.5µg/ml, 1.0µg/ml and 2.0µg/ml medium prepared ethinylestradiol for 24 h. The cells were then co-treated with combination antiretroviral drugs for a further 48 h. After the total exposure time (72 h), proliferation for each hormone concentration co-treatment was compared to untreated cells using one-way ANOVA followed by Dunnett's multi-comparison test. Data are expressed as the percentage of absolute absorbance values of treated cells compared to the absolute absorbance values for the untreated control whose cell survival is set to 100% (mean ± SEM, n=3 independent experiments). Asterisks (*) indicate data significantly different compared to the control (P<0.05).

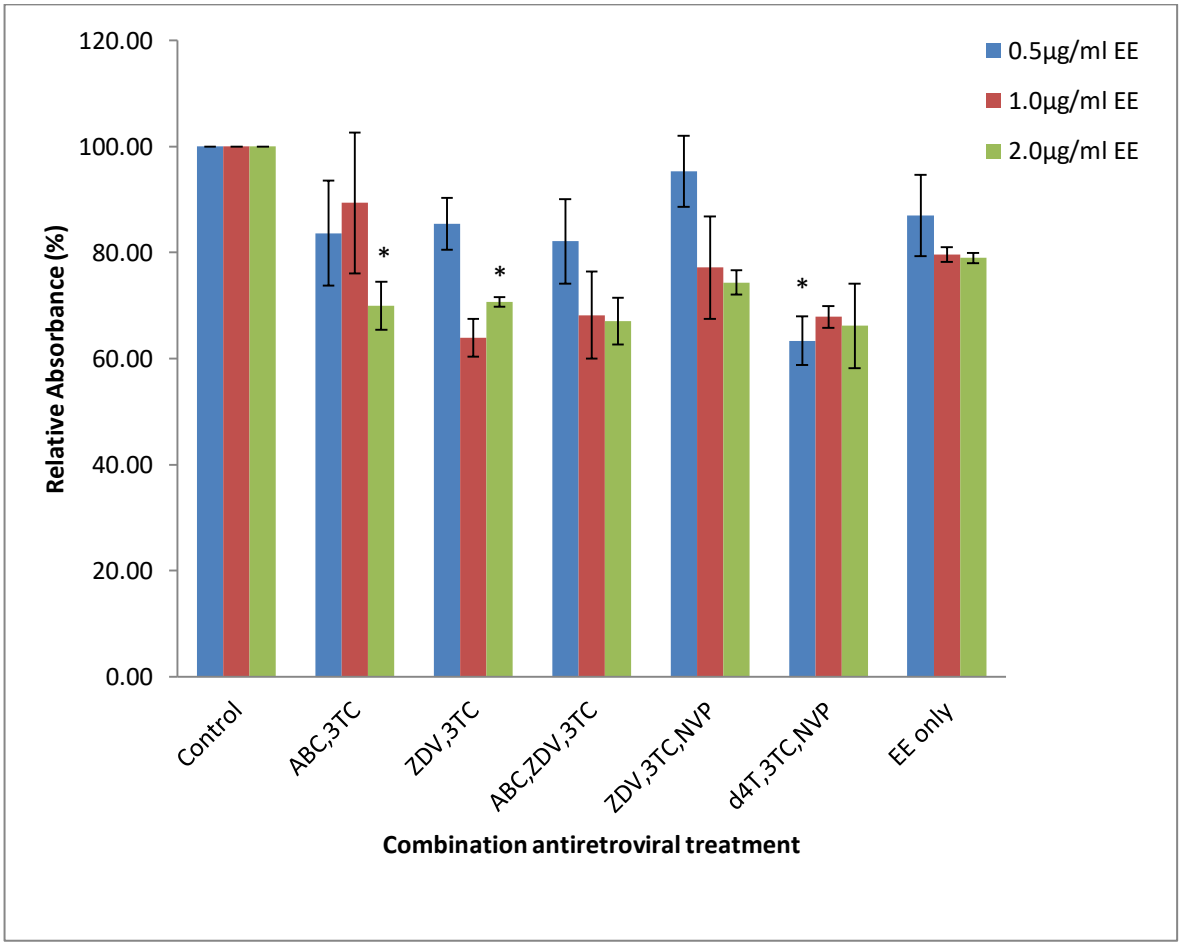

Fig 2.15: Effect of combination antiretroviral co-treatment with ethinylestradiol (EE) on HeLa cell proliferation. HeLa cells were seeded in phenol red-free DMEM for 24 h to allow attachment. The cells were pre-treated with ethinylestradiol at concentrations of 0.5µg/ml, 1.0µg/ml and 2.0µg/ml for 24 h. The cells were then co-treated with combination antiretroviral drugs for a further 48 h, thus making the total exposure time 72 h. For each concentration of ethinylestradiol, the relative absorbance for drug treated cells was compared to untreated cells by one-way ANOVA followed by Dunnett's multiple comparison tests. Data are expressed as the percentage of absolute absorbance values of treated cells compared to the absolute absorbance values for the untreated control whose cell survival is set to 100% (mean ± SEM, n=3 independent experiments). Asterisks (*) indicate data significantly different compared to the control (P<0.05).

2.4.0. Discussion

The HeLa cell is a cancerous cell line that can be used as a model of the human cervical epithelial cancer cell line. Effective therapy entails a reduction in the proliferative rate of the cancer cell. As such, it is interesting to enquire into whether or not antiretroviral drug therapy has an effect on the proliferation of the cancer cell. Furthermore, this section seeks to discuss whether or not co-administration of contraceptive hormones with highly active antiretroviral therapy affects the viability of the human cervical cell line.

Combination treatments provide a promising approach towards combating multifactorial or complex disorders (Bulusu *et al.*, 2016). The multiplicity of possible combinations and variations in individual drug concentration implies that exhaustive experimental testing is not feasible (Bulusu *et al.*, 2016., Pauzi *et al.*, 2016). The organisation of toxicity and proliferation assays, therefore, rests on the ability to predict the behaviour of drugs if administered singly, prior to examining the combinatorial effect (Pauzi *et al.*, 2016; Doudican *et al.*, 2015; Murugan *et al.*, 2016). In the present research, 50µg/ml of each nucloeside reverse trancriptase inhibitor (abacavir, zidovudine, lamivudine and stavudine) and nevirapine (a first-generation non-nucleoside reverse transcriptase inhibitor) did not impact drastically on the morphology of human cervical epithelial cancer cells during 24 h exposure, although these concentrations were cytotoxic.

The preliminary 24 h MTT proliferation assay carried out for varying concentrations was to deduce the concentrations of drugs which do not induce a drastic antiproliferative effects over 24 h. These concentrations which do not present drastic morphological transformations are ideal for toxicological and apoptosis studies (Muthusamy *et al*., 2016; Yu *et al.*, 2013). Following 24 h treatment, overall there is a concentration-dependent decrease in HeLa cell proliferation, although a static relationship was seen for cells treated with concentrations above 150µg/ml of d4T and 3TC. The results therefore show a concentration-dependent decrease in cell proliferation for ABC, ZDV and NVP-treated HeLa cells. The observed concentration-dependent decrease on treating HeLa cells with d4T and 3TC up to 150µg/ml is thus suggesting the cytostatic activity at higher concentrations.

After 24 h treatment with 50µg/ml of each antiretroviral drug in medium containing phenol red (Phr), there was no significant difference between the untreated cells and all single treatments except for 3TC. On the contrary, the 24 h treatment in Phr-devoid medium showed no significant differences for all single drug treatments characterised by relative concentrations within the $EC_{15\text{-}22}$ range for all individual treatments. This suggests the ideal

use of 50µg/ml of each drug for toxicity and apoptosis assays, since they show no drastic morphological change. The comparative differences for means observed in 24 h treatments between Phr-containing and Phr-free medium, especially with 3TC treatment, could be partially due to the oestrogenic effect of phenol red. Phr has been shown to exhibit weak oestrogenic effects in MCF-7 (Węsierska-Gądek *et al.*, 2007; Welshons *et al*., 1988) and T47D breast cancer-derived cells (Welshons *et al*., 1988). Ernst et *al.* (1989) further demonstrated that Phr exerts oestrogen-like effects *in vivo* by stimulating bone cells in mice. Such observations suggest that Phr is likely to interfere with experimental designs exploring hormone effects *in vitro*. To this end, therefore Phr-free medium is of choice in hormone-based assays (Webster *et al*., 2001; Wang *et al.*, 2004).

In the absence of hormonal contraceptive co-administration, it has been suggested that the cytostatic activity of first-generation non-nucleoside reverse transcriptase inhibitors, such as nevirapine and efavirenz, is anticancerous. Ideally NVP and ZDV (a nucleoside reverse transcriptase inhibitor) should be used in HAART combinations (BHIVA, 2008; Hecht *et al.*, 2015). In the absence of hormones, the present results concur on nevirapine and zidovudine showing antiproliferative activity in a time-dependent manner (Fig 2.10). Levonorgestrel has been shown to exhibit antiproliferative activity in a number of cancerous cells through attenuation of the apoptotic pathway (Zhao *et al*., 2015; Wildemeersch *et al*., 2015; Haoula et *al*., 2011). Co-treatment of HeLa cells with nevirapine and 1.0µg/ml, 2.0µg/ml or 4.0µg/ml levonorgestrel continues to show antiproliferative activity as there are significant differences relative to untreated cells (Fig 2.12). The same antiproliferative activity is observed for ZDV.

In vivo, abacavir (ABC) is converted into carbovir monophosphate and ultimately to carbovir triphosphate (Yuen *et al.*, 2008). While it has since been established that carbovir has potent antiretroviral activity, its toxicity limits its direct administration (McGuigan *et al.*, 2013; Yuen *et al.*, 2008). In the absence of hormones, no significant difference in cell proliferation was observed between ABC-treated cells and the untreated control. This observation concurs with McGuigan *et al.* (2013), who reported that ABC and its prodrugs do not exhibit antiproliferative activity upon lone administration. ABC treatment shows a non-significant decrease in relative percentage in cell viability upon 48 h of treatment (Fig 2.10), a situation which diminishes after 72 h of treatment. This decrease in HeLa cell viability could be due to time-dependent accumulation of carbovir, a metabolic product of ABC which is cytotoxic. The intracellular half-life of ABC ranges up to about 40 h (Yuen *et al.*, 2008; Jackson *et al.*, 2012) possibly reiterating the relative percentage decrease in

HeLa cell viability after 48 h. Co-treatment of ABC with 0.5µg/ml ethinylestradiol demonstrates an increase in HeLa cell viability with time (Fig 2.14). Co-treatment of ABC with 1.0µg/ml, 2.0µg/ml and 4.0µg/ml LNG show antiproliferative effect in HeLa cells with significant differences observed for 1.0µg/ml, 2.0µg/ml and 4.0µg/ml LNG co-treatment (Fig 2.12). This observation further concurs with antiproliferative effects of LNG on HeLa cells (Webster *et al.*, 2001). On the contrary, co-treatment of ABC with ethinylestradiol does not show significant differences (relative to the control cells) in HeLa cell viability at all exposure concentrations (0.5µg/ml, 1.0µg/ml and 2.0µg/ml), a phenomenon attributable to the established proliferative effect of EE in various cancer cells (Scaling *et al.*, 2014; Pesiri *et al.*, 2015).

Olivero *et al.* (1995) demonstrated induction of S-phase arrest in HeLa cells treated with a combination of ZDV and 3TC (a reverse transcriptase inhibitor) or those treated with ZDV alone. The dose-limiting toxicity of ZDV has been attributed to its incorporation into DNA, hence resulting in genomic instability (Olivero *et al.*, 1995). The present results show that, in the absence of contraceptive hormones, ZDV treatment resulted in a time-dependent decrease in HeLa cell proliferation over the 72 h of exposure (Fig 2.10), thus possibly implying a genomic effect as previously observed in other studies (Yu *et al.*, 2009; Olivero *et al.*, 2013). This antiproliferative effect is also evident upon ZDV co-administration with levornorgestrel, with a significant difference observed after co-treatment with 1.0µg/ml, 2.0µg/ml and 4.0µg/ml LNG. The present observations suggest that co-administration of ZDV and ethinylestradiol on HeLa cells leads to a concentration-dependent antiproliferative effect (Fig 2.14), thus demonstrating significant differences from the control with 1.0µg/ml and 2.0µg/ml EE treatment. These results are interesting, as they contradict the established proliferative effect of ethinylestradiol, thus suggesting a protective effect against cancer cell growth.

Lamivudine (3TC)-treated HeLa cells demonstrate no significant differences compared to untreated cells upon 24 h and 48 h of treatment in the absence of hormones (Fig 2.10). 72 h treatment of HeLa cells with 3TC demonstrates a significant decrease in cell proliferation. This could be a result of the gradual effect of 3TC on the S-phase arrest with increasing concentration, as discussed by Olivero *et al.* (1995). Results for 72 h co-treatment of 3TC with 1.0µg/ml, 2.0µg/ml and 4.0ug/ml levonorgestrel suggest increase in cell proliferation with increasing concentration, as indicated by the increase in relative absorbance (Fig 10.12).

In the absence of contraceptive hormones, there is a time-dependent decrease in cell viability for stavudine (d4T: a nucleoside reverse transcriptase inhibitor used in HAART regiments)-treated HeLa cells relative to untreated cells (Fig 2.10). This may suggest antiproliferative activity of d4T which concurs with observations by McGuigan *et al.* (2013) who showed that d4T had cytostatic activity against murine L1210 cells while the d4T phosphorodiamidates had anticancer activity activity against HeLa cells. In normal human mammary epithelial cell (NHMEC), d4T is associated with centromere amplification at concentrations ten-fold the plasma level following 24 h treatment (Yu *et al.*, 2009). This observation may provide a plausible explanation for the present observations, since toxicological assay concentrations higher than plasma levels were used. The World Health Organisation guidelines (2016) and British HIV Association (BHIVA), 2008) suggest a gradual withdrawal of d4T-based treatments which are still being practised in some low-resource settings, because of the perceived high d4T toxicity. The presently observed time-dependent decrease in relative absorbance after HeLa cells treatment with d4T suggests concurrence to its demonstrated anticancer properties (McGuigan *et al.*, 2013). Co-administration of levonorgestrel and stavudine to HeLa cells shows no significant difference in proliferation (Fig 2.12).

In the present section, the effect of single antiretroviral drugs on HeLa cell proliferation has been described in order to understand the mechanistic effects of contraceptive hormones on the different nucleoside reverse transcriptase inhibitors and nevirapine (a non-nucleoside reverse transcriptase inhibitor). As discussed earlier, in practice, combination antiretroviral treatment is the predominantly used intervention among HIV positive patients. Highly active antiretroviral treatment with ABC+ZDV+3TC, ZDV+3TC+NVP and d4T+3TC+NVP in the absence of hormones shows a significant time-dependent decrease in human cervical cancer cell proliferation (Figure 2.11).

Multi-drug therapy in cancer treatment has been shown to be less toxic in small combination doses while at the same time exhibiting a high therapeutic effect (Bulusu *et al.*, 2016). Pauzi *et al.* (2016) demonstrated the synergistic cytotoxic effect of bromelain and cisplastin against the MDA-MB-231 cancer cell line. Co-administration of gamitrinib and doxorubicin synergistically enhanced apoptosis in selected human prostate, kidney, cervix and lung cell lines (Park *et al.*, 2014). Whether such anticancer properties are exhibited by combination antiretroviral treatment relative to single antiretroviral drug administration has not been explored.

ZDV+3TC+NVP and d4T+3TC+NVP are highly active antiretroviral regimens that have found widespread use in most low- and medium-resource countries (WHO, 2016). In the present study, human cervical epithelial cells treated with these regimens in the absence of hormones depict the phenomenon of antiproliferative effect of combination cytotoxic or cytostatic substances, in concurrence with Park *et al.* (2014) and Pauzi *et al.* (2016). Similarly, the triple nucleoside reverse transcriptase inhibitor ABC+ZDV+3TC, which has found widespread use in low-resource settings, also depicts such a synergistic effect, as it results in a decrease in relative absorbance with time. However, this decrease is not significantly different compared to the control cells (Figure 2.11). Some epidemiological findings have associated HAART with CIN regression (Adler *et al*., 2012; Firnhaber *et al.*, 2012; Omar *et al.*, 2011; Chen *et al*., 2014; Minkoff *et al*., 2010). The presently observed decrease in HeLa cell proliferation upon ZDV+3TC+NVP and d4T+3TC+NVP administration could possibly explain these epidemiological observations suggesting cervical intraepithelial neoplasia regression on initiating HAART.

Co-treatment of ZDV+3TC+NVP and d4T+3TC+NVP (highly active antiretroviral regimens) with 2.0µg/ml LNG significantly reduces the proliferative rate of HeLa cells relative to untreated cells. On the other hand, co-administration with 4.0µg/ml LNG increases the viability of ABC+3TC+NVP and ZDV+3TC+NVP (Fig 2.13), thus suggesting the proliferative effect of LNG with increase in concentration. Co-treatment of ABC+3TC and ZDV+3TC with 1.0µg/ml, 2.0µg/ml and 4.0µg/ml LNG reduces HeLa cell proliferation, hence resulting in a significantly different proliferation rate relative to the uncontrolled cells (Fig 2.13).

Co-treatment of d4T+3TC+NVP with 0.5µg/ml ethinylestradiol shows a significant decrease in HeLa cell proliferation relative to the control cells (Fig 2.15). While lone administration of ZDV+3TC+NVP shows a significant decrease in HeLa cell proliferation (Fig 2.10), co-administration with 0.5µg/ml EE results in increased cell proliferation. The present observations therefore suggest increased proliferation with co-administration of 0.5EE in ZDV+3TC+NVP-treated HeLa cells. No significant differences in HeLa cell proliferation were observed in all treatments with combination antiretroviral treatment co-administered with 1.0µg/ml EE. 1.0µg/ml EE co-treatment with ZDV+3TC+NVP and d4T+3TC+NVP show no significant difference relative to the control cells, thus suggesting increased proliferation with 1.0µg/ml ethinylestradiol administration. Interestingly 2.0µg/ml ethinylestradiol reduced proliferation for ABC+3TC and ZDV+3TC. Treatment with 0.5µg/ml and 1.0µg/ml of ethinylestradiol demonstrates increased proliferation in

combination antiretroviral treated cells. These observations are in tandem with observations by Wang *et al.* (2004), who showed the antiapoptotic effect of oestrogen on HeLa cells, a phenomenon suggestive of cervical intraepithelial neoplasia progression and/or tumour cell proliferation.

While the antiproliferative effect is suggestive of metabolic activities that lower cell proliferation relative to untreated cells, there is need to compliment the present observations with apoptosis studies. This is because, at optimal rates, apoptosis is physiologically beneficial, while excessive apoptosis may result in pathophysiology (Dexheimer, 2013; Matt and Hofmann, 2016). Some observations have suggested that HAART causes serious non-AIDS events (SNEs) including but not limited to malignancies (Hsu *et al.*, 2013; Serrano-Villar *et al.*, 2014). It becomes imperative to investigate concurrently the effect of antiretroviral drug induced apoptosis and the effect of contraceptive hormones on human cervical epithelial cancer cell proliferation.

While the MTT assay is widely used for toxicity assays, it has its own limitations. Cells in the early stage of apoptosis may still have the ability to reduce MTT, hence possibly giving false positive results on ability to reduce the MTT (Sieuwerts *et al.*, 1995, Sylvester 2011, van Meerloo *et al*., 2011). It is thus usual to compliment proliferative assays with apoptosis assays when evaluating the effect of a treatment on cell death and proliferation. However, if time had permitted, it would have been interesting to carry more replicates of experiments to lower the standard error observed in some of the results. The present research design is premised on women continuing combined hormonal contraceptive (constituted of ethinylestradiol and levonorgestrel) use before initiating HAART. In practice, some women may initiate hormonal contraceptive use jointly with antiretroviral drugs. This may entail an alternative design typified by co-administering ethinylestradiol or levonorgestrel with antiretroviral drugs for 72 h. It is also hypothetically possible that some women will start HAART prior to hormonal contraceptive use. Such an approach warrants a design that will treat human cervical epithelial cancer cells firstly with antiretroviral drugs followed by co-treatment with levonorgestrel or ethinylestradiol. Time permitting, these alternative research designs would have been of interest.

2.4.1. Conclusion

The investigation of proliferative effects of hormonal contraceptives if co-administered with single antiretroviral drugs provided an insight into the varied mechanistic effects. Table 2.3 summarises the results for combination ARVs used in HAART treatment.

Table 2.3: Antiproliferative effect of co-administering hormonal contraceptives with combination antiretroviral drugs on HeLa cells

Treatment for drugs only	24 h	48 h	72 h
ABC+3TC	-	-	-
ZDV+3TC	+	+	-
ABC+ZDV+3TC	-	+	+
ZDV+3TC+NVP	+	++	++
d4T+3TC+NVP	-	++	++
Co-treatment with levonorgestrel	1.0µg/ml	2.0µg/ml	4.0µg/ml
ABC+3TC+LNG	++	++	++
ZDV+3TC+LNG	+	+	+
ABC+ZDV+3TC+LNG	-	+	-
ZDV+3TC+NVP+LNG	++	++	-
d4T+3TC+NVP+LNG	+	++	+
Co-treatment with ethinylestradiol	0.5µg/ml	1.0µg/ml	2.0µg/ml
ABC+3TC+EE	-	-	+
ZDV+3TC+EE	-	-	+
ABC+ZDV+3TC+EE	-	-	-
ZDV+3TC+NVP+EE	-	-	-
d4T+3TC+NVP+EE	+	-	-

HeLa cells were treated with combination antiretroviral drugs for 24, 48 and 72 h and proliferation rates compared with untreated cells. The cells were also treated with contraceptive hormones for 24 h followed by co-treatment with combination antiretroviral drugs for a further 48 h. After the total exposure time (72 h), proliferation for each hormone concentration co-treatment was compared to untreated cells. Non-significant changes are shown by (-), while (+) indicates significant changes. (++) indicates more drastic antiproliferative effect.

In the absence of hormones, there is a time-dependent decrease in HeLa cell proliferation upon treatment with ZDV+3TC+NVP and d4T+3TC+NVP. ZDV+3TC+NVP and d4T+3TC+NVP constitute part of highly active antiretroviral regimens which have found predominant use in low-resource regions including sub-Saharan Africa. The antiproliferative effect of these HAART combinations is suggestive of cytotoxicity. 1.0µg/ml, 2.0µg/ml and 4.0µg/ml LNG show antiproliferative activity upon co-treatment with ABC+3TC, ZDV+3TC and d4T+3TC+NVP. 2.0µg/ml LNG co-administration with ABC+ZDV+3TC results in decreased proliferation relative to untreated cells, thus suggesting an anticancer effect. While the reduced proliferation may suggest anticancer potential, the contrary may be true where the rate of cell death may be excessive, thereby leading to serious non-AIDS events due to induction of pathophysiological pathways. Co-administration of 0.5µg/ml ethinylestradiol with d4T+3TC+NVP demonstrates increased cell viability relative to untreated cells. Co-administration of combination antiretroviral drugs with 0.5µg/ml EE show increased cellular proliferation compared to cells treated with drug combinations only, thus suggesting a proliferative effect of EE on antiretroviral drug treated human cervical epithelial cancer cells.

Among the limitations of the MTT is the influence of test compounds that interfere with the glycolysis pathway and overestimation of metabolically active mitochondria (van Tonder *et al.*, 2015; Wang *et al.*, 2010). Early-stage apoptotic cells may be recognised as proliferating cells because they still have some ability to reduce MTT (Wang *et al.*, 2010). Further, it is important to note that cell death by apoptosis does not necessarily imply inhibition of cellular regeneration (Fan and Bergmann, 2008a; Ryoo *et al.*, 2004; Ryoo and Bergmann, 2012; Shi *et al.*, 2012). However MTT is still widely used as a preliminary indicator that there is metabolic activity taking place. It may therefore be fascinating to relate DNA damage and apoptotic cell death to HeLa viability following co-administration of antiretroviral drugs with contraceptive hormones. This may plausibly explain the relationship between cell death and cellular remodelling upon co-administration of antiretroviral drugs with either ethinylestradiol or levonorgestrel. In the next chapter, the role of nucleoside reverse transcriptase inhibitors (abacavir, zidovudine, lamivudine and stavudine) and nevirapine (a first-generation non-nucleoside reverse transcriptase inhibitor) on double-strand breaks is evaluated.

CHAPTER 3

GENOTOXICITY OF ANTIRETROVIRAL DRUGS ON CHINESE HAMSTER OVARY AND HUMAN CERVICAL CANCER EPITHELIAL CELLS

3.1. Introduction

During the cell cycle, checkpoint regulatory mechanisms ensure stability of DNA. Checkpoint failure can lead to accumulation of damaged DNA (Christmann *et al.*, 2003, Matt and Hofmann, 2016). Genotoxicity assays are important tools for determining the potential of compounds to compromise the cell's genome, ultimately resulting in health conditions such as cancer (Onwuamah *et al.*, 2014; Baumstark-Khan *et al.*, 2000). Presently, no single test is capable of detecting all possible genotoxic effects of a compound, hence the use of a battery of assays (Moreno-Villanueva *et al.*, 2011). The major limitation of most of these assays is the high percentage of false positives (Kusakabe and Tateno, 2011; Moreno-Villanueva *et al.*, 2011). This entails the need to carry out a multiple other simple but reliable tests among which the fluorometric analysis of DNA unwinding (FADU) is increasingly finding its use (Baumstark-Khan *et al*., 2000). The major advantages of this method are reproducibility, speed, lack of experimenter bias and cost effectiveness (Baumstark-Khan et *al.*, 2000; Moreno-Villanueva *et al.*, 2011).

NRTIs act by interfering with reverse transcriptase through non-competitive inhibition by attaching on the viral and host DNA (Olivero *et al.*, 2013). These drugs incorporate into nuclear and mitochondrial DNA of the host cell, thus resulting in viral DNA replication arrest and genomic instability (Olivero *et al.*, 2013). Zidovudine (ZDV) has been associated with several types of genotoxic damage through incorporation into DNA, thereby replacing thymidine (Wu *et al.*, 2013). ZDV has been shown to induce formation of micronuclei, chromosomal instability and shortening of telomeres (Wu *et al.*, 2013; Yu *et al*., 2009). Using the mouse micronucleus test, de Moraes Filho *et al.* (2016) demonstrated that the HAART regimen Combivir (ZDV+3TC) + EFV increases micronucleus frequency. Concurrently, they also demonstrated that the ratio of polychromatic erythrocyte (PCE) to normochromatic erythrocyte (NCE) was also higher for this treatment, hence suggesting that combivir + EFV is mitogenic (de Moraes Filho *et al*., 2016; Schwarzbacherová, *et al.*, 2016). Interestingly, *in vivo* studies carried out on mice with Combivir + EFV did not observe any significant DNA damage with the genotoxicity and micronucleus test (de Moraes Filho *et al.*, 2016).

Brambilla *et al.* (2012) published a review with a compendium of retrievable data on genotoxicity of 48 drugs, among which were 3TC, NVP, ZDV and ABC. Results considered met the standards of the battery of assays consisting of a gene mutation test in bacteria, an *in vitro* cytogenetic chromosomal damage test and an *in vivo* test demonstrating rodent haematopoietic cell chromosomal damage. 3TC was positive for some genotoxicity assays but negative for carcinogenicity. NVP was negative for genotoxic assays but positive for carcinogenicity assays. ZDV was positive for both genotoxic and carcinogenic assays. Similarly, Momot *et al* (2014) evaluated nucleoside excision repair in ZDV-induced chromosomal deregulation in mice and demonstrated induction of centrosome amplification as well as loss of Xpa and p53 genes. Interestingly p53 haploid insufficiency strongly protected $Xpa^{[+]}$ from ZDV-induced chromosomal amplification damage. In mice and rats, ABC was shown be carcinogenic, although no data were retrieved for genotoxicity tests (Brambilla *et al.,* 2012). Perinatal exposure to ZDV+3TC, ABC+ZDV+3TC and ZDV+3TC+NVP was shown to induce genotoxicity in newly born monkeys and such toxicity was persistent when followed up to 3 years from birth (Olivero *et al.,* 2013).

The reverse transcriptase telomerase is responsible for synthesis of DNA repeats. Studies on human HT29 cells have shown that NRTIs including ZDV, d4T and ABC inhibit telomerase, while nevirapine (a NNRTI) does not exhibit inhibitory characteristics (Hukezalie *et al.,* 2012). In Chinese hamster ovary cells (CHO), 3TC and d4T induce chromosome amplification in a concentration-dependent manner. In normal human mammary epithelial cells (NHMEC), d4T is associated with centromere amplification at concentrations ten-fold the plasma level, following 24 h treatment (Yu *et al.*, 2009). These unintended effects warrant the need for further studies on the off-target consequences of HAART (Hukezalie *et al.*, 2012). The present study thus enquires into the effect of these NRTIs and NVP on double-strand breaks in human cervical epithelial cancer cells.

Unlike nucleoside analogues, NNRTIs are not incorporated into viral DNA and they do not need to be phosphorylated in order to elicit their desired function. Rather, NNRTIs bind directly and noncompetitively to the reverse transcriptase enzyme (Barry *et al.,* 1999). Exposure of nevirapine (a non-nucleoside reverse transcriptase inhibitor of interest in this study) or zidovudine to *Allium cepa* root cells induces cytogenetic changes as shown by the mitotic index and chromosomal aberrations tests (Onwuamah *et al.,* 2014). NVP has been implicated in serious skin and liver injuries in patients undergoing antiretroviral therapeutic treatment (Kranendonk *et al.*, 2014; Madigan *et al.*, 2009). One major

metabolite of NVP, 12-hydroxy-NVP, has been shown to have sulfotransferase 1A1-dependent mutagenicity using the new SULTIA-competent Ames strains (Kranendonk *et al.,* 2014). Such observations necessitate an introspection of the potential of NVP to cause DNA strand breaks in addition to these cytogenetic and mutagenic changes.

Each of our human body cells is constantly exposed to thousands of DNA-damaging processes in a day. If uncontrolled, such damage may impair important metabolic processes, such as DNA-repair mechanisms and apoptotic removal of DNA damaged cells (Martin and Henry, 2013; Toettcher *et al.,* 2009). Common DNA damages include base and sugar alterations, single- and double-strand breaks, cross-links and base-free damages (Christmann *et al*., 2003; Dexheimer, 2013, Matt and Hofmann, 2016). Our cells have evolved inherent repair mechanisms to compensate for various types of DNA damage. Such mechanisms include base and excision repair, mismatch repair, nucleotide excision repair and double-strand break (DSB) repair (Christmann *et al.,* 2003; Dexheimer, 2013; Matt and Hofmann, 2016). DNA repair may result in removal of damaged cells by apoptosis while deregulation of DNA repair (DDR) may result in pathophysiology and disease. Examples of diseases include neurodegenerative disease, premature aging, tissue dysfunction and cancer (Matt and Hofmann, 2016). In this section, an evaluation of the effect of antiretroviral drugs on double-strand breakage in normal (CHO) and human cervical epithelial cancer cells is examined.

Double-strand breaks present one of the most hazardous types of DNA damage which often lead to cell death (Christmann, 2003; Dexheimer, 2013). Deregulation or erroneous execution of one site of DNA double-strand break repair may be sufficient to cause deletions or chromosomal aberrations resulting in cancer (Dexheimer, 2013). It is therefore intriguing to evaluate whether or not and to what extent if any do these antiretroviral drugs induce DNA strand-breaks. While DSBs repair can be attained through homologous recombination and non-homologous end joining mechanisms, it is inevitable that some cells may evade these repair mechanisms (Dexheimer, 2013; Christmann, 2003). To this effect, the invaluable role of apoptosis comes into play in programmed cell death to eliminate those cells with undesirable characteristics. The CHO epithelial cells provide a good model for non-cancerous epithelial cells while the HeLa cells have been widely used as model for human cervical epithelial cancer cells in toxicity assays (Orczyk *et al.,* 2005).

Originally designed to detect X-ray-induced DNA strand breaks, the fluorometric analysis of DNA unwinding (FADU) has since found widespread use, owing to its reproducibility

and lack of experimental bias (Birnboim, 1990; Baumstark-Khan *et al.*, 2000). FADU is now widely used for evaluating the potential of different chemicals to induce double-strand breaks in cultured cell lines, including the HeLa cells (Koczo *et al.*, 2009) and CHO cells (Baumstark-Khan *et al.*, 2000). The ability of menadione to induce strand-break in cell lines has promoted its wide as a positive control in DNA strand break assays (Koczo *et al.*, 2009). A variety of cell lysis methods, such as optical, mechanical, acoustic, electrical, and chemical lysis, are among the current techniques for single-cell lysis (Brown and Audet, 2009). In this section, the fluorometric analysis of DNA unwinding (FADU) is the method of choice for double-strand break analysis.

3.1.1. Objectives of the chapter

a. To observe the morphological changes that occur in human cervical epithelial cancer cells treated with low doses and high doses of antiretroviral drugs followed by growth in drug-free medium over a period of time.
b. To evaluate the genotoxic effect of single antiretroviral drug treatment (ABC, ZDV, 3TC, d4T and NVP) on CHO cells.
c. To evaluate the genotoxic effect of single antiretroviral drug treatment (ABC, ZDV, 3TC, d4T and NVP) on human cervical epithelial cells.

3.2.0. Materials and Methods

3.2.1. Materials

Cell culture media (DMEM without phenol red and RPMI 1640 without phenol red) were purchased from Life Technologies (United Kingdom). Antiretroviral drugs (abacavir, lamivudine, nevirapine, stavudine and zidovudine), phosphate-buffered saline (PBS), penicillin-streptomycin, sodium pyruvate, dimethylsulfoxide (DMSO), non-essential amino-acids, glutamine, charcoal-treated foetal bovine serum (FBS) were obtained from Sigma (United Kingdom). The cold trypsinisation solution (trypsin 0.2g/l, 135mM NaCl, 3mM KCl, 5mM Na_2HPO_4, 1mM KH_2PO_4, 5mM tris(hydroxymethyl)aminomethane, 0.45m*M* EDTA) was prepared in the laboratory with all reagents supplied from Sigma. Twenty-four well plates were purchased from Nunc Denmark. The CHO and HeLa cell lines were obtained curtusy of the University of East London tissue culture laboratory.

3.2.2 Cell culture and maintenance

HeLa cells were maintained as described in Chapter 2 using DMEM without phenol red. CHO cells were maintained in 75cm^2 flasks. The cells were grown in RPMI 1640 (without phenol red) supplemented with 10% charcoal-treated FBS, 1% non-essential amino acid

solution, 1% sodium pyruvate, 2mM glutamine and 1% (v/v) penicillin/streptomycin [10,000 U/ml penicillin, 10,000 µg/ml streptomycin]. The cells were incubated at 37^{o}C and 5% atmospheric CO_2. The cells were split every 3 to 4 days at approximately 75% confluence.

3.2.3 Cryopreservation of CHO cells

Cells were grown to about 75% confluence in T75 culture flasks. The cell medium was aspirated from the T75 culture flask with growing cells, the cells washed with 5ml PBS and the PBS aspirated. 2ml of trypsin/EDTA was added to cover the surface of the flask and the flask was returned to the incubator for about a minute. Trypsin was quenched by adding 8ml of medium to the flask, followed by gently pipetting up and down 4 times while washing the sides and middle part of the flask in order to harvest the highest number of cells. The cells were transferred to a 15ml centrifuge tube and spun at 1000rpm for about 5 minutes. The supernatant was removed, cells re-suspended in 1ml of fresh freezing medium and transferred to a cryotube in fresh sterile freezing medium consisting of 5% DMSO and 20% FBS in RPMI 1640. The cells were first placed at 4^{o}C for 4-6h, transferred to -80^{o}C overnight and then transferred to liquid nitrogen the next day. Upon resuscitation, up to 10 passages were used for experimental assays.

3.2.4. Effect of drug concentration on morphology of HeLa cells

In an attempt to determine the effect of antiretroviral treatment on human cervical epithelial cell morphology, 500µl of 1.0 x 10^4 cells/ml were seeded in 24-well plates and allowed to attach over 24 h. The medium was aspirated and the cells were treated with low-dose (50µg/ml) or high-dose concentrations of 300µg/ml of ABC, ZDV, 3TC and dT4. Cognisant of morphological changes observed in Chapter 2, the low- and high-dose concentrations of NVP were maintained at 50µg/ml and 200µg/ml, respectively. The reagent blank wells consisted of medium only. The vehicle control and all treatment medium were adjusted to a final concentration of 0.25% (v/v) DMSO. Drug-treated medium was replaced with fresh medium after 72 h and the cells were observed for a further 72 h thus making a total period of 144 h post treatment. The cells were treated with culture medium without ARVs for a further 288 h to assess whether the cells could accomplish normal growth. Morphological changes were followed up by an EVOS® inverted microscope (Adapted with modification from Onwuamah *et al.*, 2014).

3.2.5 Fluorimetric Analysis of DNA Unwinding

The method used is an adaptation with modification from Baumstark-Khan *et al* (2000) and Birnboim (1990). The principle of the method is dependent on the ability of some substances to introduce strand-breakage in DNA. Under alkaline conditions, the rate of DNA unwinding is dependent on the number of strand-breaks that often results from various treatments. Neutralisation of the condition by addition of acid stops the unwinding of DNA (Birnboim, 1990). Estimation of the remaining double-strand DNA is measured by using a fluorescent dye, which in the present study is Hoeschst 33258 (bisbenzamide). DNA breakages are thus inversely proportional to fluorescence. Briefly, 1000µl of $2.0x10^5$cells/ml HeLa or CHO cells were seeded in a 24-well plates and left for 48 h to allow adherence and confluence of cells. The medium was aspirated and 1000µl of working ARV solutions were reconstituted in appropriate medium (DMEM for HeLa cells and RPMI 1640 for CHO cells) to a final concentration of 50µg/ml of each drug. The cells were treated in triplicate wells. For the positive control, the cells were treated with 50µM menadione. After incubation for 1, 2 and 4 h, cells were subjected to 1.0ml cold trypsinisation (trypsine 0.2g/l, 135mM NaCl, 3mM KCl, 5mM Na_2HPO_4, 1mM KH_2PO_4, 5mM tris(hydroxymethyl)aminomethane, 0.45mM EDTA) and transferred to ice-cold Bijoux plastic tubes. CHO and HeLa cells were then treated in three groups, namely blank (B), total (T) and drug-treated (P) fluorescence (adapted from Baumstark-Khan *et al.*, 2000 and Birnboim, 1990).

3.2.5 (i) Blank fluorescence (B)

This sample measures the background fluorescence contributed by other compounds other than double-stranded DNA. This includes background fluorescence from bisbenzimide (Hoeschst 33258). This sample therefore depicts the lowest fluorescence. To determine blank fluorescence, the cell extract was sonicated under alkaline conditions in order to break all DNA and to bring about complete unwinding of the low–molecular-weight DNA fragments. 0.5ml of suspended cells were lysed by addition of 0.5ml of 0.1 Maq.NaOH. The cells were sonicated at 100W for 15 seconds and incubated at 20^oC for the whole unwinding period (30 min) and subsequently neutralized by addition of 0.5ml of 1 Maq. HCl and resonicated (After: Baumstark-Khan *et al.*, 2000 and Birnboim, 1990).

3.2.5 (ii) Total Fluorescence (T)

The T sample depicts the total double-strand DNA in untreated cells. The cell extract was sonicated under neutral conditions to prevent unwinding of the DNA. The difference T minus B (T- B) provides an estimate of the amount of double-stranded DNA (dsDNA) in untreated cell extracts. Suspended cells (0.5 ml) of the T-samples were lysed by addition of 0.5 ml of 0.1 Maq. NaOH immediately followed by neutralization through addition of 0.5 ml 1 M hydrochloric acid. The subsequent addition of acid therefore prevents DNA unwinding in these samples. The cells were then incubated at 20^{o}C for the whole unwinding period (30min) and sonicated (100W, 15s) (After: Baumstark-Khan *et al.,* 2000 and Birnboim, 1990).

3.2.5 (iii) Flourescence for drug treated cells (P)

The third sample set (P) is used to estimate the unwinding rate of drug-treated DNA. The cells were exposed to alkaline conditions that bring about partial unwinding of DNA that is dependent on the extent of breakage. Suspended cells (0.5ml) of the test samples were lysed by gentle addition of 0.5ml of 0.1 aq. NaOH without mixing, followed by incubation at 20^{o}C for 30 minutes. During this unwinding period, the alkali diffuses into the viscous lysate to give a final pH around 12.4. The tubes were shielded from light and vibrations. After DNA unwinding, the P-samples were neutralized by addition of 0.5ml of 1.0 Maq. HCl (pH 7.1–7.4). The mixture was sonicated to render the samples homogeneous, thus minimizing renaturation of DNA and reducing the fraction of DNA molecules containing both single- and double-stranded regions (After: Baumstark-Khan *et al.,* 2000 and Birnboim, 1990).

3.2.5 (iv) Measurement of double-stranded DNA (dsDNA)

500µl of fluorochrome-containing buffer (5.0 x10^{-6} M bisbenzimide in PBS) was added to all samples. This fluorochrome dye stains the cells to determine the fraction of DNA breakage. After mixing well, the relative fluorescence intensities of the samples were read by using a spectrofluorimeter operating at 350nm (excitation) and 455nm (emission). The fraction of ds-DNA (F) was calculated as follows: F = (P - B)/ (T - B) where:

(P-B) = Fluorescence of treated sample

(T-B) = Fluorescence of untreated sample (After: Baumstark-Khan *et al.,* 2000).

3.2.6 Data Analysis

Differences between the control (untreated cells) and each of the treatments were analysed by ANOVA (GraphPad Prism 7.0) followed by Dunnett's multiple comparison test ($P<0.05$).

3.3. Results

3.3.1. Morphology of drug-treated cells

By the end of 144 h, untreated cells grew well in a monolayer, while cells treated with 50µg/ml of each drug showed no morphological differences compared to control cells. After this initial 144 h treatment, control and low-dose treated cells maintained a polygonal morphology. Interestingly, high-dose treatment of cells with NVP, ZDV and ABC resulted in growth with some clumped cells (Figure 3.1). High-dose NVP-treated cells depicted a number of cells growing in clumps, with some becoming shrunken and spherical, while ABC and ZDV had fewer clusters. High-dose cells treated with 3TC and d4T did not show apparent morphological changes, although they appeared less dense than all other treatments. After further 288 h of treatment, control cells and those treated with low-dose concentrations (50µg/ml) accomplished normal morphology. Interestingly, for untreated and low-dose treated cells, as space became limiting, the cells were observed to be growing in layers characterised by a low degree of clumping. After the total exposure time, those HeLa cells previously treated with higher doses of ZDV, NVP and ABC continued to show darker nuclei, clumping and some cells with spherical morphology as space became limiting.

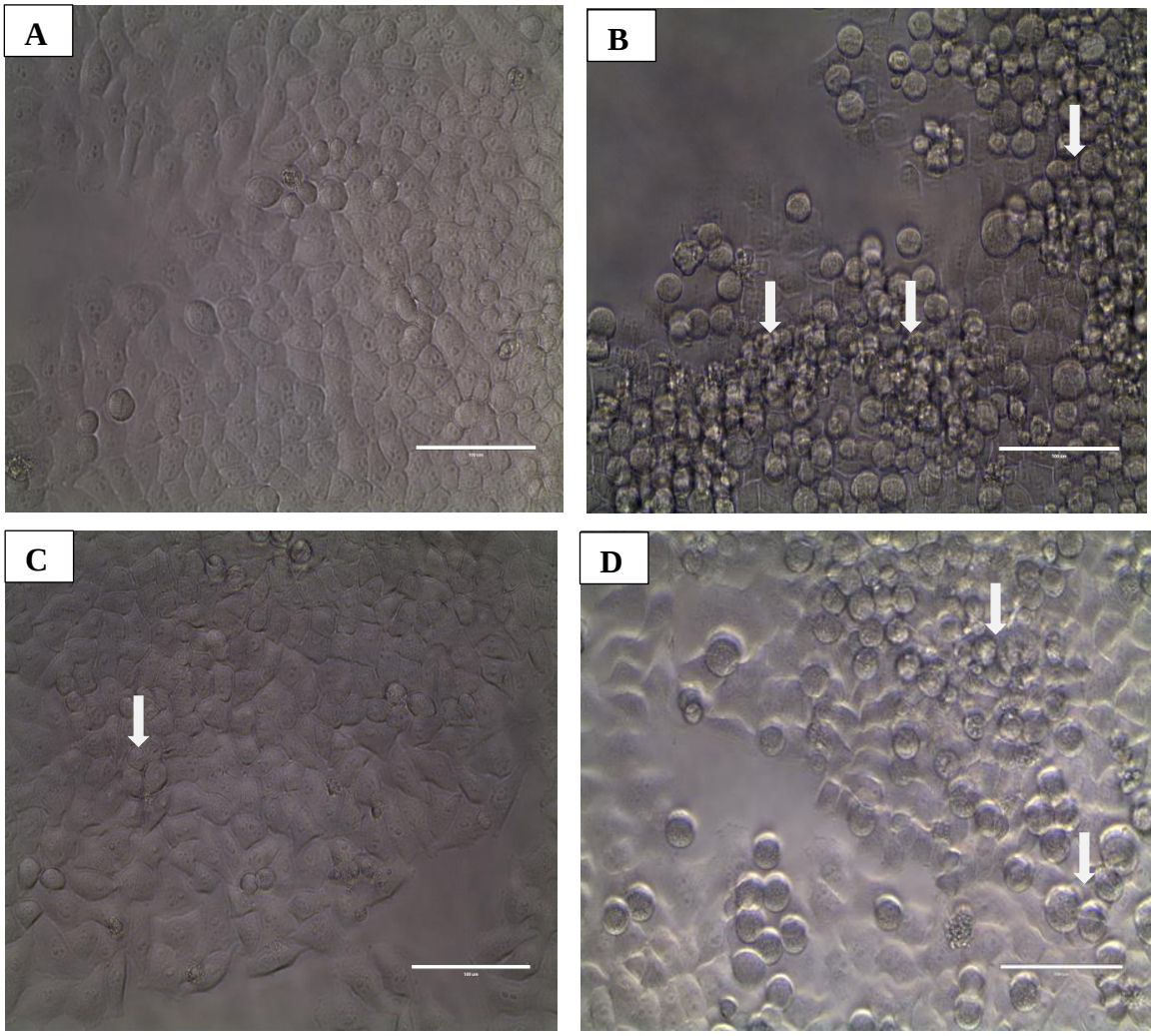

Fig 3.1: Morphological changes observed in HeLa cells treated with antiretroviral drugs for 72 h followed by culturing in drug-free medium for a further 72 h. Cells were observed under an inverted microscope scale bars represent 100µm. (A) Untreated control (B) 200µg/ml NVP (C) 250µg/ml ZDV (D) 250µg/ml ABC. Arrows indicate regions in which cells appear denser than normal. The scale bars on the right side represents 100µm.

3.3.2 FADU results for antiretroviral treatment of CHO and HeLa cells

The cells were treated with 50μg/ml of each antiretroviral drug for 1 h, 2 h, and 4 h followed by FADU analysis. The decrease in fluorescence intensity with increasing exposure time to all nucleoside reverse transcriptase inhibitors and nevirapine indicates time-dependency in DNA strand-breaks in both the non-cancerous cell line (CHO) and the human cervical epithelial cancer cells (HeLa) as shown in Figure 3.2 and Figure 3.3, respectively.

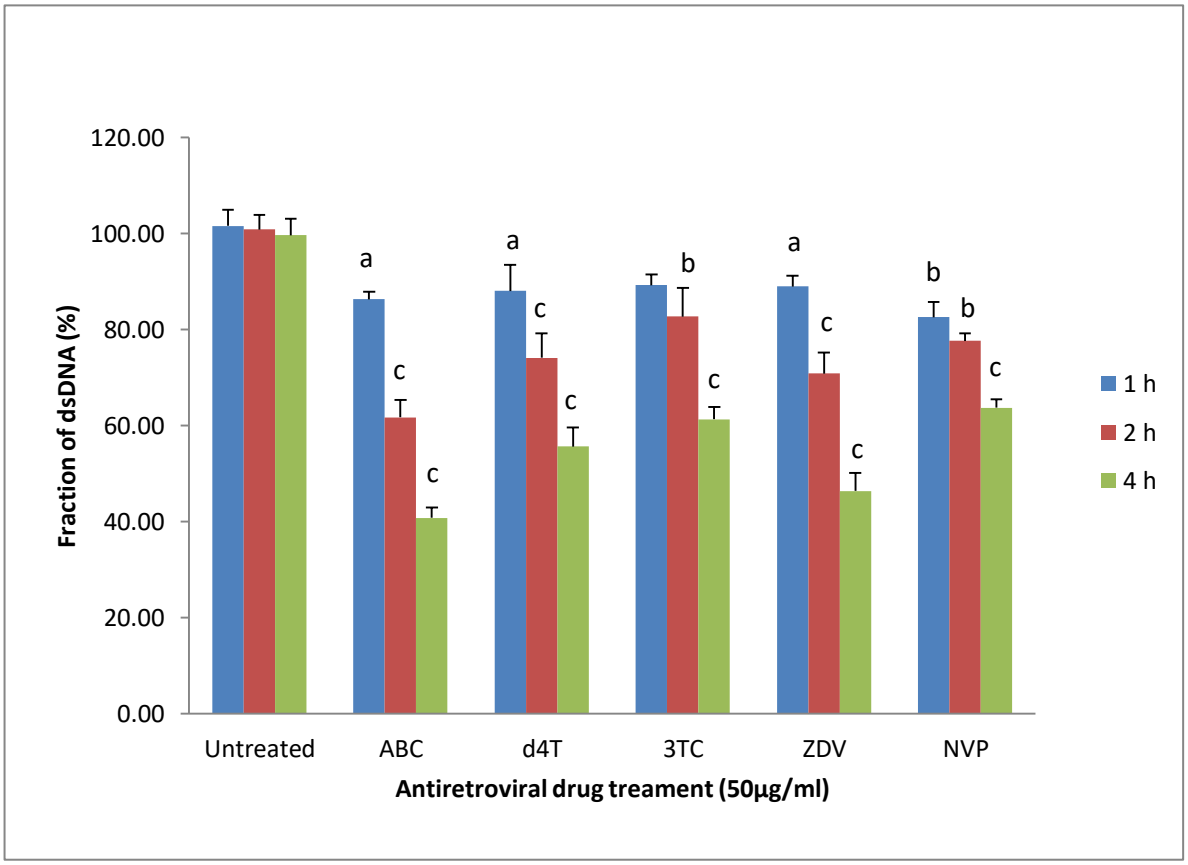

Fig 3.2: DNA strand-breaks induced by antiretroviral drugs in CHO cells. Cells were seeded and allowed to grow to confluence over 48 h. The cells were treated with 50µg/ml of each antiretroviral drug for 1 h, 2 h, and 4 h, followed by FADU analysis. There is a time-dependent DNA double-strand break in both cell lines. Data were analysed by ANOVA followed by Dunnett's multiple comparison test. Data represent mean ±SEM, n=3. The relative fluorescence of drug-treated cells was compared to the untreated cells, where a, b and c represent significant differences for $P < 0.05$, $P < 0.01$ and $P < 0.0001$ respectively.

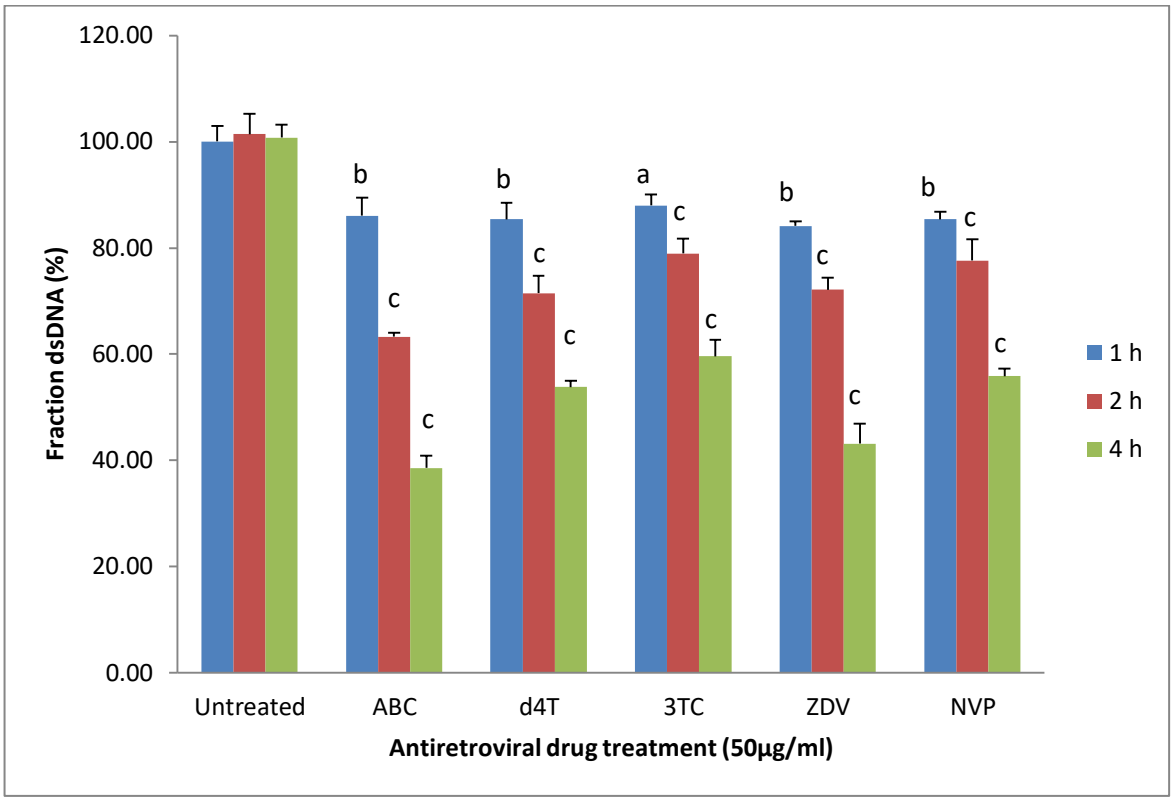

Fig 3.3: DNA strand-breaks induced by antiretroviral drugs in HeLa cells. Cells were seeded and allowed to grow to confluence over 48 h. The cells were treated with 50µg/ml of each antiretroviral drug for 1 h, 2 h, and 4 h, followed by FADU analysis. There is a time-dependent DNA double-strand break in both cell lines. Data were analysed by ANOVA followed by Dunnett's multiple comparison test. Data represent mean ±SEM, n=3. The relative fluorescence of drug-treated cells was compared to the untreated cells, where a, b and c represent significant differences for $P < 0.05$, $P < 0.01$ and $P < 0.0001$ respectively.

3.4. Discussion

A time-dependent effect of ABC, ZDV, 3TC and dT4 on double-strand breakage was observed in both Chinese hamster ovary epithelial cells and human cervical cancer epithelial cells. Treatment of cells with fresh medium for 50µg/ml of each drug treatment, followed by fresh medium, resulted in cell growth with no obvious morphological changes. At lower concentrations, no morphological differences were observed between treated cells and the untreated control for all drug treatments. This observation suggests that, although treatments of HeLa cells with 50µg/ml ABC, d4T, 3TC, ZDV and NVP induce a time-dependent double-strand DNA breakage in HeLa cells, the cells are able to accomplish cell division and growth with time. Whether or not the DNA-damaged cells are removed by apoptosis is the subject of the next chapter. However, the presently observed normal morphology with low-dose treatments is suggestive of high DNA-repair efficiency. As Dexheimer (2013) puts it, although our cells are constantly exposed to detrimental endogenous and exogenous agents, DNA-damage repair mechanisms help cells to remove different damaged cells. Alternatively or concurrently where cells have not been repaired, apoptosis (a phenomenon to be investigated in the next chapter) is of paramount importance in systematically eliminating damaged cells to ensure that they do no express undesirable characteristics such as oncogenes.

Using cell-culture techniques, it would have been interesting to carry out analysis on the formation of foci based on the BALB/c 3TC transformation assay. To this effect, the BALB/c 3T3 may prove useful and fascinating because this test is currently accepted as the only *in vitro* model used as an alternative to animal models for carcinogenesis testing (Collacci *et al.,* 2011). This has proved effective in screening substances with no apparent genotoxic evidence but presenting alerts for carcinogenicity (Colacci *et al*., 2011, Maeshima *et al*., 2009; Sasaki *et al*., 2012).

Since NRTIs are incorporated into mitochondrial and nuclear DNA of the host cell, these have a higher potential to cause genomic instability (Olivero *et al.,* 2013). The present observations concur with that phenomenon. While NRTIs lead to DSBs, nevirapine, which acts by non-competitive inhibition, also results in significant DSBs in both the CHO and HeLa cells. This observation resonates well with *in vitro* studies which showed mutagenic changes and chromosomal aberrations in *Allium cepa* cells treated with NVP (Onwuamah *et al.*, 2014). 12-Hydroxy-NVP, a nevirapine metabolite, has also been demonstrated to be mutagenic with the new SULTIA-competent Ames strains (Kranendonk *et al.*, 2014). On

the contrary, *in vivo* studies carried out on mice demonstrate that NVP did not cause any significant DNA damage in genotoxicity and the micronucleus test (de Moraes Filho *et al.*, 2016). Interestingly, NVP did not show inhibitory characteristics in HT29 cells, while the NRTIs (ABC, ZDV and d4T) inhibited human telomerase which is responsible for synthesis of DNA repeats (Hakuzalie *et al.,* 2012). Such inhibition interferes with repair of the damaged DNA. Such a lack of inhibition of the telomerase in nevirapine-treated cells is suggestive of an efficient DNA-repair mechanism which is necessary to counter deleterious effects of DSBs. Although ABC was shown be carcinogenic but with no data on genotoxicity (Brambilla *et al.,* 2012), the present results show the potential of ABC to induce DNA strand-breaks. In addition, 3TC, which was shown to be positive for some genotoxicity assays but negative for carcinogenicity (Brambilla *et al*., 2012). The present study has further shown that 3TC is able to induce DSBs in both CHO and HeLa cells.

Malignancy may be defined as the permanent transition of a tissue from normal to one which divides and proliferates limitlessly, thereby injuring adjacent cells (Stoddart, 1983). Carcinogenesis occurs through three main stages namely initiation, promotion and progression (S$_{Ø}$dring *et al.,* 2016; Stoddart, 1983; Vincent and Gatenby, 2008). The tumour microenvironment is defined by a heterogenous mass of cells and their extracellular environment to which the cells contribute (S$_{Ø}$dring *et al.,* 2016). Cultured cells are thus not equivalent to *in vivo* defined cells. In the present study, the tissue culture models used provide a primary insight into the plausible mechanism through which ARVs induce dsDNA damage. However, *in vivo* studies may be of interest in a bid to qualify the present observations.

ARVs have been shown to induce DSBs in HeLa cells. This preliminary evidence suggests the transforming potential of these drugs. Interestingly, it has since been established that transforming agents may be necessary but not always sufficient to cause malignancy (Stoddart, 1983; S$_{Ø}$dring *et al.,* 2016). Under normal conditions, cells have evolved a highly efficient mechanism for DNA repair, a phenonomenon known as DNA damage response (DDR) (Vincent and Gatenby, 2008; Dexheimer, 2013; Matt and Hofmann, 2016). While the present results depict DSBs on CHO and HeLa cells treated with NRTIs (ABC, ZDV, 3TC and d4T) of interest is whether the repair efficiency prevents the proliferation of mutational cells. Time permitting, it would have been interesting to carry out BALB/c 3TC assays to assess formation of foci and possibly the initiation/promotion potential of ARVs. Similarly, should the damaged cells be removed

by apoptosis, the process should be optimal to prevent excessive loss of cells and tissue damage. Following treatment with 50µg/ml of each antiretroviral drug, CHO and HeLa cells maintained normal morphology. This is suggestive of a mechanism which removes damaged cells and/or a high DNA-repair efficiency.

Initiators are substances that cause primary injury to the DNA (Vincent and Gatenby, 2008 Stoddart, 1983). In the present study, there is evidence that NRTIs have the potential to cause DNA damage, hence they may possibly present as initiators. It has since been established that substances that may not be carcinogenic may act as promoters for proliferation of the damaged cells. Promotion requires agents that enhance cell division. Promoters thus accelerate transformation to a malignant genotype (Stoddart, 1983; S$_{Ø}$dring *et al.*, 2016). While NRTIs have been shown to cause DSBs, whether or not other commonly used drugs such as hormonal contraceptives impact on the extent of DNA damage remains obscure. The family of simple RNA viruses to which HIV belongs have been implicated in oncogenesis (Stoddart, 1983). Viral oncogenes have been shown to be closely associated with human malignant disease and it has been established that HPV types 16 and 18 play a role in cervical epithelial cell oncogenesis (Thierry, 2009; zur Hausen, 1999). Whether or not HIV acts as a promoter of carcinogenesis in human cervical epithelial cells treated with NRTIs is beyond the scope of the present research but could also prove fascinating. This is cognate to the currently observed increase in AIDS defining cancers. Once promoted, the cells develop malignant characteristics that enable indefinite multiplication and this stage is referred to as progression (S$_{Ø}$dring *et al.*, 2016; Stoddart, 1983; Vincent and Gatenby, 2008). Since the present study is limited to cell culture experiments, such an inference into progressive malignancy is unattainable.

In separate studies described earlier, the observed genotoxic effect of ZDV lone administration (Olivero *et al.*, 2013; Yu et al., 2009) is of interest relative to the non-genotoxic observations for Combivar + EFV observed with *in vivo* studies (de Moraes Filho *et al.*, 2016). This particularly raises a question as to whether combination antiretroviral drugs reduce genotoxicity, a phenomenon to be explored in the next chapter. While there are other programmed cell death mechanisms such as necroptosis and autophagy, apoptosis is the predominant method of programmed cell death including removal of DNA damaged cells (Dexheimer, 2013; Matt and Hofmann, 2016). In the next chapter, this study therefore evaluates the extent of apoptosis in ARV-treated human cervical epithelial cells and the effect of co-treatment with contraceptive hormones. This is

cognisant of WHO guidelines that empower women on antiretroviral treatment to have the right choice of contraceptive.

3.4.1. Conclusion

Nucleotide/nucleoside reverse transcriptase inhibitors (NRTIs) act by incorporation into the viral DNA and thereby acting as chain terminators (Flepp *et al.,* 2001). The present results demonstrate that ABC, 3TC, ZDV and d4T, which fall into this class, have a potential to induce time-dependent DNA double-strand breaks. NVP, a non-nucleoside reverse transcriptase inhibitor, also induces a time-dependent DNA-strand breakage. The present results show that treatment of HeLa cells with 50μg/ml of each drug followed by culture in medium does not show obvious morphological differences compared to untreated cells. While human cells have developed specific DNA-repair systems, some cells evade these repair mechanism (Dexheimer, 2013; Matt and Hofmann, 2016). The present observations suggest that, at 50μg/ml of each drug, the DNA damage is subsequently repaired in these cells, thus enabling them to maintain normal morphology. Whether or not there are detrimental effects of this ARV treatment with time requires further analysis of cell death *via* apoptosis, a phenomenon explored in the next chapter.

CHAPTER 4

EFFECT OF CONTRACEPTIVE HORMONES ON ANTIRETROVIRAL DRUG INDUCED APOPTOSIS IN HUMAN CERVICAL CANCER EPITHELIAL CELLS

4.1. Introduction

Apoptosis is a mechanism of programmed cell death characterised by morphological and biochemical transformations that enable apoptotic cell removal by phagocytic cells (Martin and Henry, 2013). To this effect, cell removal from the tissues is in an orderly manner without disruption and leakage of cellular contents (Logue *et al*., 2009; Martin and Henry, 2013). This ensures that there is no inflammation triggered through release of immune-activating alarmins (Martin and Henry, 2013; Henry *et al.*, 2013). One major physiological change associated with cells undergoing apoptosis is transposition of phosphatidylserine (PS) from the inner to the outer surfaces of the plasma membrane (Logue *et al*., 2009; Brummatti *et al*., 2008). Pro-apoptotic signals stimulate this transposition through the aminophospholipid cation translocase *via* a calcium-dependent pathway (Logue *et al*., 2009; Matt and Hofmann, 2016). Other changes that occur during apoptosis include cell shrinkage, cleavage of chromosomal DNA into fragments, membrane blebbing, loss of electrical potential across the inner membrane of the mitochondria and transposition of cytochrome c from mitochondrial intermembrane space to the cytosol (Martin and Henry, 2013; Brummatti *et al*., 2008). Initiator caspases in apoptosis are caspases 2, 8, 9 and 10 while executioner caspases are 3, 6 and 7 (Logue *et al*., 2009; Martin and Henry, 2013). The detailed role of these caspases was outlined in Chapter 1.

Flow cytometry is dependent on the ability of laser- and arc-based flow cytometers to measure different parameters based on scatter and fluorescence. The major advantage is that it measures the parameters on each particle in the suspension rather than dependence on just the average value (Logue *et al.*, 2009, Henry *et al*., 2013). Fluorescent dyes can conjugate to surface receptors and cytoplasmic determinants, hence enabling identification of subpopulations of the cells based on their fluorescence characteristics. Annexin V binds efficiently to phosphatidylserine (PS) in the presence of Ca^{2+} ions and to a lesser extent to phosphatidylcholine and sphingomyelin (Henry *et al.,* 2013; Brummatti *et al*., 2008). Cognisant of this high affinity to PS, annexin V is used as a probe for identification and quantification of apoptotic cells (Logue *et al.,* 2009; Henry *et al.,* 2013; Brummatti *et al*., 2008). It is common practice to add propidium iodide (PI) so as to distinguish cells that

have lost plasma membrane integrity, although PI can be omitted in situations where annexin is combined to immunochemical labels (Logue *et al.,* 2009).

Cells in different phases of apoptosis exhibit differential ability of picking up annexin V and PI stains. This has necessitated the use of dot plots in four-quadrant flow cytometer analysis. Cells that are both annexin V-negative and PI-negative represent viable cells and these are located on the lower left quadrant. Cells in the early stages of apoptosis that are positive for annexin V but negative for PI constitute the lower right quadrant. These cells have externalised PS but are not yet capable of taking vital dyes such as PI. Cells in the upper right quadrant are annexin V and PI-positive. These depict cells in late apoptosis that have lost plasma membrane integrity (Logue *et al.*, 2009). The upper left quadrant has dead cells and debri that are PI-positive and annexin V-negative. Other methods of choice for apoptosis assay include the terminal deoxynucleotidyltransferase-mediated dUTP nick end labelling (TUNEL) for DNA fragmentation analysis and determination of caspases, by western blot or fluorimetry. These methods are time consuming and are prone to observer bias. On the contrary, the annexin V-binding assay is unbiased, quick, and reliable (Logue *et al.,* 2009). In this section, the method of choice is annexin V/PI staining.

Unlike apoptosis, necrosis refers to uncontrolled cell death. It is characterised by sudden cell death, loss of membrane integrity and release of alarmins (Martin and Henry 2013). While necrosis is not typically associated with caspase activation, an exception is pyroptosis, in which cell death is a result of activation of caspases (1, 4 and 5). These are inflammatory caspases (Martin and Henry, 2013). On the other hand, necroptosis is another form of programmed cell death whose activation can be induced by death ligands, such as TNFα and Apo2/TRIAL (Matt and Hofmann, 2016). Necroptosis is characterised by lack of activation of caspases but instead receptor-interacting kinases 1 and 3 (RIPK1 and RIPK3) activate the process (Matt and Hofmann, 2016). Normally, DNA damage that is not repaired results in cell removal by apoptosis. However, as outlined in the previous chapter, DNA damage may alter important processes such as DNA replication or transcription (Dexheimer, 2013; Lowe and Lin, 2000; Toettcher *et al.,* 2009).

Genomic and non-genomic signals mediated by cell and nuclear membrane receptors have been associated with oestrogen-induced cell proliferation. Oestrogen-dependent mitochondrial oxidative phosphorylation and gene transcription support the notion of mitochondria as oestrogen targets (Chen *et al.*, 2003; Felty and Roy, 2005). While

mitochondria provide a major source of oxygen species in epithelial cells, oestrogen redox cycling in the absence of mitochondria also generates ROS. Antioxidants inhibit oestrogen-dependent cell growth (Chen *et al.*, 2003; Felty and Roy 2005). Both oestrogens and ROS target PKC, MEK, ERK A-Raf, Akt and transcription factors AP-1, NF-κB and CREB involved in oestrogen-dependent cell proliferation (Felty and Roy, 2005). Ethinylestradiol enhances nuclear genome transcription and inhibits transforming growth factor beta in (TGFβ)-induced apoptosis in hepatic cells (Chen *et al.*, 2003). While this phenomenon is suggestive of oestrogen-induced proliferative diseases including cancer, there is paucity of information regarding the role of ethinylestradiol on HeLa cell apoptosis upon co-administration with antiretroviral drugs.

Treatment of normal human cervical epithelial cells (hECEs) with 10nM 17β-oestradiol blocks apoptosis induced by P2X7-receptor ligands. 17β-Oestradiol attenuates apoptotic effects in hECEs immortalised with HPV-16. In both normal and cervical cancer cells (HT3 and CaSki), the antiapoptotic effects of 17β-oestradiol involve attenuation of caspase-9 and terminal caspase-3 activity (Wang *et al.*, 2004). Ethinylestradiol is a derivative of 17β-oestradiol and an important constituent of some combined oral contraceptive pills (Fruzzeti and Bitzer, 2010). It is therefore intriguing to evaluate the effect of ethinylestradiol on antiretroviral drug-induced proliferation in human cervical epithelial cells. Webster *et al.* (2001) demonstrate that oestrogen and progesterone can increase apoptosis of E2 and E7 protein-induced apoptosis.

4.1.1. Objectives of the chapter

a. To evaluate the antiapoptotic effect of single antiretroviral drugs in human cervical epithelial cancer cells.
b. To determine the effect of co-administering levonorgestrel or ethinylestradiol on single antiretroviral drug-induced apoptosis in human cervical epithelial cells.
c. To evaluate the antiapoptotic effect of combination antiretroviral drugs in human cervical epithelial cancer cells.
d. To determine the effect of co-administering levonorgestrel or ethinylestradiol on combination antiretroviral drug-induced apoptosis in human cervical epithelial cells.

4.2.0. Materials and Methods

4.2.1 Materials

The Annexin V-FITC Apoptosis detection Kit (BMS 500FI/300CE) was obtained from eBioscience. Antiretroviral drugs (abacavir, lamivudine, nevirapine, stavudine and zidovudine), camptothecin, and phosphate-buffered saline (PBS) were obtained from Sigma (United Kingdom). T25 culture flaks were purchased from Nunc Denmark. The HeLa cell line was obtained curtusy of the University of East London tissue culture research laboratory.

4.2.2 Cell culture and maintenance

HeLa and Chinese hamster ovary cells are adherent cells whose culture and maintenance were as described in Chapter 2 and Chapter 3 respectively.

4.2.3 Treatment and harvesting of cells for annexin-V staining

Cells were treated and harvested as adapted from BD Bioscience's method for Annexin V staining of adherent cells for flow cytometry. Briefly, approximately 5.0 x 10^5cells were seeded in T25 cell culture flasks and left overnight to attach. The medium was replaced with single and combination antiretroviral drugs at final concentrations of 50µg/ml for each drug. The cells were treated with antiretroviral drugs only or co-administered with either 0.5µg/ml ethinylestradiol (EE) or 4.0µg/ml levonorgestrel (LNG) for 48 h. 5µM Camptothecin served as a positive control for apoptosis induction. Vehicle-treated cells with 0.25% (v/v) DMSO constituted the negative control. The final DMSO concentration in all cell treatments was maintained at 0.25% (v/v). After treatment, the cell culture medium was aspirated. 1.0ml of trypsin was added to the culture flask and left for 2 min at room temperature in order to detach adherent cells. Where needed, the sides of the flask were gently tapped to facilitate detachment of adherent cells. 4ml of medium were added and the contents gently mixed by pipetting up and down. The contents were transferred to 15ml centrifuge tubes and centrifuged at 1000 revolutions per minute (rpm) for 3 minutes. The supernatant was discarded and cells were resuspended in 5ml PBS.

4.2.4 Annexin V-FITC protocol

HeLa cells were harvested and washed in PBS. About 2-5 x 10^5 cells/ml were suspended in 200µl binding buffer. 5µl of annexin V-FITC was added to 195µl cell suspension, mixed and incubated in the dark for 10 minutes on ice. The cells were then washed with 200µl binding buffer and suspended in 190µl binding buffer. 10µl of propidium iodide was added

and the samples analysed for apoptosis using a BD Accuri™ C6 Flow Cytometer. The general procedure is outlined in Figure 4.1.

4.2.5 Flow cytometry analysis

Firstly, unstained control cells were run with the voltages adjusted to place the unstained samples in the first quadrant of the dot plots. The voltage was fixed at this stage and was not changed. Each single colour (Annexin V-FITC and PI) control was run to adjust compensation and remove spectral overlap from interfering FL channels. The experimental samples were run by measuring Annexin V-FITC emission on the FL1 channel and the PI emission on FL2.

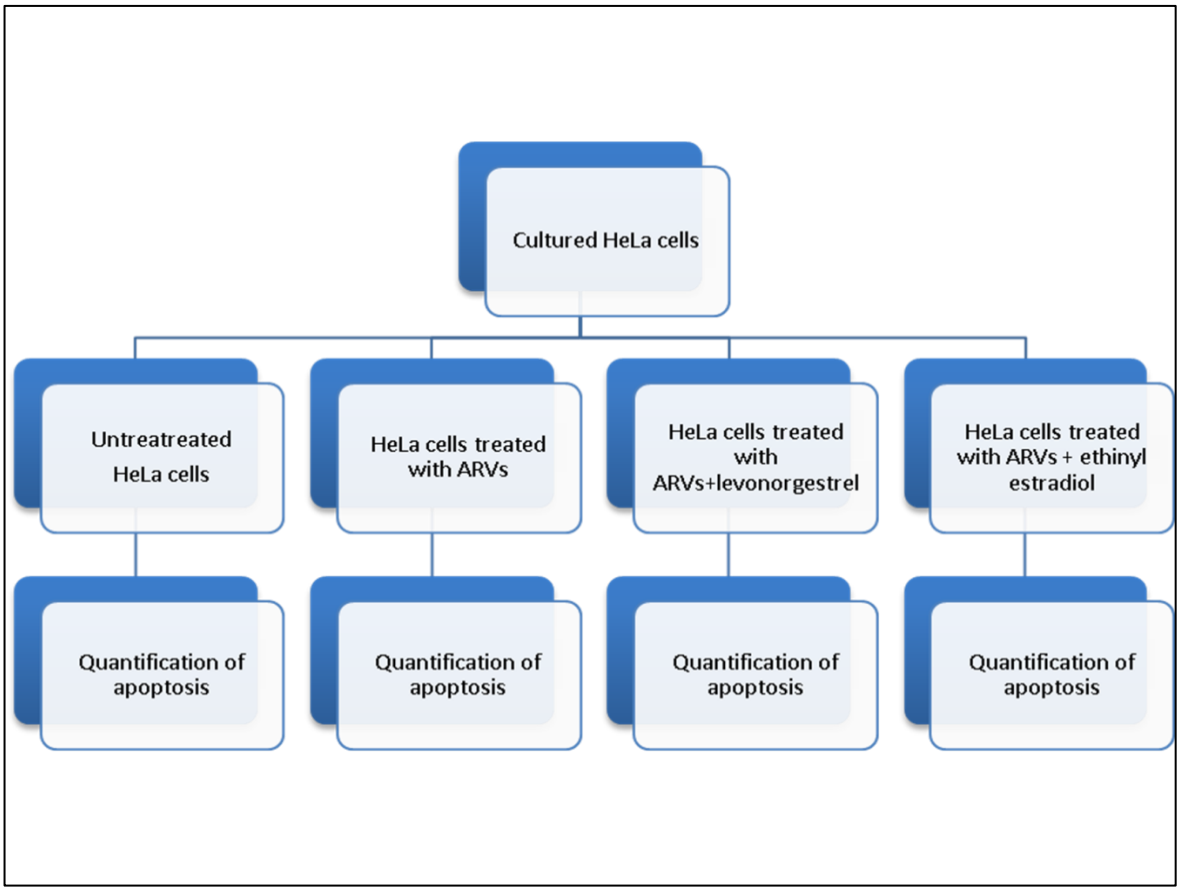

Fig 4.1: Outline of the experimental design for investigating the effect of antiretroviral drug co-administration with contraceptive hormones on human cervical epithelial cancer cell apoptosis. HeLa cells were treated with single antiretroviral drugs or a combination of ARVs at final concentrations of 50µg/ml for each drug. Cells were treated with drugs only or with drugs and ethinylestradiol (EE) at 0.5µg/ml or with drugs and levonorgestrel (LNG) at 4.0µg/ml for 48 h. Four-way dot plots into live, early apoptotic, late apoptotic, and dead cells quantified the cells.

4.2.5. Data Analysis

Descriptive statistics were computed on Excel. For comparison among different treatments, data was analysed by ANOVA followed by Tukey's multiple comparison test ($P \leq 0.05$).

4.3.0. Results

The experimental results for apoptosis analysis are presented in this section.

4.3.1 Annexin-V/PI staining for apoptosis

Using the conventional four-way dot plots, the present study determined the fractions for live, early apoptotic, late apoptotic, and necrotic cells after treatment of HeLa cells with single and combination antiretroviral drugs for 48 h. For nucleoside reverse-transcriptase inhibitors, the working stock was diluted in medium because all the four drugs (ABC, ZDV, 3TC and d4T) are water-soluble. NVP is soluble in DMSO. The final vehicle concentration for DMSO in the medium was adjusted to 0.25% for single antiretroviral treatment, combination antiretroviral treatment and untreated control.

4.3.2 Controls and treatment with hormones only

HeLa cells treated with 5µg/ml camptothecin, 4.0µg/ml levonorgestrel, 0.5µg/ml ethinylestradiol or left in untreated medium were incubated for 48 h. Table 1 presents the descriptive statistics for three different treatments (n=3) while Figure 4.2 depicts four-way dot plots for untreated control, positive control, levonorgestrel-treated and ethinylestradiol-treated cells. Fig 4.2 also depicts the statistical comparison among controls and hormone treated cells. Untreated cells undergo a reduced rate of apoptosis with no late apoptotic and dead cells, while camptothecin induced drastic apoptosis (Table 1).

Table 4.1: Descriptive statistics for treatment of HeLa cells with controls and contraceptive hormones

Cell type (%)	Live	Early apoptotic	Late apoptotic	Dead
Untreated	89.43±2.06	10.57±2.06	0.00±0.00	0.00±0.00
+ve control	25.83±2.06	68.97±2.29	4.47±0.31	0.73±0.06 *
LNG only	85.53±1.00	11.37±2.19	1.13±0.31	1.30±0.10
EE only	85.67±1.46	10.50±1.28	1.30±0.10	2.50±0.10

HeLa cells were treated with 5µg/ml camptothecin, levonorgestrel (4.0µg/ml), ethinylestradiol (0.5µg/ml) or left in the untreated medium and incubated for 48 h. Cells were stained with annexin V and PI. Four-way dot plots into live, early apoptotic, late apoptotic and dead cells quantified the cells. Data represent mean ± SD (n=3). Asterisk indicate significant differences in apoptosis (early + late apoptosis) compared to the untreated control using Tukey's multiple comparison test (P<0.05).

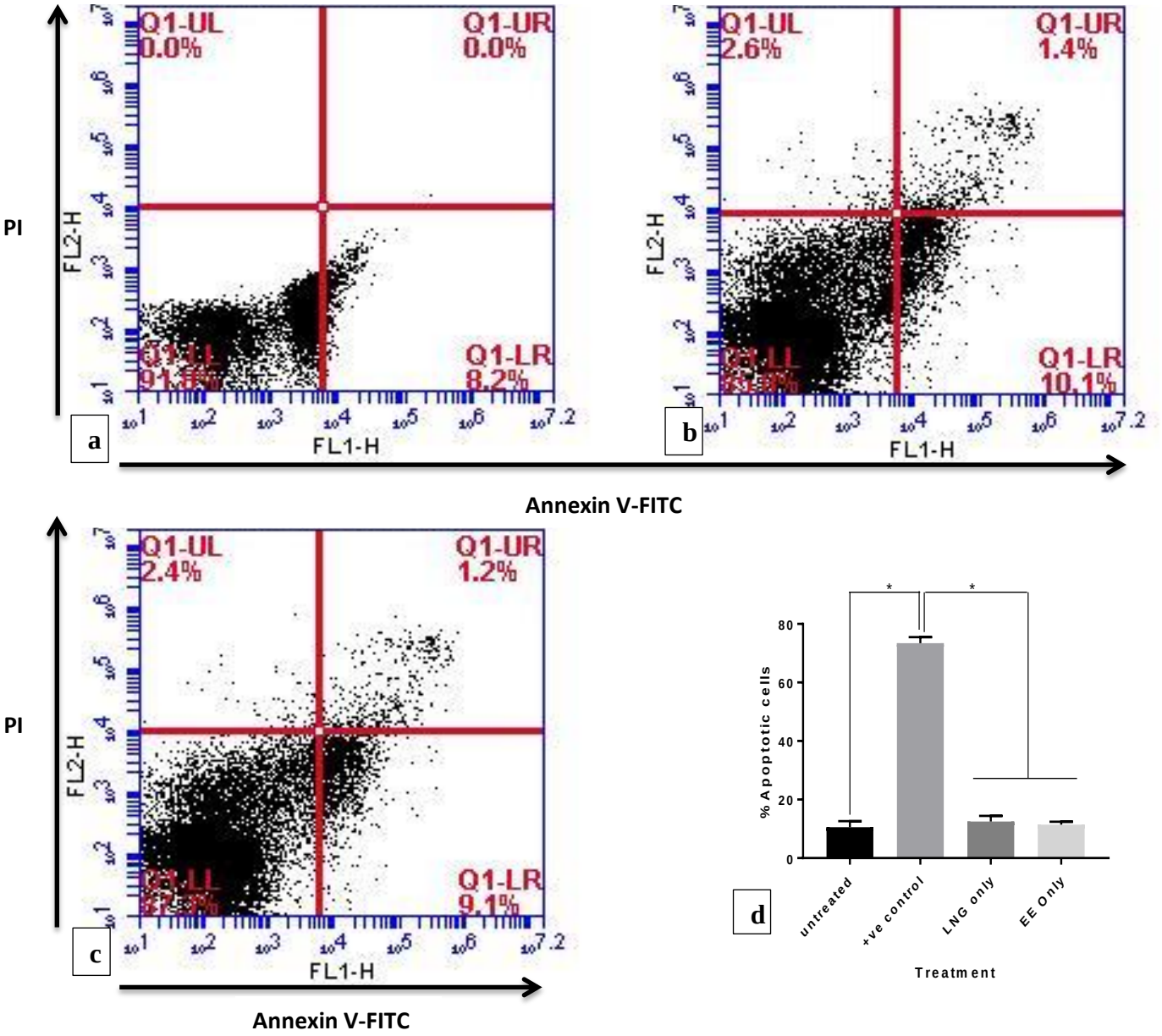

Fig 4.2: Flow cytometry analysis for hormone treatments and controls. The four-way dot plots depict single representatives (n=1) of untreated HeLa cells [a], HeLa cells pre-treated with 4.0µg/ml levonorgestrel (LNG) [b] and 0.5µg/ml ethinylestradiol (EE) [c]. Untreated cells constitute the negative control. The cells were incubated for 48 h followed by staining with annexin V and PI. Annexin V and PI-negative cells in lower left quadrant (Q1-LL) depict viable cells in the population (n=1). Cells in the lower right quadrant (Q1-LR) are in the early stages of apoptosis. These HeLa cells are annexin V-positive and PI-negative. Cells located in the upper right quadrant (Q1-UR) are positive for both annexin V and PI. These cells are in late stages of apoptosis (secondary necrosis). Cells in the upper left quadrant (Q1-UL) represent dead cells. (d): Effect of levonorgestrel and ethinylestradiol on HeLa cell apoptosis. For each individual experiment, apoptotic cells are a sum of early and late apoptosis. Data were analysed by one-way ANOVA. The Tukey's test adjusted for multiple comparisons among different treatments. Data represent means ± SD of 3 independent experiments (*P≤0.05).

4.3.3. Flow cytometry results for single-drug antiretroviral treatment

HeLa cells were treated with different antiretroviral drugs at final concentrations of 50μg/ml of each single antiretroviral drug. Cells were treated with drugs only or with drugs and ethinylestradiol at 0.5μg/ml or with drugs and levornogestrel at 4.0μg/ml for 48 h. Four-way dot plots into live, early apoptotic, late apoptotic and dead cells quantified the cells (Figures 4.3-4.7). For statistical comparison, rate of apoptosis was considered as the sum of early and late apoptotic cells in each individual treatment (n=3). The one-way ANOVA non-parametric approach analysed the data. To assess the differences among the different treatments, the Tukey's multi-comparison test was applied. Significant differences were reported where $P \leq 0.05$. Table 4.2 summarises descriptive statistics for each single drug treatment of the HeLa cells in three independent experiments.

Table 4.2: Descriptive statistics for treatment of HeLa cells with single antiretroviral drugs for apoptosis assay

Treatment	% Live cells	%Early apoptotic cells	%Late apoptotic cells	%Dead cells
Untreated	89.43±2.06	10.57±2.06	0.00±0.00	0.00±0.00
ABC	61.43±0.78	27.17±0.21	5.13±0.56	6.37±0.35 *
ABC+LNG	58.50±1.32	27.90±0.87	6.47±0.32	7.17±0.23 *
ABC+EE	70.47±0.82	27.57±0.64	1.37±0.15	0.60±0.10 *
ZDV	57.33±0.61	31.50±0.44	5.63±0.55	5.57±0.25 *
ZDV+LNG	69.90±1.93	21.40±1.28	5.37±0.49	3.33±0.21 *
ZDV+EE	63.77±2.23	30.67±2.25	3.13±0.06	2.50±0.10 *
3TC	56.27±2.47	23.20±0.89	7.97±1.23	12.33±0.58 *
3TC+LNG	74.40±2.74	16.93±2.12	4.03±0.40	4.67±0.35 *
3TC+EE	58.87±2.12	33.07±1.99	3.83±0.12	4.23±0.06 *
d4T	53.90±3.33	27.13±0.46	11.93±1.46	7.70±0.24 *
d4T+LNG	64.13±2.24	24.00±1.42	7.70±0.53	4.17±0.26 *
d4T+EE	81.00±1.99	16.03±1.33	1.30±0.17	2.00±0.08 *
NVP	48.70±3.24	31.83±1.29	6.80±0.90	12.03±1.85 *
NVP+LNG	71.10±2.89	21.60±2.49	2.80±0.30	4.50±0.36 *
NVP+EE	68.63±1.16	22.93±0.85	2.03±0.21	6.40±0.70 *

HeLa cells were pre-treated with single antiretroviral drugs with and without levonorgestrel (LNG) or ethinylestradiol (EE) for 48 h. The cells were stained with annexin V and PI. Four-way dot plots into live, early apoptotic, late apoptotic, and dead cells quantified the cells. Data represent mean ± SD (n=3). Asterisks indicate significant differences in apoptosis (early + late apoptosis) compared to the untreated control using Tukey's multiple comparison test (P<0.05).

Compared to untreated cells, treatment with ABC alone significantly increases apoptosis ($P \leq 0.05$). Co-treatment with ethinylestradiol significantly reduces ABC-induced apoptosis, while levonorgestrel co-administration did not show an effect (Figure 4.3). The decrease in apoptosis with ethinylestradiol co-administration suggests the possibility of increased risk of mutation of the DNA-damaged cells following treatment with abacavir. Treatment of HeLa cells with ZDV alone or its co-administration with either levonorgestrel and ethinylestradiol increases apoptosis compared to untreated cells (Figure 4.4). Co-administration of ZDV and levonorgestrel drastically decreases the apoptotic population, while ethinylestradiol co-administration has no effect on ZDV-induced apoptosis. This shows an increased risk of zidovudine DNA-damaged cells having to mutate and escape apoptosis, hence leading to continuous growth of the cancerous cells. HeLa cells undergo increased apoptosis after 3TC treatment, compared to untreated cells. Levonorgestrel downregulates 3TC-induced apoptosis, while ethinylestradiol co-administration does not show any significant difference (Figure 4.5), thus suggesting increased risk of evasion of apoptosis with levonorgestrel administration.

Co-administration of levonorgestrel or ethinylestradiol with stavudine (d4T) significantly reduces d4T-induced apoptosis, a phenomenon that may explain decreased toxicity. Ethinylestradiol results in a more drastic decrease in d4T-induced apoptosis compared to levonorgestrel (Figure 4.6). While NVP increases apoptosis in HeLa cells levonorgestrel and ethinylestradiol down regulates NVP-induced apoptosis (Fig 4.7). Cognisant of the observed increased DNA damage upon treatment of human cervical epithelial cancer cells with stavudine and nevirapine noted in Chapter 3, these present results depict an increased risk of mutation and apoptosis evasion with both levonorgestrel and ethinylestradiol co-administration with either stavudine or nevirapine.

The observed results show the varied effect of contraceptive hormones on antiretroviral drug-induced apoptosis. These results outline the mechanistic effect of single antiretroviral drugs and hormones on human cervical epithelial cancer proliferation. However, in practice, antiretroviral drugs are administered in combination. The next section therefore explores the effect of hormonal contraceptives on combination antiretroviral drug-induced apoptosis.

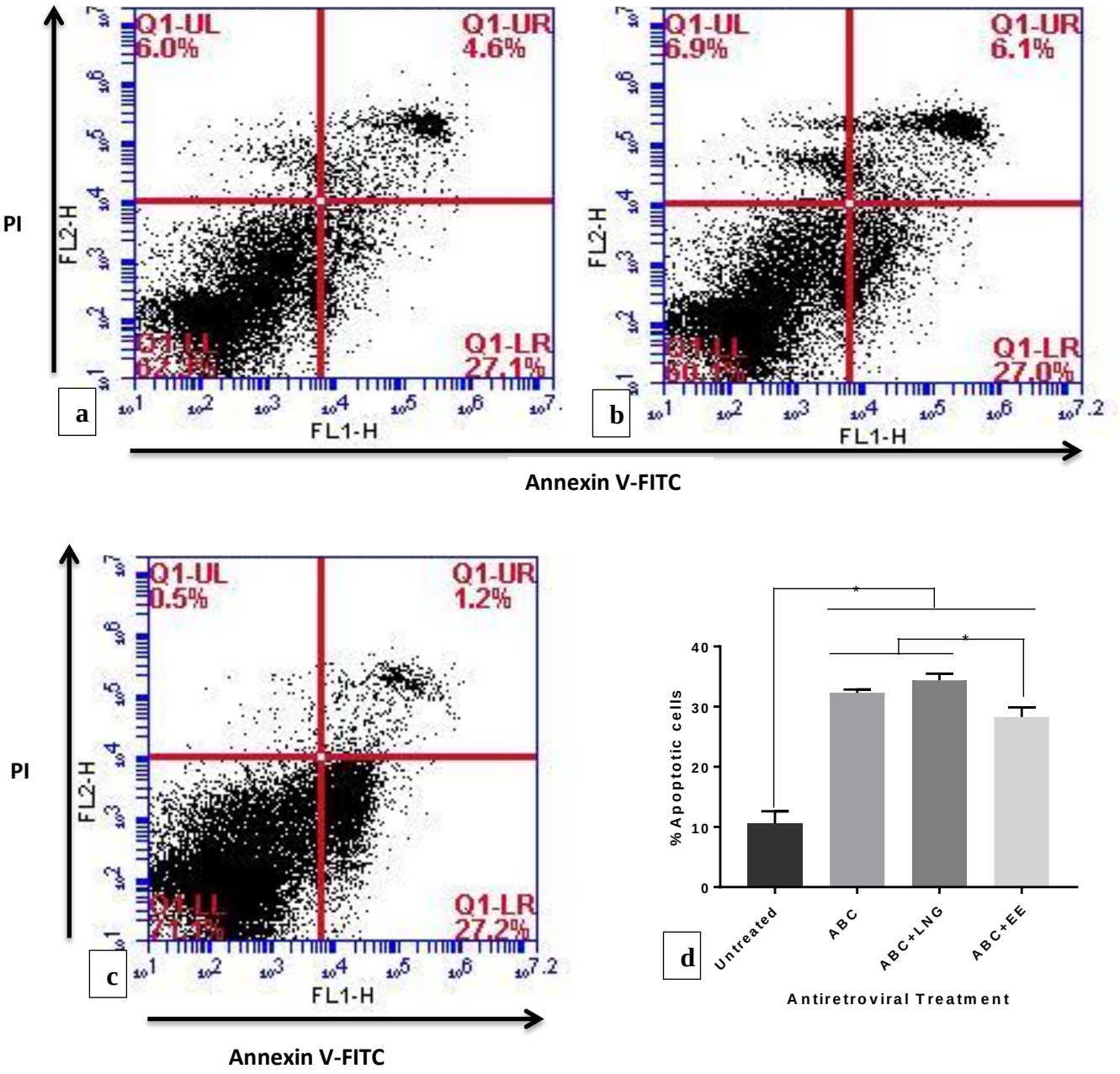

Fig 4.3: Flow cytometry analysis of HeLa cells pre-treated with abacavir (ABC) with or without levonorgestrel (LNG) or ethinylestradiol (EE). Treatment was with ABC only (a), ABC with LNG (b) or ABC with EE (c) for 48 h followed by staining with annexin V and PI. Annexin V and PI negative cells (Q1-LL) depict viable cells in the population (n=1). Cells in the lower right quadrant (Q1-LR) are in the early stages of apoptosis. These HeLa cells are annexin V-positive and PI-negative. Cells located in the upper right quadrant (Q1-UR) are positive for both annexin V and PI. These cells are in late stages of apoptosis. Cells in the upper left quadrant (Q1-UL) represent dead and necrotic cells. (d): Effect of LNG and EE on ABC-induced apoptosis in HeLa cells. Apoptotic cells are a sum of both early and late apoptotic cells. Data were analysed by one-way ANOVA. The Tukey's test adjusted for multiple comparisons among different treatments. Data represent means ±SD of 3 independent experiments (P≤0.05).

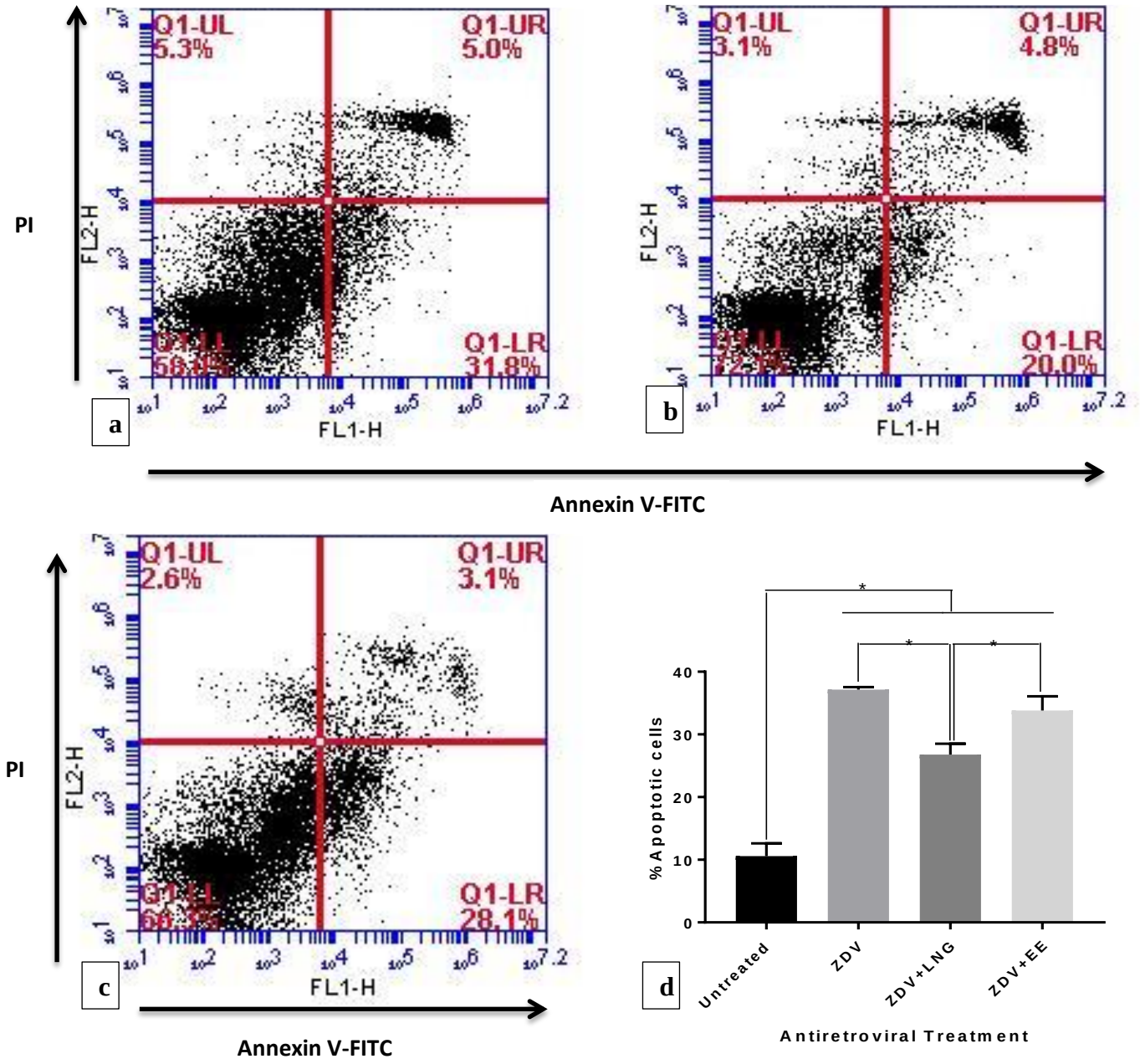

Fig 4.4 Flow cytometry analysis of HeLa cells pre-treated with zidovudine (ZDV) with or without levonorgestrel (LNG) or ethinylestradiol (EE). Treatment was with ZDV only (a), ZDV with LNG (b) or ZDV with EE (c) for 48 h followed by staining with annexin V and PI. Annexin V and PI negative cells (Q1-LL) depict viable cells in the population (n=1). Cells in the lower right quadrant (Q1-LR) are in the early stages of apoptosis. These HeLa cells are annexin V-positive and PI-negative. Cells located in the upper right quadrant (Q1-UR) are positive for both annexin V and PI. These cells are in late stages of apoptosis. Cells in the upper left quadrant (Q1-UL) represent dead and necrotic cells. (d): Effect of LNG and EE on ZDV-induced apoptosis in HeLa cells. For each individual experiment, apoptotic cells are a sum of both early and late apoptosis. Data were analysed by one-way ANOVA. The Tukey's test adjusted for multiple comparisons among different treatments. Data represent mean ± SD of three independent experiments (*P≤0.05).

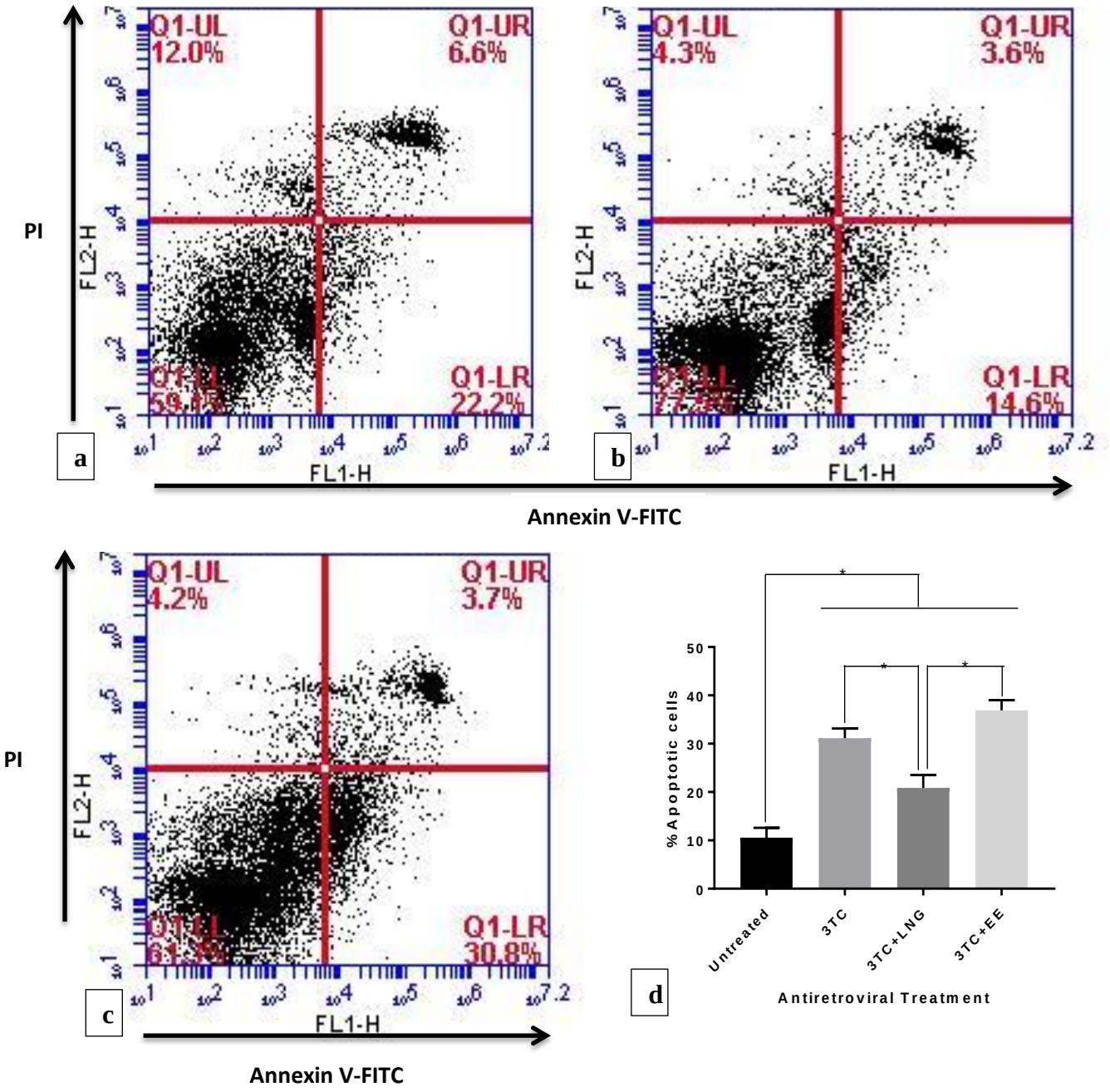

Fig 4.5 Flow cytometry analysis of HeLa cells pre-treated with lamivudine (3TC) with or without levonorgestrel (LNG) or ethinylestradiol (EE). Treatment was with 3TC only (a), 3TC with LNG (b) or 3TC with EE (c) for 48 h followed by staining with annexin V and PI. Annexin V and PI negative cells (Q1-LL) depict viable cells in the population (n=1). Cells in the lower right quadrant (Q1-LR) are in the early stages of apoptosis. These HeLa cells are annexin V-positive and PI-negative. Cells located in the upper right quadrant (Q1-UR) are positive for both annexin V and PI. These cells are in late stages of apoptosis. Cells in the upper left quadrant (Q1-UL) represent dead and necrotic cells. (d): Effect of LNG and EE on 3TC-induced apoptosis in HeLa cells. For each individual experiment, apoptotic cells are a sum of both early and late apoptotic cells. Data were analysed by one-way ANOVA. The Tukey's test adjusted for multiple comparisons among different treatments. Data represent means ±SD of three independent experiments (*P≤0.05).

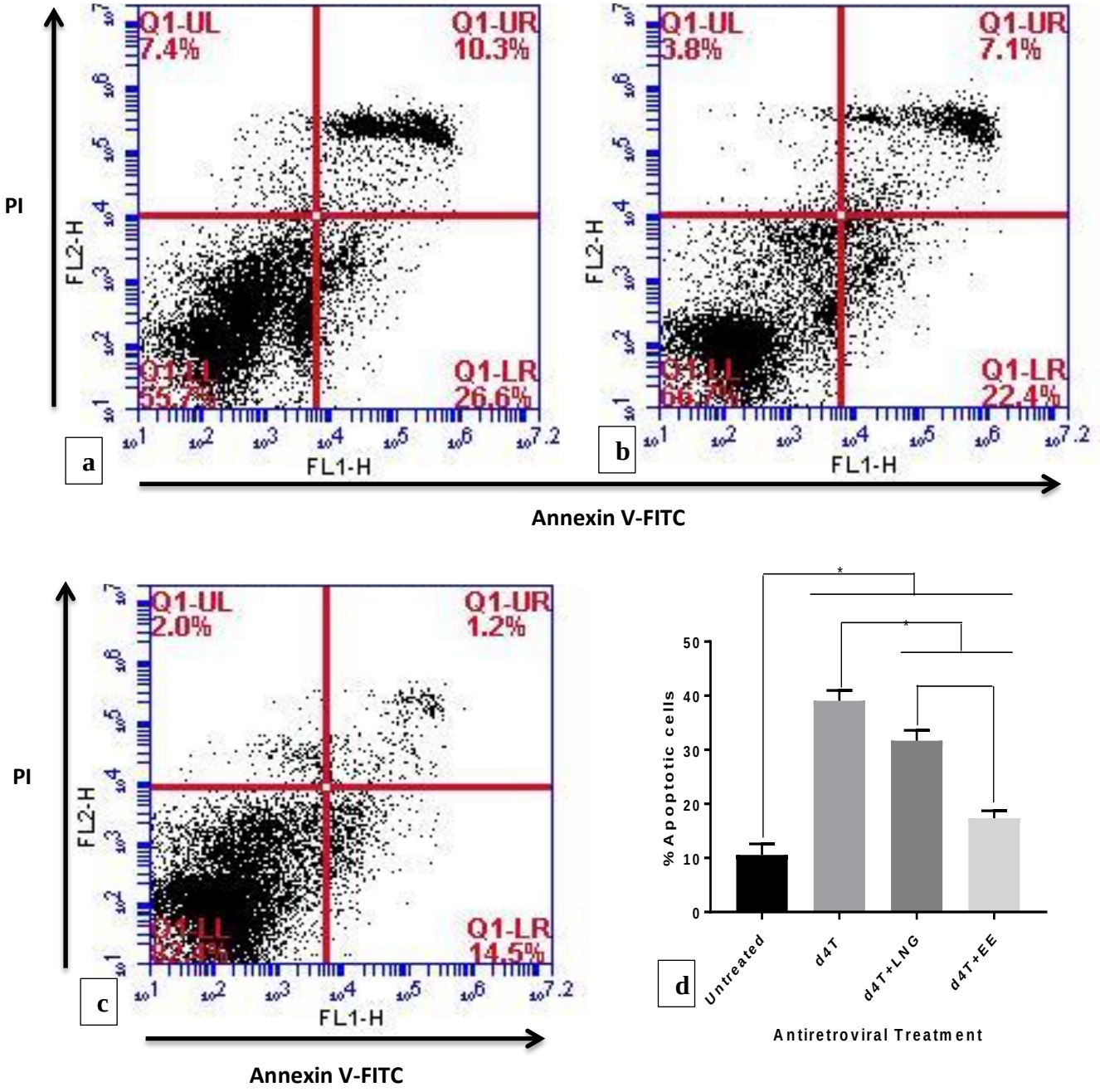

Fig 4.6 Flow cytometry analysis of HeLa cells pre-treated with stavudine (d4T) with or without levonorgestrel (LNG) or ethinylestradiol (EE). Treatment was with d4T only (A), d4T with LNG (B) or d4T with EE (C) for 48 h followed by staining with annexin V and PI. Annexin V and PI negative cells (Q1-LL) depict viable cells in the population (n=1). Cells in the lower right quadrant (Q1-LR) are in the early stages of apoptosis. These HeLa cells are annexin V-positive and PI-negative. Cells located in the upper right quadrant (Q1-UR) are positive for both annexin V and PI. These cells are in late stages of apoptosis. Cells in the upper left quadrant (Q1-UL) represent dead and necrotic cells. D: Effect of LNG and EE on d4T-induced apoptosis in HeLa cells. For each individual experiment, apoptotic cells are a sum of both early and late apoptotic. Data were analysed by one-way ANOVA. The Tukey's test adjusted for multiple comparisons among different treatments. Data represent means ±SD of three independent experiments (*P ≤0.05).

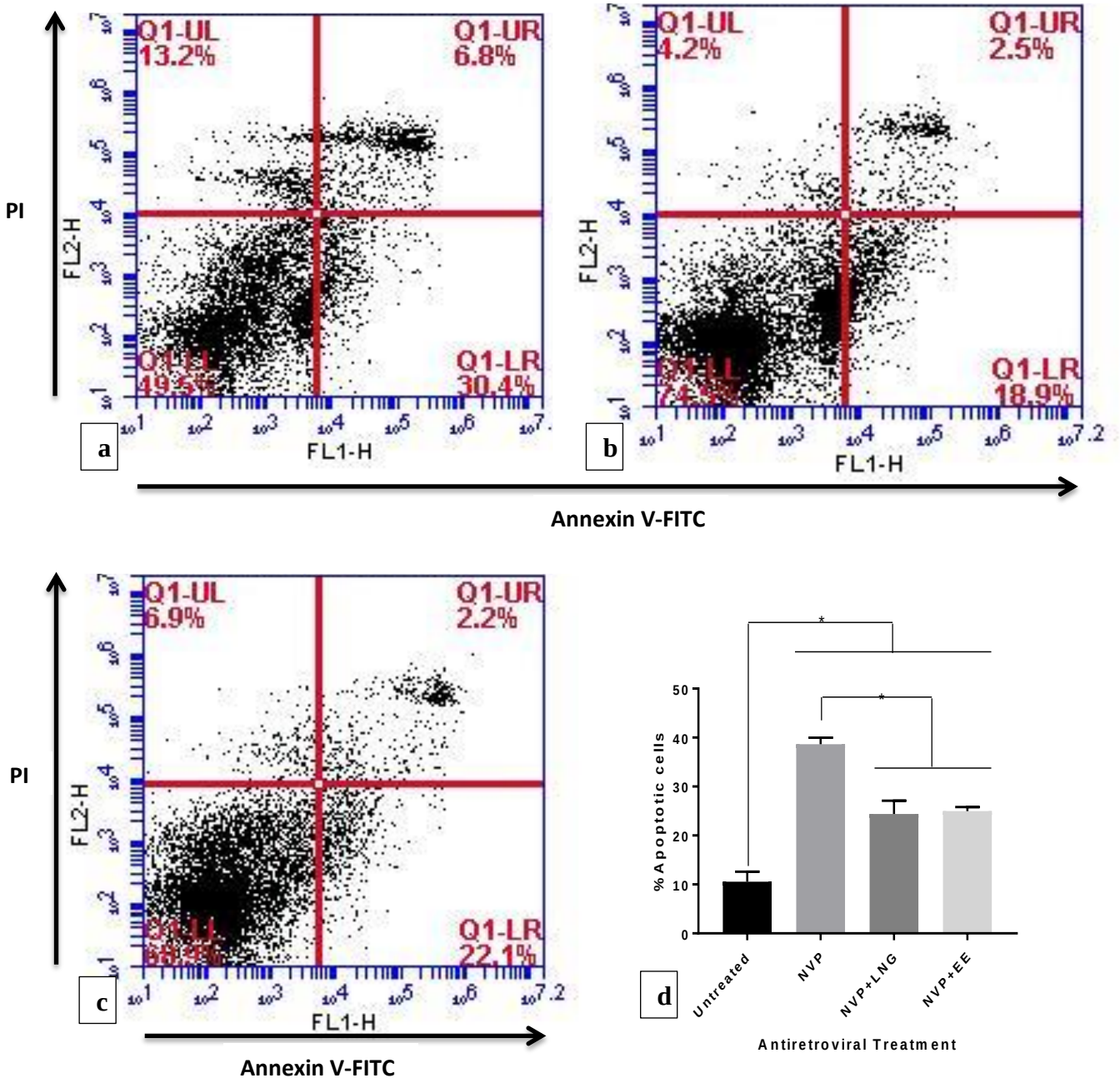

Fig 4.7 Flow cytometry analysis of HeLa cells pre-treated with nevirapine (NVP) with or without levonorgestrel (LNG) or ethinylestradiol (EE). Treatment was with NVP only (a), NVP with LNG (b) or NVP with EE (c) for 48 h followed by staining with annexin V and PI. Annexin V and PI negative cells (Q1-LL) depict viable cells in the population (n=1). Cells in the lower right quadrant (Q1-LR) are in the early stages of apoptosis. These HeLa cells are annexin V-positive and PI-negative. Cells located in the upper right quadrant (Q1-UR) are positive for both annexin V and PI. These cells are in late stages of apoptosis. Cells in the upper left quadrant (Q1-UL) represent dead and necrotic cells. (d): Effect of LNG and EE on NVP-induced apoptosis in HeLa cells. For each individual experiment, apoptotic cells are a sum of bot early and late apoptotic cells. Data were analysed by one-way ANOVA. The Tukey's test adjusted for multiple comparisons among different treatments. Data represent means ±SD of three independent experiments (*P≤0.05).

4.3.4. Flow cytometry results for combination antiretroviral treatment

HeLa cells were treated with different combinations of antiretroviral drugs at final concentrations of 50µg/ml for each single drug. Cells were treated with combination drugs only or with combination drugs and ethinylestradiol at 0.5µg/ml or with drugs and levornogestrel at 4.0µg/ml for 48 h. Four-way dot plots into live, early apoptotic, late apoptotic and dead cells quantified the cells (Figures 4.8 to 4.12). For statistical comparison, apoptotic cells were considered as the sum of early and late apoptotic cells in each individual treatment (n=3). Data were analysed by the one-way ANOVA followed by Tukey's multi-comparison test. Significant differences were reported where $P \leq 0.05$. Table 4.3 summarises descriptive statistics for each combination antiretroviral drug treatment of the HeLa cells in three independent experiments.

Table 4.3: Descriptive statistics for treatment of HeLa cells with combination antiretroviral drugs for apoptosis assay

Treatment	% Live cells	%Early apoptotic cells	%Late apoptotic cells	%Dead cells
Untreated	89.43±2.06	10.57±2.06	0.00±0.00	0.00±0.0
ABC+3TC	69.77±1.76	18.07±0.35	8.17±1.27	4.00±0.20 *
ABC,3TC+LNG	59.83±1.29	30.90±0.79	6.80±0.35	2.47±0.15 *
ABC,3TC+EE	71.20±2.25	23.57±2.31	4.03±0.06	1.20±0.00 *
ZDV+3TC	78.27±1.69	14.37±1.02	2.37±0.40	5.03±0.29 *
ZDV,3TC+LNG	51.10±0.40	34.43±3.79	10.10±0.89	4.77±0.15 *
ZDV,3TC+EE	55.43±2.54	38.03±2.53	2.97±0.06	3.53±0.06 *
ABC+ZDV+3TC	74.80±0.75	18.57±0.55	3.03±0.21	3.67±0.25 *
ABC,ZDV,3TC+LNG	61.73±0.35	29.00±0.26	6.10±0.26	2.97±0.25 *
ABC,ZDV,3TC+EE	60.10±1.91	35.13±1.89	3.77±0.12	1.00±0.10 *
ZDV,3TC,NVP	83.47±2.40	10.53±1.82	1.93±0.38	4.07±0.23
ZDV,3TC,NVP+LNG	57.63±4.21	31.40±3.99	7.53±0.45	3.43±0.12 *
ZDV,3TC,NVP+EE	66.33±1.31	27.93±1.27	2.87±0.06	2.87±0.06 *
d4T+3TC+NVP	76.40±2.82	15.63±2.44	2.00±0.26	5.97±0.15 *
d4T,3TC,NVP+LNG	53.20±2.12	34.37±1.69	9.87±0.35	2.57±0.15 *
d4T+3TC,NVP+EE	71.37±0.38	22.13±0.06	2.73±0.21	3.77±0.15 *

HeLa cells were pre-treated with combination antiretroviral drugs with and without levonorgestrel (LNG) or ethinylestradiol (EE) for 48 h. The cells were stained with annexin V and PI. Four-way dot plots into live, early apoptotic, late apoptotic and dead cells quantified the cells. Data represent mean ± SD (n=3). Asterisks indicate significant differences in apoptosis (early + late apoptosis) compared to the untreated control using Tukey's multiple comparison test (P<0.05).

Relative to single antiretroviral drugs, the present results depict a reduction in the rate of apoptosis with combinatorial treatment. This non-drastic apoptosis suggests a protective effect as too-drastic apoptosis may result in cell loss and possibly tissue damage. Compared to untreated cells, treatment with a double combination of ABC+3TC significantly increases apoptosis. Co-treatment with levonorgestrel drastically increases ABC+3TC-induced apoptosis while ethinylestradiol co-treatment does not have a significant effect on AB+3TC-induced apoptosis (Figure 4.8). Treatment of HeLa cells with ZDV+3TC significantly increased apoptosis compared to untreated cells (Figure 5.9). Co-treatment with both levonorgestrel and ethinylestradiol increases ZDV+3TC-induced apoptosis.

ABC+ZDV+3TC treatment significantly increases the rate of apoptosis relative to untreated cells. HeLa cells undergo further increased apoptosis after treatment with ABC+ZDV+3TC in the presence of either 4.0µg/ml levonorgestrel or 0.5µg/ml ethinylestradiol (Figure 4.10). ZDV+3TC+NVP treatment does not significantly affect HeLa cell apoptosis relative to untreated cells (Fig 4.11). The decreased apoptosis suggests evasion of human cervical epithelial cancer cell death in the presence of this regimen. However, co-treatment of ZDV+3TC+NVP with 4.0µg/ml and 0.5µg/ml ethinylestradiol drastically increases apoptosis in ZDV+3TC+NVP-treated cells ($P<0.05$). There is a significant difference in apoptosis between d4T+3TC+NVP-treated cells and untreated cells. 4.0µg/ml levonorgestrel drastically increases d4T+3TC+NVP-induced apoptosis. 0.5µg/ml ethinylestradiol also increases apoptosis in d4T+3TC+NVP-treated cells, although this is at a much lower percentage compared to 4.0µg/ml levonorgestrel (Fig 4.12). Overall, these results show that there is a significant increase in ABC+ZDV+3TC- and d4T+3TC+NVP-induced apoptosis in human cervical epithelial cancer cells. However, treatment with ethinylestradiol and levonorgestrel drastically increases this combination antiretroviral drug-induced apoptosis.

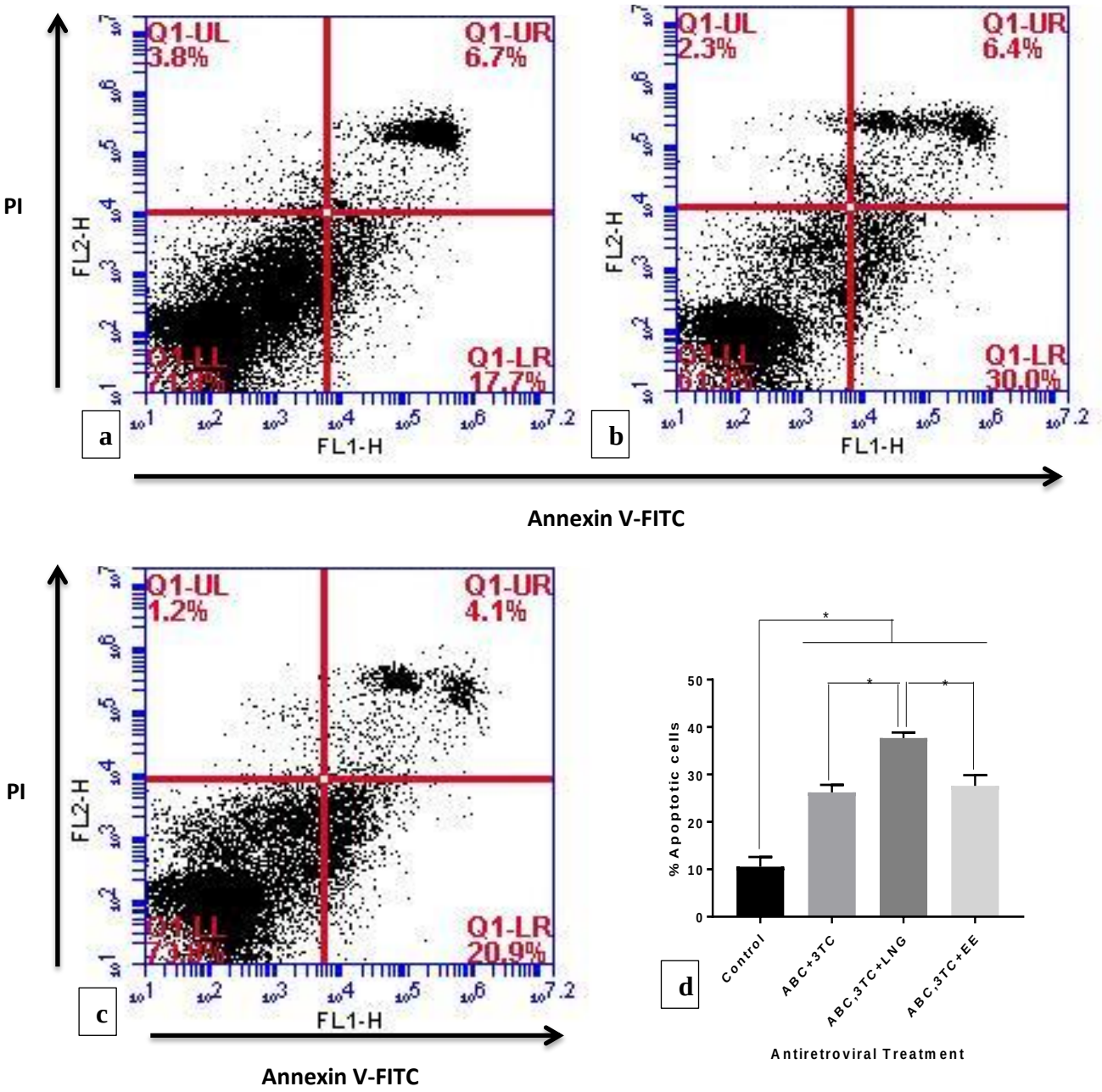

Fig 4.8: Flow cytometry analysis of HeLa cells pre-treated with a combination of ABC and 3TC with or without levonorgestrel (LNG) or ethinylestradiol (EE). Treatment was with ABC+3TC (a), ABC+3TC with LNG (b) or ABC+3TC with EE (c) for 48 h followed by staining with annexin V and PI. Annexin V and PI negative cells (Q1-LL) depict viable cells in the population (n=1). Cells in the lower right quadrant (Q1-LR) are in the early stages of apoptosis. These HeLa cells are annexin-V positive and PI-negative. Cells located in the upper right quadrant (Q1-UR) are positive for both annexin V and PI. These cells are in late stages of apoptosis. Cells in the upper left quadrant (Q1-UL) represent dead and necrotic cells. (d): Effect of LNG and EE on ABC+3TC-induced apoptosis in HeLa cells. For each individual experiment, apoptotic cells are a sum of both early and late apoptotic cells. Data were analysed by one-way ANOVA. The Tukey's test adjusted for multiple comparisons among different treatments. Data represent means ± SD of three independent experiments (P≤0.05).

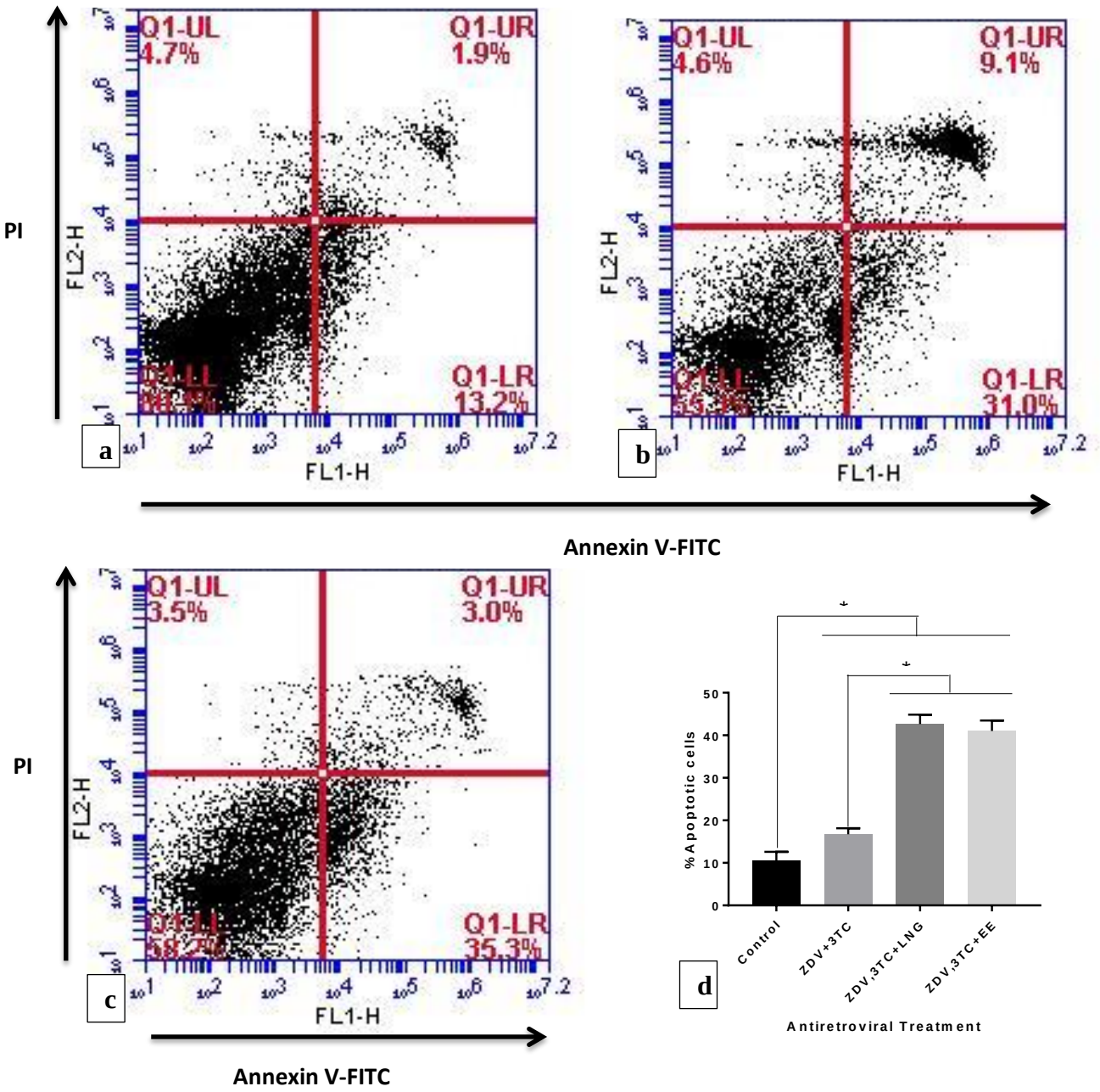

Fig 4.9: Flow cytometry analysis of HeLa cells pre-treated with a combination of ZDV and 3TC with or without levonorgestrel (LNG) or ethinylestradiol (EE). Treatment was with ZDV+3TC (a), ZDV+3TC with LNG (b) or ZDV+3TC with EE (c) for 48 h followed by staining with annexin V and PI. Annexin V and PI negative cells (Q1-LL) depict viable cells in the population (n=1). Cells in the lower right quadrant (Q1-LR) are in the early stages of apoptosis. These HeLa cells are annexin-V positive and PI-negative. Cells located in the upper right quadrant (Q1-UR) are positive for both annexin V and PI. These cells are in late stages of apoptosis. Cells in the upper left quadrant (Q1-UL) represent dead and necrotic cells. (d): Effect of LNG and EE on ZDV+3TC-induced apoptosis in HeLa cells. For each individual experiment, apoptotic cells are a sum of both early and late apoptotic cells. Data were analysed by one-way ANOVA. The Tukey's test adjusted for multiple comparisons among different treatments. Data represent means ± SD of three independent experiments ($P \leq 0.05$).

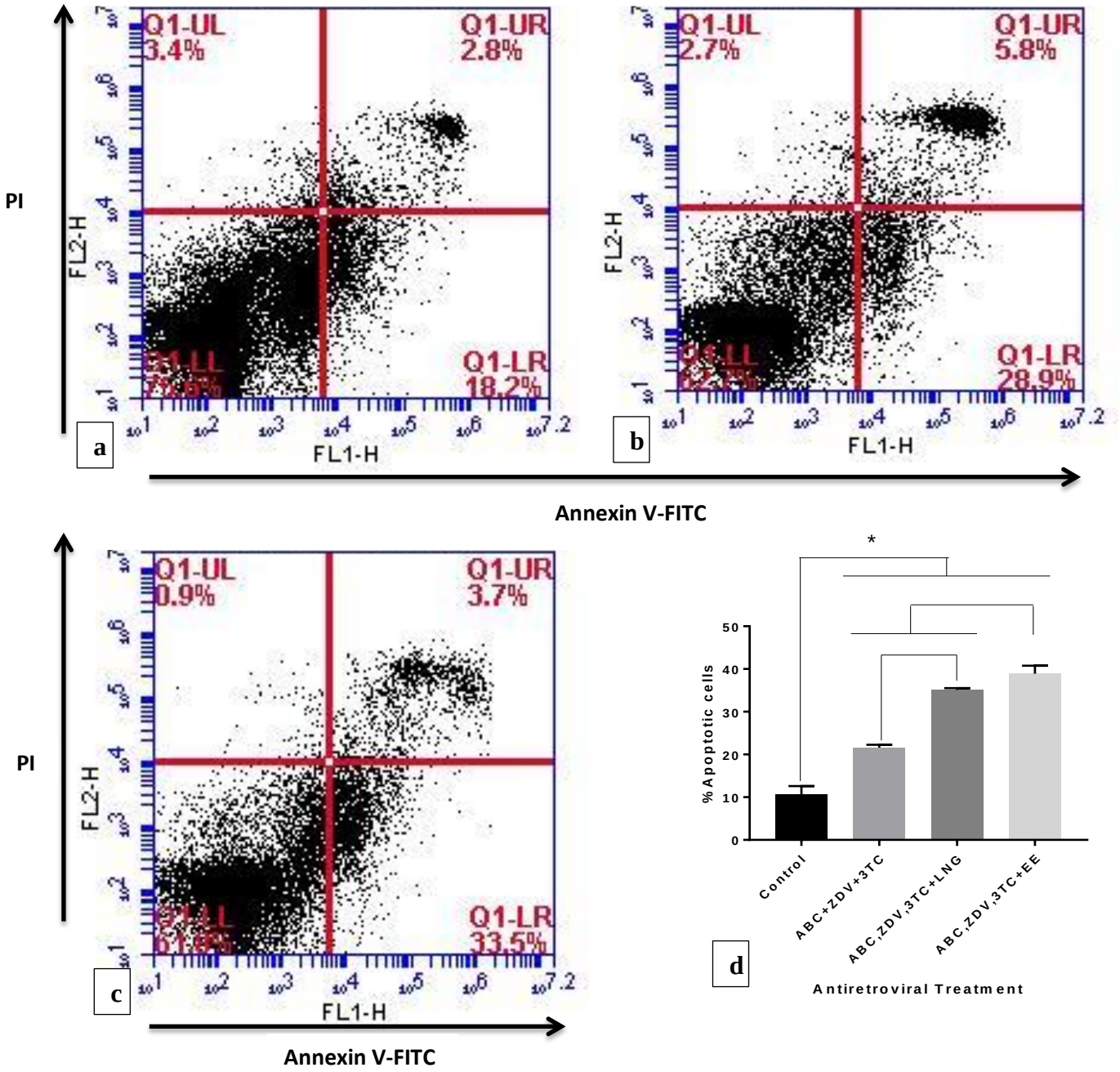

Fig 4.10: Flow cytometry analysis of HeLa cells pre-treated with a combination of ABC, ZDV and 3TC with or without levonorgestrel (LNG) or ethinylestradiol (EE). Treatment was with ABC+ZDV+3TC (a), ABC+ZDV+3TC with LNG (b) or ABC+ZDV+3TC with EE (c) for 48 h followed by staining with annexin V and PI. Annexin V and PI negative cells (Q1-LL) depict viable cells in the population (n=1). Cells in the lower right quadrant (Q1-LR) are in the early stages of apoptosis. These HeLa cells are annexin V-positive and PI-negative. Cells located in the upper right quadrant (Q1-UR) are positive for both annexin V and PI. These cells are in late stages of apoptosis. Cells in the upper left quadrant (Q1-UL) represent dead and necrotic cells. (d): Effect of LNG and EE on ABC+ZDV+3TC-induced apoptosis in HeLa cells. For each individual experiment, apoptotic cells are a sum of both early and late apoptotic cells Data were analysed by one-way ANOVA. The Tukey's test adjusted for multiple comparisons among different treatments. Data represent means ±SD of three independent experiments (P≤0.05).

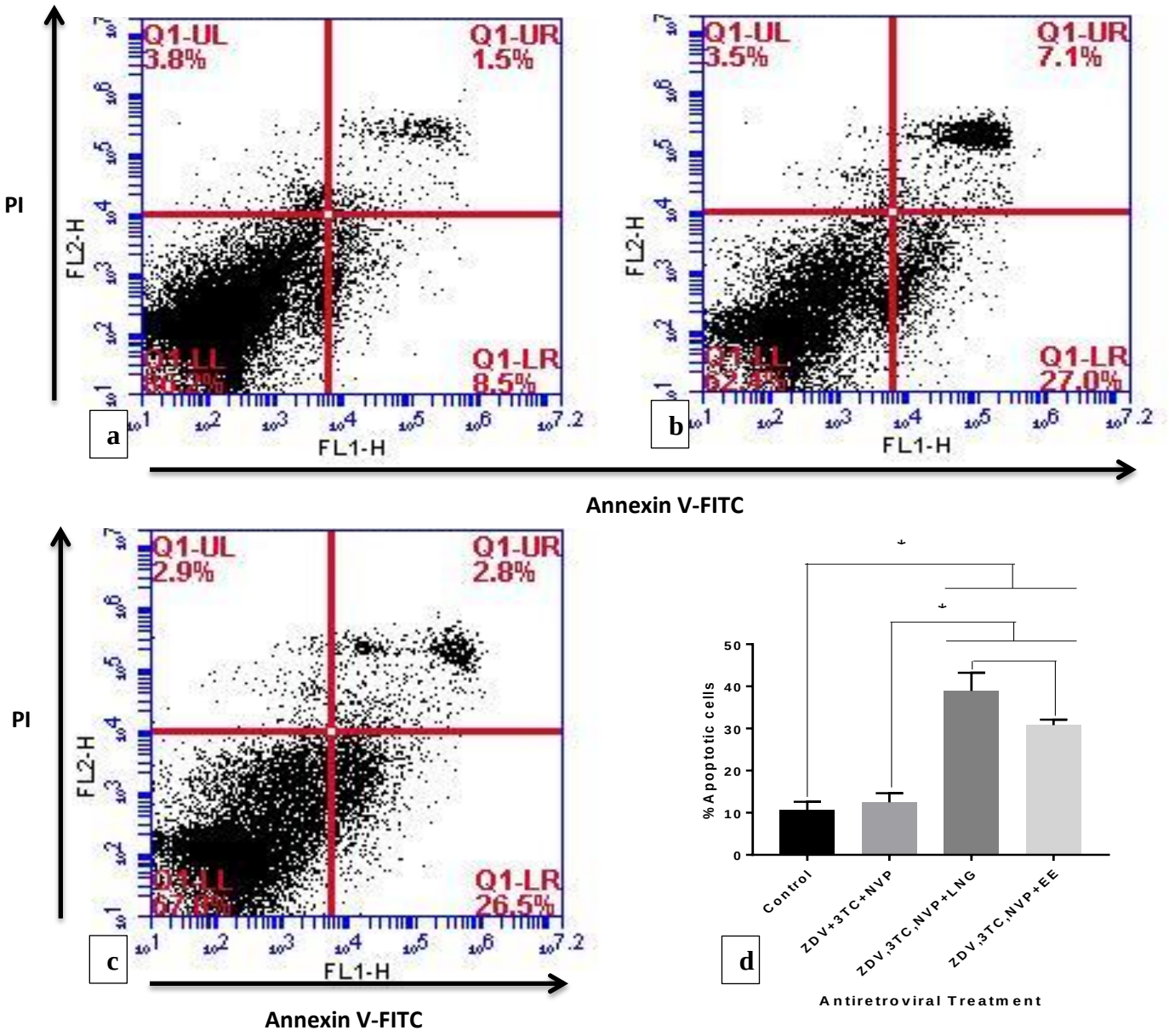

Fig 4.11: Flow cytometry analysis of HeLa cells pre-treated with a combination of ZDV, 3TC and NVP with or without levonorgestrel (LNG) or ethinylestradiol (EE). The cells were treated by ZDV+3TC+NVP (a), ZDV+3TC+NVP with LNG (b) or ZDV+3TC+NVP with EE (c) for 48 h followed by staining with annexin V and PI. Annexin V and PI negative cells (Q1-LL) depict viable cells in the population (n=1). Cells in the lower right quadrant (Q1-LR) are in the early stages of apoptosis. These HeLa cells are annexin V-positive and PI-negative. Cells located in the upper right quadrant (Q1-UR) are positive for both annexin V and PI. These cells are in late stages of apoptosis. Cells in the upper left quadrant (Q1-UL) represent dead and necrotic cells. (d): Effect of LNG and EE on ZDV+3TC+NVP-induced apoptosis in HeLa cells. For each individual experiment, apoptotic cells are a sum of both early and late apoptotic cells. Data were analysed by one-way ANOVA. The Tukey's test adjusted for multiple comparisons among different treatments. Data represent means ±SD of three independent experiments (*P≤0.05).

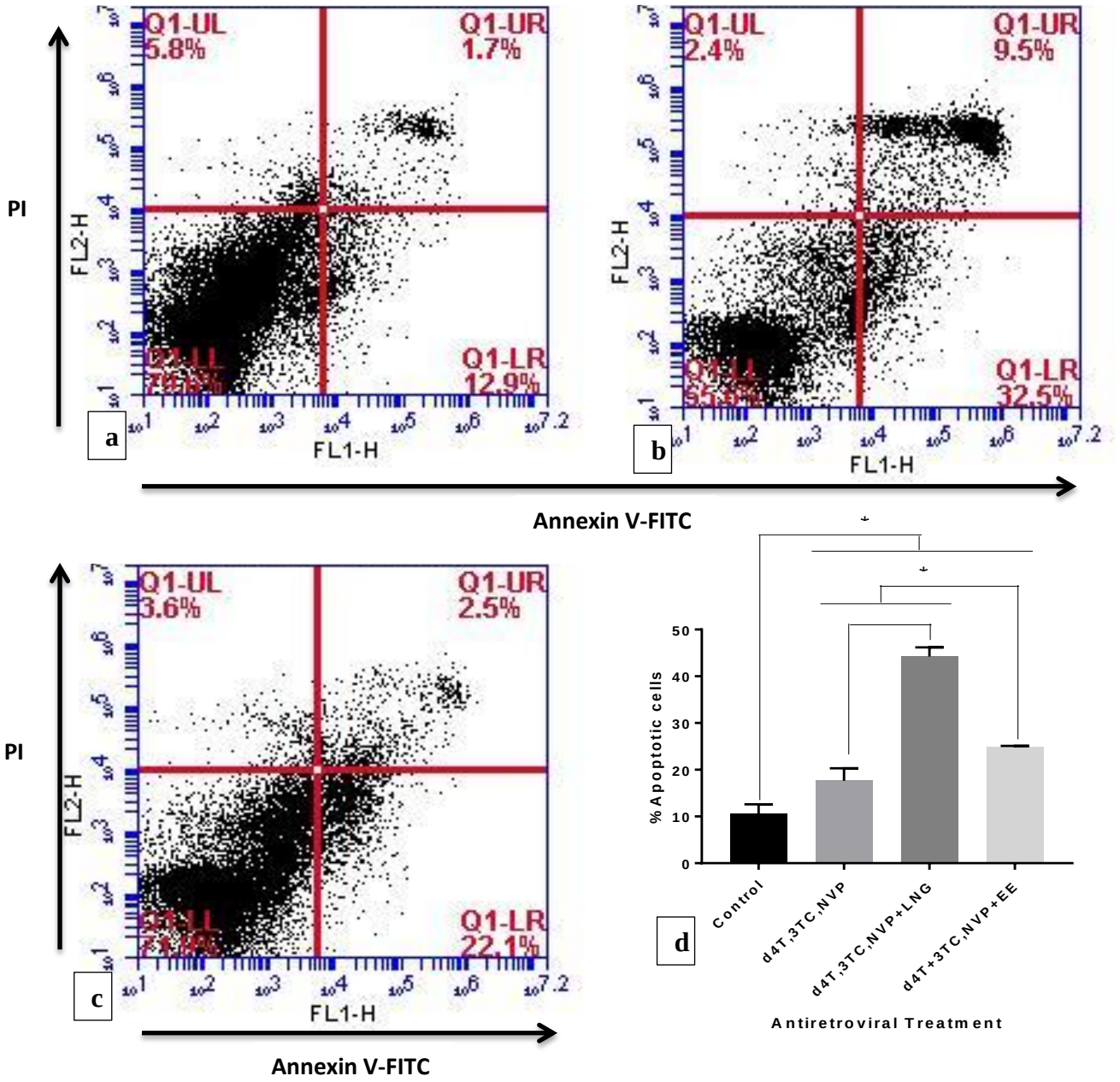

Fig 4.12: Flow cytometry analysis of HeLa cells pre-treated with a combination of d4T, 3TC and NVP with or without levonorgestrel (LNG) or ethinylestradiol (EE). The cells were treated by d4T+3TC+NVP (A), d4T+3TC+NVP with LNG (B) or d4T+3TC+NVP with EE (C) for 48 hr followed by staining with annexin V and PI. Annexin V and PI negative cells (Q1-LL) depict viable cells in the population (n=1). Cells in the lower right quadrant (Q1-LR) are in the early stages of apoptosis. These HeLa cells are annexin V-positive and PI-negative. Cells located in the upper right quadrant (Q1-UR) are positive for both annexin V and PI. These cells are in late stages of apoptosis. Cells in the upper left quadrant (Q1-UL) represent dead and necrotic cells. D: Effect of LNG and EE on d4T+3TC+NVP-induced apoptosis in HeLa cells. For each individual experiment, apoptotic cells are a sum of both early and late apoptotic cells. Data were analysed by one-way ANOVA. The Tukey's test adjusted for multiple comparisons among different treatments. Data represent means ± SD of three independent experiments (*P≤0.05).

4.4. Discussion

The overlap of some early apoptotic cells into the bottom left quadrant suggest signal spill-over from the FITC into the PI read out channel. This could also have been influenced by the gating strategy, hence possibly contributing to a false-negative in some early apoptotic cells. Most flow cytometers detect PI fluorescence in the FL2 channel. However it is recommended to detect the PI signal in the FL3 emission, as this minimises the compensation needed to correct for signal spill-over (Immunochemistry Technologies, n.d.). The present study detected PI fluorescence in the FL2 channel. False negative results for early apoptotic cells could also have resulted from excessive incubation periods in some independent experiments.

Treatment of HeLa cells with single 50µg/ml of each single antiretroviral drug was shown to induce apoptosis drastically in human cervical epithelial cancer cells. In Chapter 3, it was demonstrated that all the five antiretroviral drugs (abacavir, zidovudine, lamivudine, stavudine and nevirapine) have genotoxic effects in HeLa and Chinese hamster ovary cells. It was shown that NRTIs induce double-strand breakage. This increased apoptosis is possibly due to membrane-associated activation of apoptotic response to DNA damage-induced cell death. This could be attributed to deregulation of DNA response coupled with intrinsic stimuli of mitochondrial apoptotic pathway associated with genotoxic stress (Matt and Hofmann, 2016; Czabotar *et al.*, 2014). In Chapter 3, it was shown that nevirapine, which acts by non-competitive inhibition of DNA polymerase, also induces HeLa cell double-strand breaks. NVP administration also significantly increases apoptosis compared to untreated cells, a phenomenon that concurs with its reported carcinogenicity (Brambilla *et al.,* 2012). These observations therefore suggest that human cervical epithelial cells are inevitably exposed to some form of DNA damage from the antiretroviral drugs. If uncontrolled, such damage may impair important metabolic processes such as DNA-repair mechanisms and apoptotic removal of DNA-damaged cells (Toettcher *et al.*, 2009). Our cells have evolved inherent repair mechanisms to compensate for various types of DNA damage. Apoptosis is programmed cell death, which aids removal of damaged cells, hence the observed increase in apoptosis following exposure to antiretroviral drugs.

The present results demonstrate that treatment with ABC alone drastically increases apoptosis (early + late apoptosis) in HeLa cells. Although co-administration with EE significantly reduces ABC-induced apoptosis (from 32% to 29%), the relative rate of apoptosis is still drastically higher compared to untreated cells whose mean percentage is

11%. In their compendium, Brambilla *et al.* (2012) highlight that ABC was shown to be carcinogenic. In addition to inhibition of telomerase in HT29 cells (Hakuzalie *et al.*, 2012), in Chapter 3, it was shown that ABC induces double-strand breaks in HeLa cells. The present results suggest that, in the advent of these unwanted effects, the apoptotic pathway is triggered to get rid of unrepaired cells. However, as shown in Chapter 2, over a period of 72 h there is no significant difference in proliferation between MTT proliferation assays for ABC-treated and untreated cells. These results therefore suggest apoptosis-induced proliferation by adjacent HeLa cells in response to cell cycle arrest as they initiate apoptosis. Alternatively, this could be due to compensatory proliferation (CP) as mitogens are secreted in response to cells lost by apoptosis (Ryoo *et al.*, 2004; Fan and Bergmann, 2004). This is therefore suggestive of regenerative hyperplasia as cells become excessively mitogenic in a bid to replace lost cells.

A number of studies are in concurrence with the observed proliferative effects of oestrogens on cancer cells. As outlined earlier, Mosli *et al.* (2013) demonstrated that *in vitro* exposure of non-transformed prostate epithelial cells (BPH-1) to catechol oestrogens (CEs) had a neoplastic effect. They further demonstrated that the CE 4-hydroxyestradiol (4-OHE_2) showed more carcinogenicity relative to the parent hormone 17β-oestradiol (E2). It has also been shown that oestrogen has proliferative effects in normal and MCF-7 breast cancer cells (Scaling *et al.*, 2014; Pesiri *et al.*, 2015). In MC7 10A cells, oestrogen stimulation in the presence of GPER-selective agonist G-1 was shown to increase the mitogenic index (Scaling *et al.*, 2014).

As observed in Chapter 2, upon co-administration of ABC with 1.0µg/ml, 2.0µg/ml and 4.0µg/ml LNG, the reduction in cell viability suggests antiproliferative activity. In a related study, LNG was shown to inhibit human endometrial stromal cell (HESC) and human endometrial glandular cell (HEGC) proliferation while inducing apoptosis in both cell lines (Zhao *et al.*, 2015). The levonorgestrel intra-uterine system (LNG-IUS) has since been shown to induce regression of complex endometrial hyperplasia (Wildemeersch *et al.*, 2015; Haoula et *al.*, 2011; Zhao *et al.*, 2015). LNG-IUS down-regulates expression of PRA, PRB, ER-α and ER-β (Vereide *et al.*, 2006; Gomes *et al.*, 2009). Complex atypical hyperplasia was also shown to decrease after progestin therapy (Gunderson *et al.*, 2014; Kudesia *et al.*, 2014). This suggests that, the observed decrease in enzymatic reduction of MTT results from the increased rate of apoptosis. Interestingly, at 0.5µg/ml and 1.0µg/ml of EE, ABC-treated HeLa cells show no significant difference in reduction of MTT,

relative to untreated cells. The present results suggest that LNG does not have an effect on apoptosis induced cellular remodelling in ABC-treated cells, while EE increases HeLa cell viability.

Compared to untreated cells, ZDV significantly increases apoptosis in HeLa cells from 11% to 37%. While EE does not alter the rate of ZDV-induced apoptosis, LNG significantly decreases the rate of ZDV-induced apoptosis to 27%. This rate still seems a bit higher, as rates of 15-20% seem sufficient for cancer cell elimination and/or antiproliferative activity. As shown in Chapter 3, ZDV also induces a time-dependent DNA double-strand breakage in both HeLa and CHO cells. ZDV has been shown to inhibit telomerase in human HT29 cells (Brambilla *et al.*, 2012). Furthermore, ZDV was also shown to exhibit cytogenetic changes in *Allium cepa* root cells (Onwuamah *et al.*, 2014). Compounded with the well-established genotoxic effect of ZDV (Momot *et al.*, 2014, Yu *et al.*, 2009), the presently-observed drastic apoptosis associated with its lone administration seems a natural elimination method for cells with undesired genetically modified characteristics. In Chapter 2, this study evaluated the role of EE and LNG on HeLa cell viability. Lone administration of ZDV was shown to have a time-dependent decrease in reduction of MTT. LNG co-administration maintains the anti-proliferative effects at all concentrations (1.0µg/ml, 2.0µg/ml and 4.0µg/ml), thus depicting apoptosis-dependent decrease in MTT reduction. Upon co-administration of ZDV with 0.5µg/ml EE, HeLa cells do not depict a difference in reduction of MTT, relative to untreated cells. Cognisant of the significant increase observed in HeLa cells apoptosis upon this co-treatment of ZDV with 0.5µg/ml EE, these observations are suggestive of EE resulting in improved viability of HeLa cells as they regenerate themselves (Ryoo *et al.*, 2004). Further increases in EE concentration to 1.0µg/ml and 2.0µg/ml significantly reduce the rate of proliferation, thus suggesting increased cell loss and possibly tissue damage.

Lone administration of 3TC drastically increases apoptosis in HeLa cells from 11% to 31%. Co-administration with EE does not show a difference in the rate of apoptosis while LNG drastically reduces 3TC-induced apoptosis to 21%. McGuigan *et al.* (2013) demonstrated the cytostatic activity of 3TC on HeLa cells. As shown in Chapter 3, the potential of 3TC to induce double-strand breaks in HeLa and CHO cells was also demonstrated. The present observations on lone 3TC administration suggest apoptotic removal of genotoxically damaged HeLa cells cognisant of the role of apoptosis in removal of DNA-damaged cells (Matt and Hofmann, 2016). From cell viability assays in Chapter 2,

administration of 3TC only depicts a time-dependent decrease in cell proliferation. Co-administration of 3TC with LNG does not show a difference in rate of reduction of MTT relative to the control. Since the present results have shown a significant increase in the rate of apoptosis, this non-significant relationship therefore suggests cellular remodelling for compensatory proliferation. Similarly, the increase in cell viability upon 3TC co-administration with 0.5µg/ml and 1.0 EE suggests EE-induced cellular remodelling and possibly regenerative hyperplasia.

In Chapter 3, it was shown that d4T induces double-strand breaks in both HeLa and CHO cells. This suggests that eversion of DNA-repair triggers the observed drastic apoptosis observed in these HeLa cells treated with d4T. Co-treatment with both 4.0µg/ml LNG and 0.5µg/ml EE reduces d4T-induced apoptosis from a mean of 39% to 31% and 17% respectively. While EE-induced apoptosis seems optimal (<20%) for beneficial inhibition of cancer cell proliferation, the MTT proliferation assay (Chapter 2) depicts no difference if compared to untreated cells. This is suggestive of EE-induced proliferation and mitogenic activity. Alternatively, it is important to note that cells in early apoptosis may still have the potential to reduce MTT in the assay and thus may be quantified as live cells (Brummati, 2008; Ryoo and Bergmann, 2012; Shi *et al.*, 2012). In addition to too-drastic apoptosis, co-treatment with LNG similarly suggests apoptosis induction of cellular remodelling in d4T-treated cells as shown by no significant differences observed in the MTT proliferation assay.

In Chapter 3, a time-dependent induction of double-strand breaks in both HeLa and CHO cells treated with NVP was observed. Its metabolite, 12-hydroxy-NVP, has been shown to be mutagenic (Kranendonk *et al.*, 2014). The present results depict a drastic increase in HeLa cell apoptosis upon treatment with NVP, possibly following deregulation of DNA repair for some excessively damaged cells. Both EE and LNG decrease NVP-induced apoptosis in HeLa cells. From Chapter 2, lone administration of 50µg/ml NVP shows a time-dependent decrease in reduction of MTT. Similarly, co-treatment with 0.5µg/ml EE and all concentrations of LNG (1.0µg/ml, 2.0µg/ml and 4.0µg/ml) also depict significant antiproliferative activity, thus suggesting that 0.5µg/ml EE and 4.0µg/ml LNG reduces NVP-induced apoptosis. This reduction from 39% to 25% and 24%, respectively, may suggest attainment of optimal apoptosis levels sufficient to eliminate cancer cells without too drastic cell loss and possibly tissue damage.

17β-Oestradiol has antiapoptotic effects in normal and cancer cell lines (HT3 and CaSki, respectively) through attenuation of caspase-9 and terminal caspase-3 activity (Wang et *al.*, 2004). Our results showed that ethinylestradiol, a derivative of 17β-oestradiol, also significantly reduces ABC, d4T and NVP-induced apoptosis. On the contrary, no significant change was observed for ZDV and 3TC co-treatment with ethinylestradiol. While this later observation may suggest non-attenuation of caspases 3 and 9 described for 17β-oestradiol, an evaluation of the mechanism(s) of apoptotic pathways in single drug co-administration with ethinylestradiol could be interesting. Webster *et al.* (2001) demonstrated an increased level of apoptosis induction by HPV types 16E2 and E7 proteins in the presence of progesterone. Interestingly, in the present study, levonorgestrel (a synthetic progestogen) had no significant effect on ABC-induced apoptosis, while it significantly decreases 3TC, ZDV, d4T and NVP-induced apoptosis. While investigation of the mechanism for this decrease in apoptosis was beyond the scope of this research, such an investigation may offer a plausible *in vitro* explanation of cancerous cell viability and regeneration following co-administration of antiretroviral drugs and combined oral contraceptive hormones.

The present results also demonstrate the varied effect of contraceptive hormones (levonorgestrel and ethinylestradiol) on antiretroviral drug-induced apoptosis in HeLa cells. These results demonstrate that EE but not LNG decreases ABC-induced apoptosis in HeLa cells. On the hand, LNG but not EE decreases ZDV- and 3TC-induced apoptosis in HeLa cells. Both EE and LNG reduce d4T- or NVP-induced apoptosis in HeLa cells. The observed decrease in apoptosis following co-administration of contraceptive hormones suggests their association with increased risk for cells to mutate and escape apoptosis. EE has a more drastic effect than LNG on d4T-induced apoptosis. This suggests that different contraceptive hormones exhibit different potential of altering antiretroviral-induced apoptosis. This also suggests apoptosis dependence on the nature of the antiretroviral drug that induces the baseline apoptosis. Similarly, this allows understanding of varying neoplastic and cancer cell proliferation with differing antiretroviral drug treatments. The problem remains that of establishing the optimum levels of apoptosis. This is because, in all single-drug treatments (with or without hormone co-treatment), apoptosis is increased in HeLa cells compared to the control cells. Cognisant of the fact that too much apoptosis may be pathological while optimum rate is beneficial (Matt and Hofmann, 2016; Logue *et al.*, 2009; Brummatti *et al.*, 2008), there is need to outline the desired balances for induction of apoptosis.

While discussion of single-drug effect is important for understanding the mechanistic effect of antiretroviral toxicity, combination antiretroviral treatment is the modern day approach to HIV/AIDS treatment, since single ARV treatment has been widely discontinued due to development of viral resistance (WHO, 2010; BHIVA, 2008). In practice, administration of these drugs is predominantly in triple combinations as discussed previously. Alternatively, the double combinations ABC+3TC and ZDV+3TC can be administered in combination with a protease inhibitor (AIDSinfo, 2017).

One may argue that the results from Figures 4.9, 4.10, 4.11 and 4.12 become of marginal interest in the light of the combinatorial effect (that is, more drug treatment leads to more cell death). It is however imperative to note that combinatorial, not single, antiretroviral treatment is the modern-day preferred treatment method (AIDSinfo, 2017). Overall, results for combination antiretroviral treatment demonstrate a decreased effect on apoptosis relative to single antiretroviral treatment. To this effect, the effect of contraceptive hormones on highly active antiretroviral drug-induced apoptosis becomes of interest. While these results may suggest evasion of apoptosis compared to single drug treatment, the combinations ABC+3TC, ZDV+3TC, ABC+ZDV+3TC and d4T+3TC+NVP still depict a relatively high rate of apoptosis compared to untreated HeLa cells. However, there is a drastic decrease in apoptosis, resulting in no significant difference in apoptosis between ZDV+3TC+NVP-treated and untreated cells. Co-administration of both 4.0μg/ml levonorgestrel and 0.5μg/ml ethinylestradiol drastically increased triple combination antiretroviral drug (ABC+ZDV+3TC, d4T+3TC+NVP and ZDV+3TC+NVP)-induced apoptosis. Similarly, the two hormones increase ZDV+3TC-induced apoptosis in HeLa cells. While 4.0μg/ml levonorgestrel co-treatment significantly increases ABC+3TC-induced apoptosis in HeLa cells, there are no differences observed following 0.5μg/ml ethinylestradiol co-treatment. The present results suggest increased human cervical cancer cell death by apoptosis following co-administration of all combination antiretroviral drugs (ABC+3TC, ZDV+3TC, ABC+ZDV+3TC, d4T+3TC+NVP and ZDV+3TC+NVP) with levonorgestrel.

While combination treatments increase apoptosis relative to untreated cells in the absence of hormones, of interest is their overall lower apoptotic effects compared to single antiretroviral treatments. With the exception of ABC+3TC, that induces more than 20% apoptosis, the rest of the treatments cause average apoptosis percentages below 20%.

Curiously, this study thus reported lower toxicities for combination antiretroviral drug treatment compared to single drug treatment. Bulusu *et al.* (2016) maintain that drug combination therapy has demonstrated more effective cell growth inhibition than administration of either agents alone in cancer treatment. During cancer treatment, multi-drug therapy was shown to be less toxic in small combination doses, while at the same time exhibiting a high therapeutic effect (Bulusu *et al.,* 2016). The observed decrease in cervical neoplastic transformation and reduced cancer cell proliferation among some patients undergoing HAART (Adler *et al*., 2012; Finhaber *et al*., 2012; Omar *et al.*, 2011; Minkoff *et al*., 2010) could be due to the additive and/or synergistic effect of the constituent antiretroviral drugs. However, in the present study, it is interesting to note that the combination ZDV+3TC+NVP does not alter the rate of apoptosis in the absence of contraceptive hormones. These results therefore envisage that, in the absence of hormonal contraceptives, *in vitro* evidence suggests that ZDV+3TC+NVP treatment results in evasion of apoptosis thus leading to growth of the human cervical epithelial cell. Co-administration of ZDV+3TC+NVP with both ethinylestradiol and levonorgestrel increase the rate of apoptosis thus suggesting a protective effect of both hormones through increased elimination of the cancerous cells.

The combination drug ABC+3TC is commonly used in first-line treatment simultaneously with a protease inhibitor (BHIVA, 2008). In this *in vitro* study, it was shown that ABC+3TC induces apoptosis in human cervical epithelial cancer cells. As demonstrated in Chapter 2, there is a time-dependent decrease in HeLa cell viability upon lone administration of ABC+3TC-treated cells as depicted by the MTT assay. Interestingly, as demonstrated in Chapter 2, co-administration of 0.5µg/ml or 1.0µg/ml of EE does not significantly alter cell viability (from MTT) of ABC+3TC treated cells, despite the high percentage rate of apoptosis observed with co-treatment for 48 h. Induction of apoptosis does not necessarily imply inhibition of cellular remodelling (Shi *et al.,* 2012). Apoptosis induction may lead to mitogen stimulation which, in turn, stimulates proliferation of adjacent cells in a bid to replace lost cells through compensatory proliferation (CP) also known as apoptosis-induced proliferation (Ryoo and Bergmann, 2012; Fan and Bergmann, 2008b). As discussed earlier, a number of studies have also demonstrated the proliferative effect of oestrogens on different cancer cells (Pesiri et *al.*, 2015; Scaling *et al*., 2014; Atawia *et al.*, 2013).

This *in vitro* observation concurs with some epidemiological observations suggesting no effect of HAART on neoplasia progression (Ezechi *et al*., 2014; Shrestha *et al*., 2010; Sirera *et al*., 2008). It is however imperative to note that, in the MTT assay, cells in early apoptosis still have the ability to reduce MTT, possibly resulting in false positive proliferative assay (Shi *et al.,* 2012; Brummati *et al.,* 2008). Although the MTT assay is relatively faster (Sieuwerts *et al*., 1995; Sylvester 2011) complimenting this assay with the viable cell count would have gone some way to improve the viability analysis. On the contrary, it has since been established that cervical cancer is a hormone dependent tumour that may be activated by oestrogens that act as cofactors (Practice Committee of American Society for Reproductive Medicine, 2008; Jiang *et al.*, 2016).

Modern day approaches in combined hormonal contraception is to reduce the amount of oestrogens in COCs as much as is possible without affecting efficacy of the contraceptive pill (Blackburn *et al*., 2000). Otherwise, apoptosis-dependent regeneration of cells may lead to regenerative hyperplasia (Ryoo and Bergmann, 2012). Co-administration with 4.0µg/ml LNG further increases apoptosis in ABC+3TC-treated cells and this is associated with decreased cell proliferation, hence suggestive of increased cancer cell death. However, it may be important to titrate for LNG concentrations that do not depict too drastic apoptosis *in vivo*, cognisant of the established protective effect of progestin against endometrial hyperplasia (Vereide *et al.,* 2014; Gomes *et al.,* 2009). Similarly, the levonorgestrel intra-uterine system (LNG-IUS) has since been shown to induce regression of complex endometrial hyperplasia (Wildemeersch *et al.,* 2015; Haoula *et al.,* 2011; Zhao *et al.,* 2015).

Combivir (ZDV+3TC) is another double antiretroviral combination used in combination ARV treatment in conjunction with a protease inhibitor. In the present study, administration of this combination significantly increases apoptosis of human cervical epithelial cells from a mean of 11% to 17 ($P<0.05$). The MTT proliferation assay for ZDV+3TC-treated cells described in Chapter 2 depicts a time-dependent decrease in proliferation. These observations suggest a predominantly apoptotic-dependent antiproliferative activity of ZDV+3TC devoid of compensatory proliferation. If maintained within optimal levels, apoptosis is physiologically beneficial in removal of unwanted cells, while excessive apoptosis may lead to pathophysiology such as loss of cells and tissue damage (Matt and Hofmann, 2016; Dexheimer, 2013). In the absence of contraceptive hormones, the present results suggest a protective effect of ZDV+3TC against the

proliferation of human cervical epithelial cancer cells. Co-administration with 4.0µg/ml LNG or 0.5µg/ml EE drastically increases ZDV+3TC induced apoptosis ($P<0.05$) to averages of 45% and 41% respectively. The proliferation assay for ZDV+3TC co-administration with 4.0µg/ml LNG depicts no difference in viability compared to untreated cells (Chapter 2). On the contrary, no difference is observed in the reduction of MTT between untreated cells and those exposed to 0.5µg/ml EE co-treatment with ZDV+3TC. In view of the high rate of apoptosis in these 0.5EE treated cells, this present observation suggests cellular remodelling activity of EE. If uncontrolled, this compensatory proliferation could be one way in which EE acts as a co-factor for continuous human cervical epithelial cell proliferation characterised by regenerative hyperplasia.

The combination ABC+ZDV+3TC is a triple NRTI commonly used in HIV/AIDS treatment. Upon its lone administration, the regimen ABC+ZDV+3TC significantly increases the rate of apoptosis in HeLa cells ($P<0.05$) from 11% to 21% . In Chapter 2, it was demonstrated that treatment of HeLa cells with this combinatorial regimen depicts a time-dependent decrease in viable cells, hence suggesting the beneficial anticancer effect of its lone administration. Upon co-administration with either 4.0µg/ml LNG or 0.5µg/ml EE, the corresponding MTT reduction assays in Chapter 2 show no statistical difference compared to untreated cells, thus suggesting that apoptosis did not inhibit remodelling. This again suggests apoptosis-induced remodelling by those cells which are undergoing apoptotic changes (Ryoo and Bergmann, 2012; Jiang and Wang, 2016). In the present experimental set-up, it remains obscure whether this perceived contraceptive hormone-stimulated cellular regeneration remains under control to avoid hyperplasia and possibly cancer.

For triple combination treatments, overall in the absence of hormones, only the HAART regimen ZDV+3TC+NVP did not significantly change the rate of apoptosis if compared to untreated control cells. This suggests that the antiproliferative effect of ZDV+3TC+NVP observed with the MTT assay in Chapter 2 is predominantly due to inhibition of proliferation relative to untreated cells, since inability to reduce MTT is an indicator of reduced cellular viability or metabolic activity (Sieuwerts *et al*., 1995; Sylvester 2011; van Meerloo *et al*., 2011; Shi *et al.,* 2012). One way of such inhibition could be through the cell cycle arrest. These observations concur with the decrease in cervical neoplastic transformation and reduced cancer cell proliferation among some patients undergoing HAART (Adler *et al*., 2012; Firnhaber *et al*., 2012; Omar *et al.*, 2011; Chen *et al*., 2014;

Minkoff *et al* 2010). Bulusu *et al.* (2016) and Murugan *et al.* (2016) postulate that the additive and/or synergistic effect of combinatorial drug delivery may be beneficial through reduction in toxicity of individual drugs. Co-administration with either levonorgestrel or ethinylestradiol drastically increases apoptosis in ZDV+3TC+NVP-treated human cervical epithelial cells ($P<0.05$), thus suggestive of increased cancer cell loss. This increased toxicity upon co-administration of hormonal contraceptives is suggestive of causality of cervical intraepithelial neoplasia progression or other serious non-AIDS events observed with some cohort studies (Lillo et *al.*, 2001; McKenzie *et al*., 2011).

The HAART regimen d4T+3TC+NVP has been among the recommended ARVs in low-resource settings until recently, when the World Health Organisation recommended withdrawal of d4T due to its observed toxicity (WHO, 2010). However the regimen still finds use in low-resource countries. Lone administration of d4T+3TC+NVP increases HeLa cell apoptosis from 11% to 18%, a percentage moderate enough to impact non-drastic cell loss with limited cell tissue damage. Coupled with the time-dependent decrease in HeLa cell proliferation as shown in Chapter 2, this is suggestive of the beneficial antiproliferative effect of this combinatorial treatment. Co-treatment with 0.5µg/ml EE and 4.0µg/ml LNG further significantly increases apoptosis to 25% and 44%, respectively. This is also supported by a decrease in HeLa cell MTT viability assay in Chapter 2 where there is a corresponding loss of MTT reduction rate at these co-administered concentrations. The too-drastic loss of cells following this co-administration of hormones is suggestive of increased cell loss and possibly tissue damage.

Although combination ARV treatment with regimens ZDV+3TC, ABC+ZDV+3TC and ZDV+3TC+NVP was shown to induce genotoxicity in new-born monkeys (Olivero *et al.*, 2013), overall the present results demonstrate higher toxicity for single-drug treatment compared to combination treatment. The range of apoptosis (early + late) in single drug treatment is 31-39% (n=5, mean=35.6%) while the range for combination treatment is 12-26% (n=5; mean=18.9%). In separate *in vivo* studies carried out on mice, Combivir + EFV did not observe any significant DNA damage with the genotoxicity and micronucleus test (de Moraes Filho *et al.*, 2016). Such reduced toxicity results in limited but beneficial elimination of cancerous cells through apoptosis and/or cell cycle arrest. In the absence of contraceptive hormones, our results for combination ARV treatment of HeLa cells suggest anticancer activity as depicted by the time-dependent decrease in cell proliferation observed in Chapter 2. On the contrary, the higher rate of apoptosis observed with single-

drug treatment is suggestive of increased loss of cells and possibly tissue damage. These observations are in support of the established reduced toxicity of combination ARV treatment, compared to single drug therapy which was used prior to 2000 (BHIVA, 2008; WHO, 2010).

4.4.1. Conclusion

Table 4.4 below summarises the effect of antiretroviral drug co-treatment with levonorgestrel and ethinylestradiol on human cervical epithelial cancer cell apoptosis.

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Table 4.4: Effect of antiretroviral drug co-treatment with hormones on HeLa cell apoptosis

Single drug	No hormone	Effect of LNG on drug induced apoptosis	Effect of EE on drug induced apoptosis
ABC	++	++	++
ZDV	++	++	++
3TC	++	+	++
d4T	++	++	+
NVP	++	+	+
Combination drug	**No hormone**	**Effect of LNG on drug induced apoptosis**	**Effect of EE on drug induced apoptosis**
ABC+3TC	+	++	++
ZDV+3TC	+	++	++
ABC+ZDV+3TC	+	++	++
ZDV+3TC+NVP	-	++	++
d4T+3TC+NVP	+	++	+

The table summarises the effect of single and combination antiretroviral drug co-treatment with levonorgestrel and ethinylestradiol on HeLa cell apoptosis following a 48 h treatment. The results show the comparison of each treatment to the untreated control where: (+) represents significant differences with ≤ 25% increase in apoptosis (early+late) compared to the untreated control, while (++) shows a more drastic increase in the rate of apoptosis (> 25% compared to the untreated control) and (-) demonstrates no significant difference between the drug-treated cells and the untreated control. The rate of apoptosis among drug-treated cells and the control was compared using Tukey's multiple comparison test ($P \leq 0.05$).

While single-drug treatment evaluation provides an insight into the mechanistic approach to understanding the role of hormonal contraceptives on antiretroviral drug-induced apoptosis, combination antiretroviral therapy is of interest, as it is the predominantly administered form of therapy. Single NRTIs and NVP drastically induce HeLa cell apoptosis compared to combination ARV treatment. In the absence of contraceptive hormones, the present results depict a protective effect of combination antiretroviral treatment against the proliferation of human cervical epithelial cancer cells through moderate rates of increased apoptosis relative to untreated cells. NTRIs combination drug-induced apoptosis is drastically increased with 4.0µg/ml levonorgestrel co-treatment. Similarly 0.5µg/ml EE drastically increases all combination drug-induced apoptosis, with the exception of cells treated with ABC+3TC. This too drastic increase in human cervical epithelial cancer cell apoptosis is suggestive of increased cell loss and possibly tissue damage.

Curiously, it was observed that, despite the drastic induction of apoptosis by EE, ABC+3T, ZDV+3TC, ABC+ZDV+3TC and ZDV+3TC+NVP did not significantly differ to the untreated control in their effects on the reduction of MTT. This is suggestive of a cellular remodelling effect of EE and possibly regenerative hyperplasia. Treatment with LNG only demonstrates such non-proliferative difference upon co-administration with ABC+ZDV+3TC and ZDV+3TC+NVP. This suggests that the increased cytotoxic activity of LNG in combination with other regimens (ABC+3T, ZDV+3TC and d4T+3TC+NVP) does not positively impact on tissue regeneration through proliferation or compensatory hyperplasia but it rather results in increased human cervical cancer cell loss. Interestingly, at 2.0µg/ml, LNG showed antiproliferative activity with all drug combination, thus suggesting a protective effect against the human cervical epithelial cancer cell proliferation.

CHAPTER 5

ANTIRETROVIRAL DRUG-INDUCED TRANSFORMATION OF U937 CELLS TO ADHERENT CELLS

5.1.0. Introduction

The increased use of highly active antiretroviral therapy (HAART) has resulted in dramatically increased survival of HIV-infected individuals. A downside of the success of antiretroviral therapy (ART) is the premature onset and increased risk of certain inflammatory age-related diseases associated with low levels of chronic immune activation (Hsu *et al.*, 2013). Such activation is thought to contribute to what is described as serious non-AIDS events (SNAEs) (Mafuva and Morgan, 2016, Hsu and Sereti, 2013). SNAEs include malignancies, cardiovascular events, bone diseases as well as renal and hepatic diseases (Hsu and Sereti, 2013; Serrano-Villar *et al.*, 2014; Lapadula *et al.*, 2015). HIV, microbial translocation, Hepatitis B virus (HBV) and Hepatitis C virus (HCV) may lead to immune activation (Hsu *et al.*, 2013; Lapadula *et al*., 2013).

HIV infection may result in a decline in CD4 T cells (immunodeficiency, hence directly contributing to SNAEs). Antiretroviral toxicities have also been suggested in direct contribution to SNAEs (Hsu *et al.*, 2013). Blood monocytes contain CD4 molecules (Zhen *et al*., 2014). The expression of CD4 molecules in human monocytes enables the use of the CD4 count as a standard diagnosis in quantifying monocytes (Zhen *et al*., 2014; Filion *et al*., 1990). The normal CD4 cell count in a healthy individual ranges from 500-1500cells/mm^3. HIV infection results in CD4 levels below 500 cells/mm^3. HAART treatment should be initiated as soon as one is diagnised as HIV^+ (AIDSinfo, 2017).

Acute myeloid leukaemia (AML) results from an increased number of myeloid cells whose maturation has been arrested, hence resulting in haematopoietic insufficiency (Le Dieu *et al*., 2009). Induction of differentiation in AML cells may provide an alternative therapeutic strategy (Meshkini *et al*., 2009, Parihar *et al*., 2009). A number of compounds have been shown to differentiate AML cells towards granulocyte and/or macrophage morphology (Studzinski, 2001; Deng *et al*., 2014). In addition to differentiation, all-*trans*-retinoic acid and phorbol 12-myristate 13-acetate (PMA) cause apoptosis in leukaemia cells (Meshkini *et al.*, 2009). To this effect, PMA has found widespread use as a positive control in monocyte differentiation studies (Meshkini *et al*., 2009, Parihar *et al*., 2009). There is paucity of information regarding the role of antiretroviral treatment on monocyte differentiation, as this may provide a therapeutic strategy for the cancer cells. An

introspection into this may provide a plausable explanation for SNAEs associated with antiretroviral therapy or the ARV potential anticancer activity envisaged in Chapter 4.

The U937 cell line, a derivative of a histolytic lymphoma, is widely used in differentiation studies involving immune activation, such as monocyte conversion to macrophages and dentric cells. Differentiation can be induced by different stimuli including PMA, other chemical compounds and hormones (Minafra *et al.*, 2011; Song *et al,* 2015; Vrana *et al.*, 1999). PMA-dependent transformation of U937 cells is both concentration- and time-dependent. U937 conversion into macrophages or dendritic cells in the presence of PMA is dependent on the *in vitro* micro-environment (Garcìa *et al.*, 1999; Martinez & Gordon, 2014; Studzinski, 2001). For example, Garcìa *et al.* (1999) demonstrated that in the presence of PMA, NF-kappa B (NFκB) proteins that regulate immune responses, inflamatory responses, cellular growth as well as apoptosis, U937 differentiation is inhibited. Similarly PU.1, a transcription protein containing oligonucleotides, inhibited differentiatiation (Garcìa *et al.*, 1999). Whether or not combination ARVs elicit differentiation pathways requires further evaluation. Seeding densities have been shown to affect growth with cell counts below $2x10^4$/ml depicting poor cellular differentiation for HL60 cells (Studzinski, 2001). These observations demonstrate that variations in the *in-vitro* culture environment affect the monocyte differentiation. Optimal seeding densities of $3x10^5 \pm 1x10^5$ cells/ml have been recommended for monocyte differentiation studies, although some other variations have been successful (Vrana *et al.*, 1999; Studzinski, 2001).

Macrophages are adherent cells that constitute a heterogenous group of cells associated with innate immunity. Hao *et al.* (1999) describe uncharacterised adherent cells derived from U937 as 'macrophage-like' cells. M1 macrophages eliminate pathogens, virus-infected cells and cancer cells. These M1 macrophages are associated with reactive oxygen species (ROS) and inducible nitric oxide (iNOS) production (Housinkveld and van der Burg, 2011). M2 macrophages have been associated with tumour promotion. These M2 macrophages polarise T cells, hence dampening immune response (Housinkveld and van der Burg, 2011; Martinez and Gordon, 2014). While classification of the transformed adherent cells by immunological methods is beyond the scope of this research, it is interesting to enquire into the ability of the transformed cells to produce reactive oxygen species. The present study evaluates the ability of antiretroviral transformed U937 cells to produce the superoxide anion ($\bullet O_2^-$). The superoxide anion has since been associated with

regulation of apoptosis, proliferation and immune response (Hancock *et al*., 2000; Choi *et al.*, 2006).

Reactive oxygen species (ROS) are a heterogenous group that includes radicals such as the superoxide anion ($\bullet O_2^-$), hydroxyl radical (OH•) and nitric oxide (NO•). Non-radical ROS include hydrogen peroxide (H_2O_2), hydroxide ion (OH^-), singlet oxygen ($^1O_2$) and peroxy nitrite ($OONO^-$). ROS can exhibit both intra- and extracellular messanger properties that include induction of apoptosis, gene expression and cell signalling (Hancock *et al.*, 2000; Vara and Pula, 2014). ROS can regulate cell proliferation, cell differentiation and immune response (Hancock *et al.*, 2000). The superoxide anion, together with other reactive oxygen species such as H_2O_2, has been predominantly asssociated with oxidative burst (Hancock *et al.*, 2000, Choi *et al*., 2006). There should be physiological balances between reactive oxygen species produced as an immune response and their degradation. Superoxide dismutase (SOD) catalyses the following reaction:

$$2(\bullet O_2^-) + 2H_2O \rightarrow 2H_2O_2 + O_2$$

Catalase, a member of the glutathione peroxidases, then catalyses the following reaction:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Respiratory burst, which is increased generation of reactive oxygen species, has been associated with NADPH-catalysed production of oxygen species by phagocytic cells. High ROS levels may lead to DNA damage and/or oxidative stress (Meshkini *et al*., 2009) thus possibly associated with SNAs. It has since been established that PMA-induced differentiation of U937 to macrophages results in increased nitroblue tetrazolium (NBT) reduction. To this effect, the modified colorimetric nitroblue tetrazolium (NBT) assay has been used to determine quantitatively the production of superoxide by phagocytic cells. This method is ideal for determination of even trace amounts superoxide anions produced in monocytes and macrophages (Choi *et al.*, 2006; Deng *et al.*, 2014).

U937 Differentiation can be induced by various agents that induce signal transduction pathways, such as hormones and PMA (Minafra *et al*., 2011; Song *et al.*, 2015; Vrana *et al.*, 1999). There are limited *in vitro* studies on single and combination antiretroviral-induced premonocyte differentiation into adherent cells. In this section, the role of single and combination antiretroviral treatment on U937 differentiation into adherent cells with 'macrophage-like' characteristics and ultimately the effect of this drug treatment on the ability to reduce NBT was assessed.

5.1.1. Objectives of the chapter

a. To evaluate the morphological changes on U937 cells after 72 h single and combination antiretroviral treatment.
b. To evaluate single and combination ARV potential to transform premonocyte U937 cells into adherent cells following 72 h treatment.
c. To measure the amount of intracellular superoxide anion produced duing 72 h treatment of adherent cells with single and combination antiretroviral treatment.

5.2. Materials and methods

5.2.1 Materials

Cell culture medium (RPMI-1640 without phenol red) was purchased from Life Technologies (United Kingdom). Antiretroviral drugs [abacavir (ABC), lamivudine (3TC), nevirapine (NVP), stavudine (d4T) and zidovudine (ZDV)], nitroblue tetrazolium (NBT), penicillin-streptomycin, sodium pyruvate, dimethylsulfoxide (DMSO), superoxide dismutase, glutamine, charcoal-treated foetal bovine serum (FBS), 7ml Bijou bottles, and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma (United Kingdom). Twenty-four-well plates were purchased from Nunc Denmark. The U937 cell line was obtained courtesy of the University of East London tissue culture research laboratory.

5.2.2 U937 cell culture and maintenance

U937 cells were grown in RPMI 1640 supplemented with 10% FBS, 2mM glutamine, 1% sodium pyruvate and 1% antibiotic solution with 10000U of penicillin and 10mg/ml streptomycin. The cells were incubated at 37^{o}C and 5% atmospheric CO_2. The suspended cells were split every 3 to 4 days.

5.2.3 Cryopreservation of U937 cells

The non-adherent U937 cells were transferred to a 15ml centrifuge tube. The cells were then spun at 1000 rpm for 5 min and the cells reconstituted by the freezing medium [complete medium supplemented with 5% (v/v) DMSO]. The supernatant was removed, cells re-suspended in 1ml of fresh freezing medium consisting of 5% DMSO and 20% FBS in RPMI-1640. The cells were transferred into a cryotube. They were stored overnight at -80^{o}C and transferred to liquid nitrogen. Upon resuscitation, up to 10 passages were used for experimental assays to ensure that the cells still maintain inherent characteristics.

5.2.4 Transformation of U937 to adherent cells

500µl of $2.0x10^5$cells/ml were seeded in 24-well plates. The cells were left for 24 h at 37^oC and 5% CO_2 to allow adaptation of cells to the environment. 500µl of 100µg/ml ABC, ZDV, d4T, 3TC and NVP were added thus bringing the final concentration in each well to 50µg/ml and the total volume to 1.0ml. Conditioned medium was normalised to 0.25% (v/v) DMSO. The vehicle control consisted of conditioned medium normalised to 0.25% (v/v) DMSO. U937 cells were also treated with the following combinations of antiretroviral drugs: ABC+3C, ZDV+3TC, ABC+ZDV+3TC, ZDV+3TC+NVP and d4T+3TC+NVP. 500µl of medium with a final volume of 100µg/ml of each antiretroviral drug in the combination were added, thus bringing the final concentration of each drug to 50µg/ml. The cells were seeded in triplicate for each drug combination. One well was used to determine the cell count in non-adherent (floating cells), while the second was used to determine the number of adherent cells using the trypan blue cell counting technique. The number of dead cells was also estimated concurrently in both adherent and non-adherent cells. The third served as a reserve in case of an error in processing one of the wells. The cells were then incubated for 72 h and the number of adherent cells was estimated. The experiment was repeated three times (n=3). Morphological transformation was assessed using the EVOS® inverted microscope (Method adapted with modification from Vrana *et al.*, 1999).

5.2.5 Non adherent and adherent cell estimation

Suspended non-adherent cells (1.0ml) were collected from each treatment well after 72 h of treatment and transferred to a Bijou bottle. Each treatment well was washed twice with 500µl of warm medium (37^oC) and the medium transferred into a Bijou bottle. This brought the total volume of suspended cells to 2ml. The number of cells were estimated using the trypan blue method. To estimate adherent cells, suspended cells were removed and the wells washed twice with 500µl of warm PBS, which was decanted. 200µl of trypsin-EDTA were added and the plates were gently shaken. The plates were placed in an incubator for 2 minutes. 800µl of medium was added and the 1.0ml with detached cells added to a bijou bottle. The wells were washed again twice with 500µl of medium, thus bringing the total volume of cells in each Bijou bottle for each treatment to 2.0ml. The number of adherent cells was then estimated using the trypan blue exclusion assay described below.

5.2.6 The trypan blue dye exclusion assay

Adherent, floating and dead cells were determined by the trypan blue/haemocytometer method 72 h post treatment. Dead cell count depicts the sum of dead cells derived from both adherent and floating cells. The percentage of adherent cells was calculated relative to the total live cell count. Briefly, under sterile conditions, 100µl of the suspended cells was added to a micro-tube and these were diluted with an equal volume of trypan blue. A moistened coverslip was slid backwards and forwards on the haemocytometer chamber until Newton's rings were seen on the coverslip. Both sides of the chamber were filled with the suspended cells by capillary action. The cells were viewed under an EVOS® inverted microscope while being counted using a tally counter. The number of cells was multiplied by the dilution factor. The total cell count in each of the wells was estimated by multiplying the cell count by 2.0 ml (the total volume of the suspended cells in each well).

5.2.7. Nitroblue tetrazolium assay procedure

The assay procedure was adapted from Choi *et al.* (2006) with modifications as depicted in Deng et *al.* (2014) and Meshkini *et al*. (2009). Briefly, 2.0 x 10^5 U937 cells were suspended in RPMI 1640 medium supplemented with 10% FBS and 10 000U of penicillin and 10mg/ml streptomycin (Sigma). 500µl per well of the suspended U937 cells were seeded in in a 24 well plate and incubated at 37^{o}C and 5% CO_2 for 24 hours. After 24 h incubation, 500µl of 100µg/ml of ABC, ZDV, d4T, 3TC and NVP were added in triplicate wells bringing the final concentration of each drug to 50µg/ml. Two sets of wells were treated with PMA at a final concentration of 10ng/ml so as to promote differentiation into macrophages. Untreated cells were exposed to medium only. The cells were then incubated at 37^{o}C and 5% CO_2 for 72 h. Concurrently, treatment with antiretroviral drug combinations, namely ABC+3TC, ZDV+3TC, ABC+ZDV+3TC, ZDV+3TC+NVP, d4T+3TC+NVP, was also carried out. 500µl of 2.0x10^5cells/ml were seeded in 24-well plates. The plates were incubated for 24 h at 37^{o}C and 5% CO_2. 500µl of medium with a final volume of 100µg/ml of each antiretroviral drug in the combination were added thus bringing the final concentration of each drug to 50µg/ml. The treated cells were incubated for 72 h with morphological observations carried out after 24, 48 and finally 72 h.

The medium and the suspended cells were aspirated. Suspended cells and the whole medium were removed and the cells washed once with PBS. The wells were incubated with 100µl of 2mg/ml NBT and 100µl of 600ng/ml PMA for 1 hour. One set of PMA transformed cells was treated with 100µl of 2mg/ml NBT, 600ng/ml of PMA and 30µg/ml

superoxide dismutase (SOD) to constitute the control. After incubation, the cells were washed twice with warm PBS, then once with methanol and air dried to ensure that extracellular NBT is completely removed. 120µl of 2Maq. KOH was added to solubilize cell membranes followed by addition of 140µl of DMSO to dissolve the blue formazan with gentle shaking for 10 minutes at room temperature. 100µl of the dissolved formazan was transferred from each of the triplicate wells to a 96-well plate and the absorbance read at 620nm (After: Choi *et al*., 2006 and Lin et *al.*, 2014). To cater for differences in the total transformed cells, cell numbers were normalised and the relative absorbance compared per $1.0x10^5$ (100 000) cells.

5.2.8. Data Analysis

Descriptive statistics were computed on Excel. The total number of viable cells (adherent + non-adherent) for each treatment was compared to vehicle treated cells using the unpaired t-test. Differences between the control and each of the the treatments for NBT assay were analysed by ANOVA (Graphpad Prism 7.0) followed by Dunnet's multiple comparison test ($P \leq 0.05$).

5.3.0. Results

5.3.1 Morphology of antiretroviral- and PMA-treated cells

Following 72 h of exposure to single antiretroviral treatment, the morphological appearance of the cultured cells was captured using an EVOS® inverted microscope at 24 h intervals. After 24 h of treatment with 10ng/ml PMA, granule formation was depicted with trace amount of cells attached on the cell culture plates. After 48 h, the majority of the PMA treated cells were attached to the well plates. After 72 h, PMA-treated cells a typical appearance characterised by some lobular and spherical nuclei, vacuole-containing cytoplasm and were granulated (Figure 5.1). These cells were less dense and more sparsely distributed. The cell density on seeding was $0.2x10^6$ increasing to $0.37x10^6$ 72 h post treatment, thus suggesting that the lower density of PMA-treated cells relative to the control is due to differentiation rather than cytotoxicity. The negative control group treated cells with medium only and the vehicle-treated cells (0.25% v/v DMSO) were undifferentiated, characterised by round large nuclei with defined round and regular cell margins.

Following ZDV treatment, attachment of some of the cells became apparent after 48 h and, by 72 h, there were a number of distinctly larger granulated attached cells with spherical

nuclei (Figure 5.2). A higher proportion of cells (86%) were adherent, with most assuming an amorphous and granular structure for ZDV. The ZDV wells had more granulated cells than did d4T ABC, 3TC and NVP (Figure 5.3). By 72 h post-treatment, fewer granule-containing cells were observed among ABC, 3TC and NVP treated cells. No apparent granulation was observed in the untreated cells. At 48 h post-treatment, combination-treated cells with ABC+ZDV+3TC, ZDV+3TC+NVP and d4T+3TC+NVP began to attach on the culture plate while other cells remained in suspension. By 72 h post-treatment, the triple combination-treated cells appeared larger with dark granules and lobular nuclei (Figure 5.2). In comparison, very few granulated cells were observed among ABC+3TC and ZDV+3TC treated cells (Figure 5.3). Overall these observations show a time-dependent induction of differentiation in U937-treated cells. A high degree of granulation characterised with lobular nuclei was observed for ZDV only and triple combination (HAART)-treated cells (Figure 5.1). Fewer lobular nucleated and fewer granulated cells for d4T, 3TC, NVP, ABC, ABC+3TC and ZDV+3TC are shown in Figure 5.3.

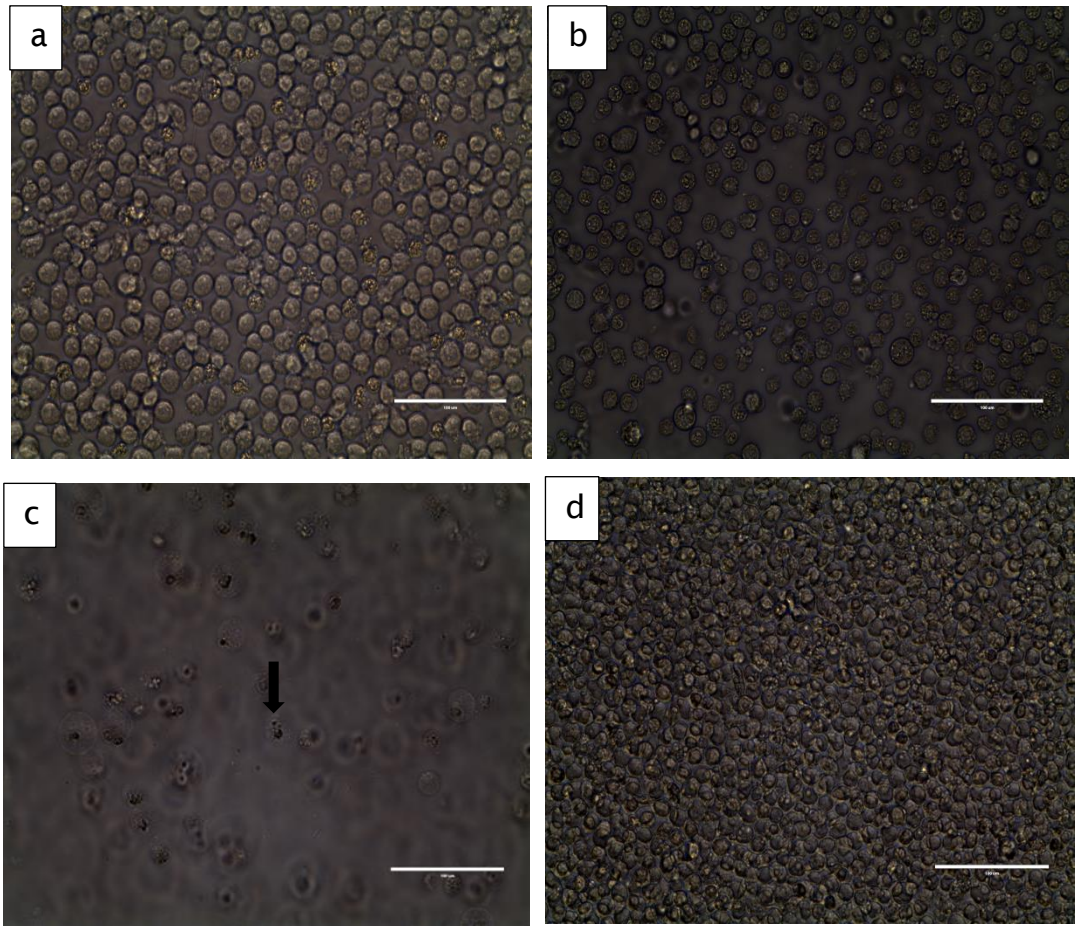

Fig 5.1: Morphological appearance of U937 cells over a 72 h treatment with PMA. Cells were treated with 10ng/ml PMA and incubated at 37^{O}C and 5% CO_2 for 72 h. The photographs were captured with an EVOS® microscope after 24 h PMA treatment (a), 48 h PMA treatment and 72h PMA treatment (c). Untreated cells were captured after 72 h treatment (d). The PMA-treated cells decreased in density with time while untreated cells were more dense. The black arrow indicates a lobular nucleus in a PMA-treated cell. The scale bars represent 100µm.

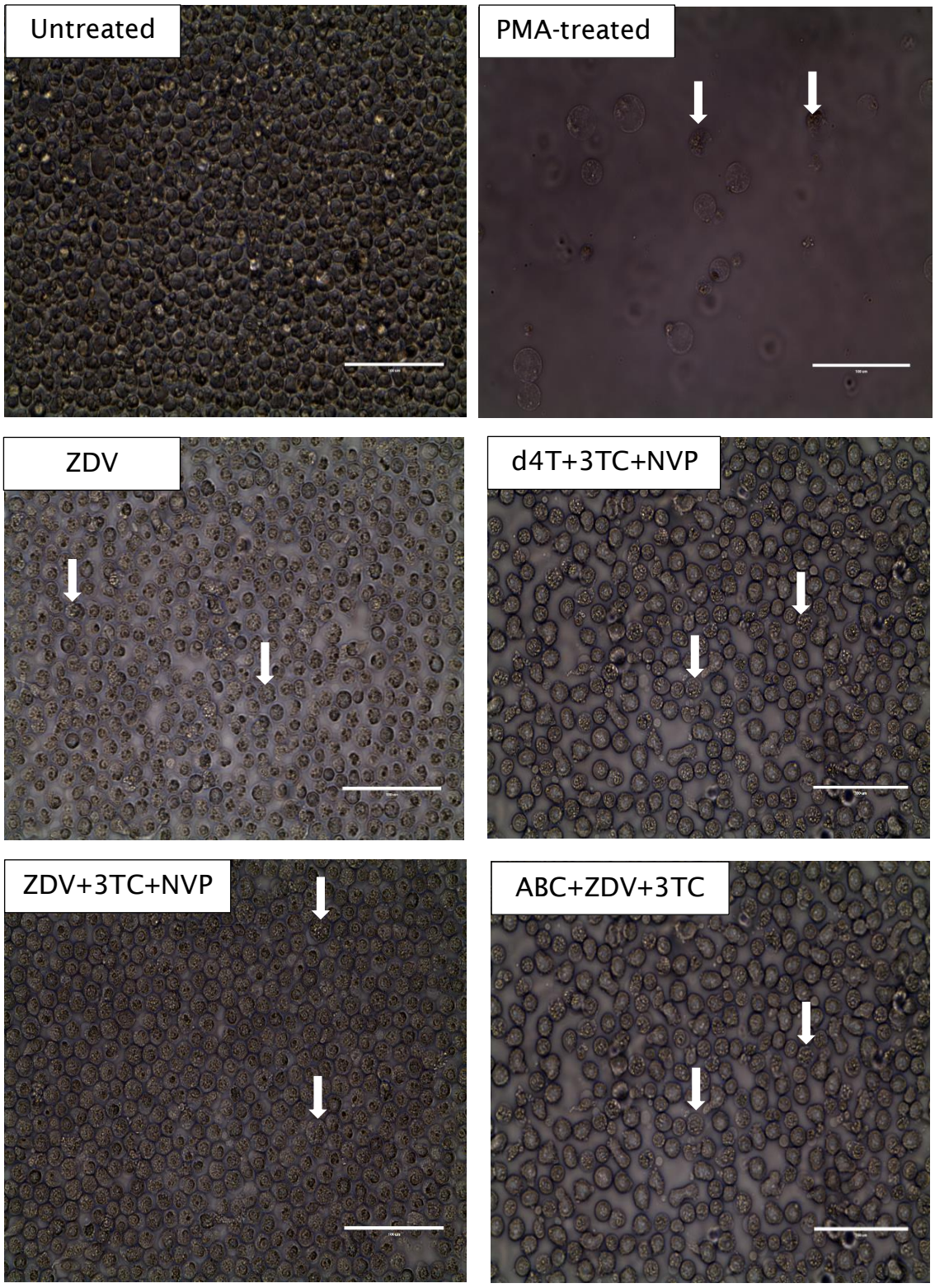

Fig 5.2: Morphological appearance of cells after 72 h treatment with PMA, ZDV, d4T+3TC+NVP, ZDV+3TC+NVP and ABC+ZDV+3TC. Cells were treated with PMA or combination drugs at a final concentration of 50µg/ml for each drug and incubated at 37^{O}C and 5% CO_2 for 72 h. A high degree of granulation was observed for ZDV, d4T+3TC+NVP and ZDV+3TC+NVP stimulated cells. ABC+ZDV+3TC treatment resulted in a mixture of granular and agranular cells. Arrows indicate typical transformed cells with some distinct granulated morphology and a lobular nucleus. The scale bars represent 100µm (After: Mafuva and Morgan, 2016).

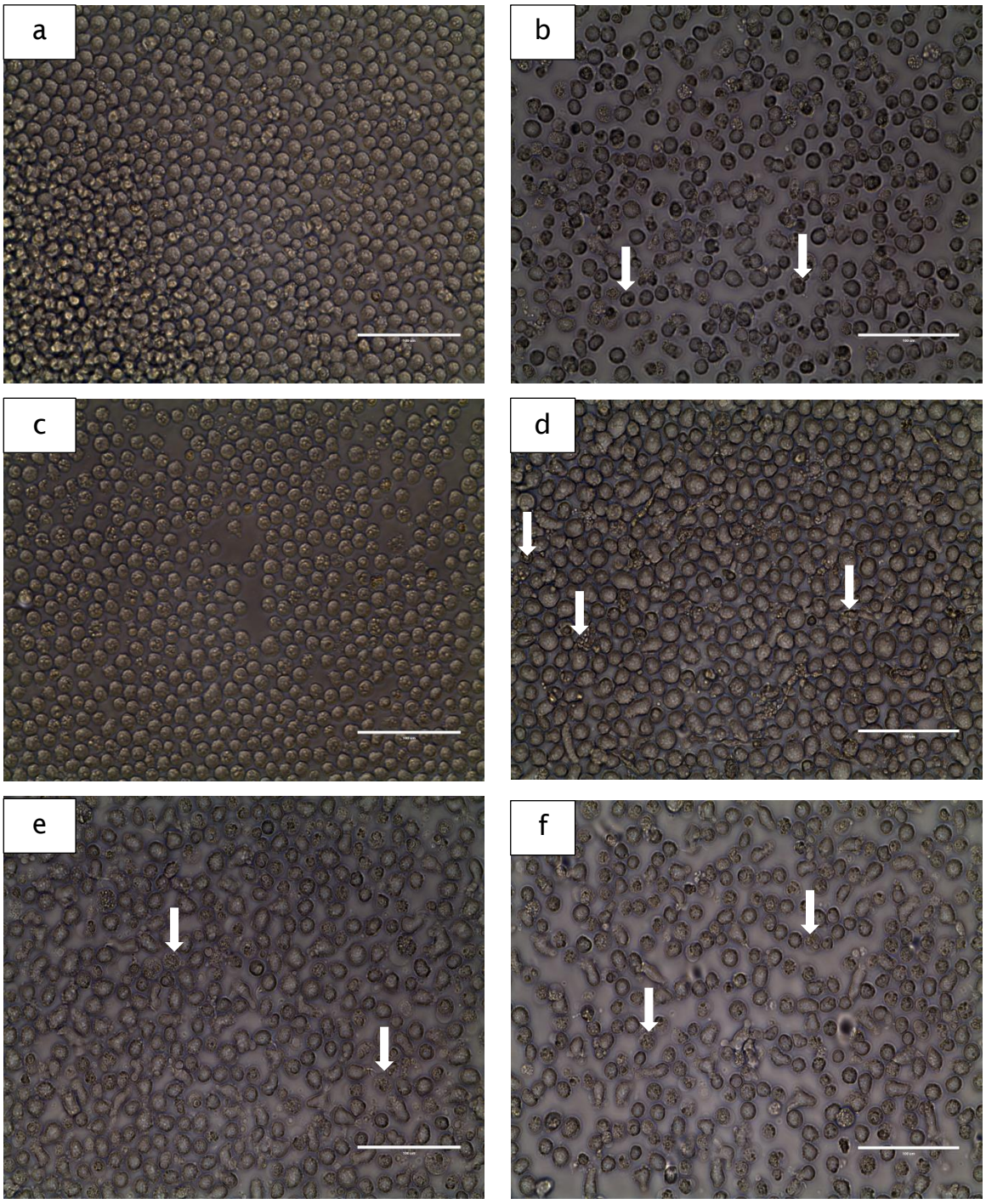

Fig 5.3: Morphological appearance of cells after 72 h treatment with d4T, 3TC, NVP, ABC, ABC+3TC and ZDV+3TC. Cells were treated with single or combination drugs at a final concentration of 50µg/ml for each drug and incubated at 37°C and 5% CO_2 for 72 h. (a) cells treated with d4T do not depict obvious morphological difference from untreated cells in Figure 5.1. (b) cells treated with 3TC with some appearing darker as shown by arrows (c) Cells treated with NVP depicting small spherical cells without obvious morphological transformation characteristics. (d) cells treated with with ABC, arrows indicate lytic cells (e) cells treated with ABC+3TC arrows indicate granular morphology (d) ZDV+3TC treated cells with arrows depicting granular cells. The scale bars represent 100µm (After: Mafuva and Morgan, 2016)

5.3.2 Transformation of U937 cells to adherent cells

Table 5.1 and Figure 5.4 present the descriptive statistics and graphical presentation for single drug-treated cells, respectively. The total number of viable cells for each treatment was compared to the total live cells in the vehicle-treated cells using the one-way ANOVA followed by Dunnett's multiple comparison test ($P \leq 0.05$). Adherence from single drug treatment was 3.2% for ABC, 86.0% for ZDV, 21.0% for lamivudine 3TC, 47.2% for dT4 and 12.5% for NVP (Fig 5.4) (Mafuva and Morgan, 2016). The total cell counts for ZDV, 3TC and d4T are lower than those for ABC and NVP (Table 5.1). However the number of dead cells is lower for ZDV ($3.0x10^4$ cells), 3TC ($3.0x10^4$ cells) and d4T ($4.0x10^4$ cells) if compared to both ABC ($9.0x10^4$ cells) and NVP ($1.2x10^5$ cells). ZDV and 3TC dead cell counts estimates are similar to untreated cells control ($3.0x10^4$ cells) and 0.25% DMSO-treated cells ($3.0x10^4$ cells).

Table 5.2 and Figure 5.5 present the descriptive statistics and graphical presentation for combination drug treatment, respectively. The total number of viable cells for each treatment was compared to the total live cells in the vehicle-treated cells using one-way ANOVA followed by Dunnett's post-test ($P \leq 0.05$). The percentage adherence was 13.5% for ABC+3TC, 7.9% for ZDV+3TC, 40.1% for ABC+ZDV+3TC, 42.1% for ZDV+3TC+NVP and 39.9% for d4T+3TC+NVP (Mafuva and Morgan, 2016). The number of dead cells was higher in all 5-combination treatments when compared to untreated and DMSO vehicle-treated cells. Overall, the number of dead cells is lower for triple combination-treated U937 cells (ABC+ZDV+3TC=$9.0X10^4$ cells and d4T+3TC+NVP=$8.0X10^4$ cells), compared to the double combinations ABC+3TC and ZDV+3TC which each depict $1.1x10^5$ and $2.1x10^5$ dead cells respectively. On the contrary, ZDV+3TC+NVP result in increased cell death ($1.9x10^5$ cells) relative to ZDV+3TC.

Table 5.1 Descriptive statistics for U937 cells pre-treated with single antiretroviral drugs

Cell Type ($x10^6$)	untreated	vehicle	+ve (PMA)	ABC	ZDV	3TC	d4T	NVP
Non-adherent	1.96 ±0.07	1.98 ±0.09	0.04 ±0.04	1.53 ±0.08	0.19 ±0.18	0.75 ±0.08	0.57 ±0.06	1.12 ±0.08
Adherent	0.00 ±0.01	0.00± 0.00	0.33 ±0.06	0.05 ±0.02	1.17± 0.06	0.20± 0.04	0.51± 0.08	0.16 ±0.04
Dead	0.05 ±0.01	0.03± 0.02	0.03 ±0.07	0.09 ±0.02	0.03± 0.02	0.03± 0.02	0.04 ±0.04	0.12 ±0.04
Total viable	1.96 ±0.07	1.98 ±0.09	0.37 ±0.06	1.58 ±0.11	1.36 ±0.24	0.95 ±0.12	1.08± 0.14	1.28 ±0.07
Adherent (%)	0.00	0.00	89.19	3.16	86.03	21.05	47.22	12.50

U937 cells were treated with 10ng/ml PMA as the positive control (+ve) or treated with single antiretroviral drugs or left untreated (-ve) for 72 h. Total live cells represent the sum of adherent and non-adherent cells for each individual treatment. The numbers represent the estimated total cell count (mean ± SD, n=3) (After: Mafuva and Morgan, 2016)

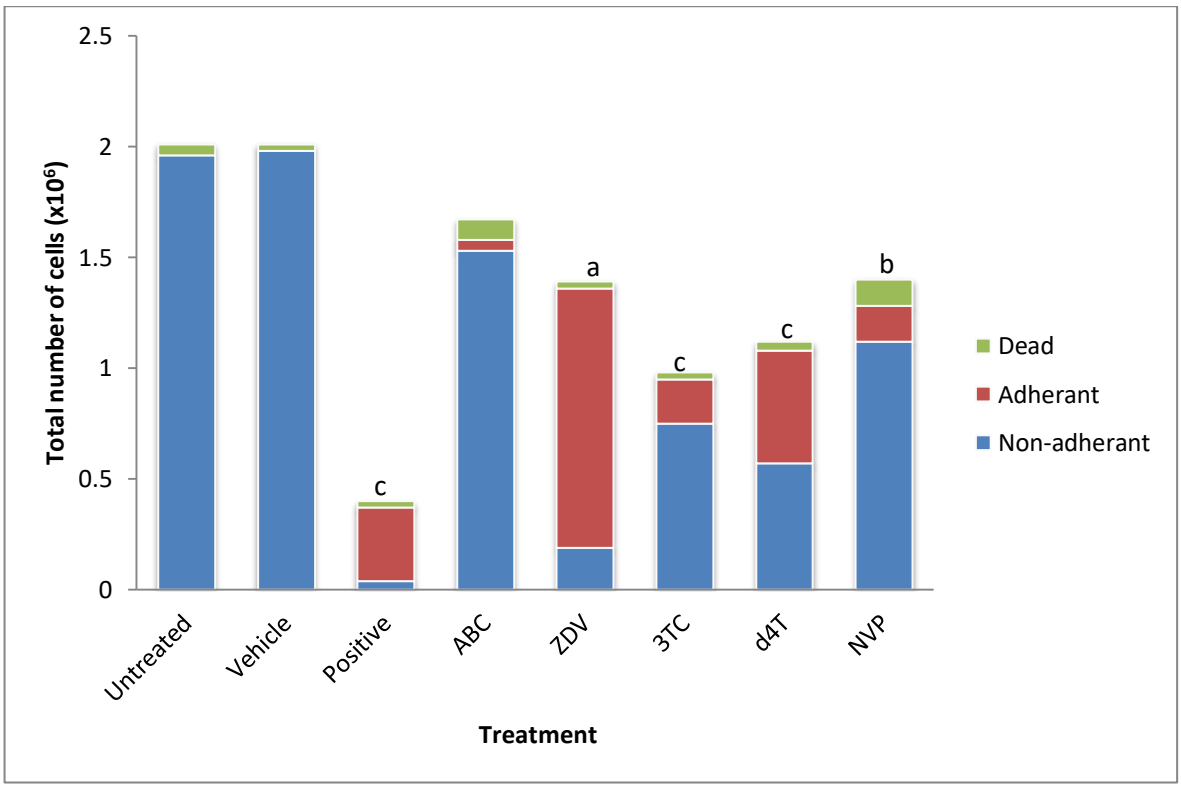

Fig 5.4: Differential conversion of U937 to adherent cells by single antiretroviral treatment. U937 cells were treated with 10ng/ml PMA as the positive control (+ve), single antiretroviral drugs, or left untreated (negative control) for 72 h. Vehicle cells or treated with 0.25% DMSO for 72 h. The numbers represent the estimated cell count (mean ± SD, n=3). The total number of viable cells (adherent + non-adherent) for each treatment was compared to vehicle treated cells by the one-way ANOVA followed by the Dunnett's multiple comparison. Data that are statistically significant are indicated by a, b, and c where, (a<0.05, b<0.01, c<0.001) (After: Mafuva and Morgan, 2016)

Table 5.2 Descriptive statistics for U937 cells pre-treated with combination ARDs.

Cell type ($x10^6$)	-ve	vehicle	+ve (PMA)	ABC+ 3TC	ZDV+ 3TC	ABC+ZDV +3TC	ZDV+3TC +NVP	d4T+3TC +NVP
Non-adherent	2.03 ±0.12	1.99 ±0.12	0.07 ±0.06	1.68 ±0.04	1.40 ±0.12	1.12 ±0.08	0.84 ±0.12	1.07 ±0.14
Adherent	0.00 ±0.00	0.00 ±0.00	0.35 ±0.03	0.25 ±0.06	0.12 ±0.04	0.75 ±0.06	0.61 ±0.08	0.71 ±0.17
Dead	0.04 ±0.02	0.04 ±0.04	0.04 ±0.04	0.11 ±0.02	0.21 ±0.16	0.09 ±0.02	0.19 ±0.06	0.08 ±0.04
Total viable	2.03 ±0.12	1.99 ±0.12	0.42 ±0.04	1.93 ±0.05	1.52 ±0.14	1.87 ±0.12	1.45 ±0.16	1.78 ±0.06
Adherent (%)	0.00	0.00	83.33	12.95	7.89	40.11	42.07	39.89

U937 cells were treated with 10ng/ml PMA as the positive control (+ve) or treated with combination antiretroviral drugs or left untreated (-ve) for 72 h. Total live cells represent the sum of adherent and non-adherent cells for each individual treatment. The numbers represent the estimated total cell count (mean ± SD, n=3) (After: Mafuva and Morgan, 2006)

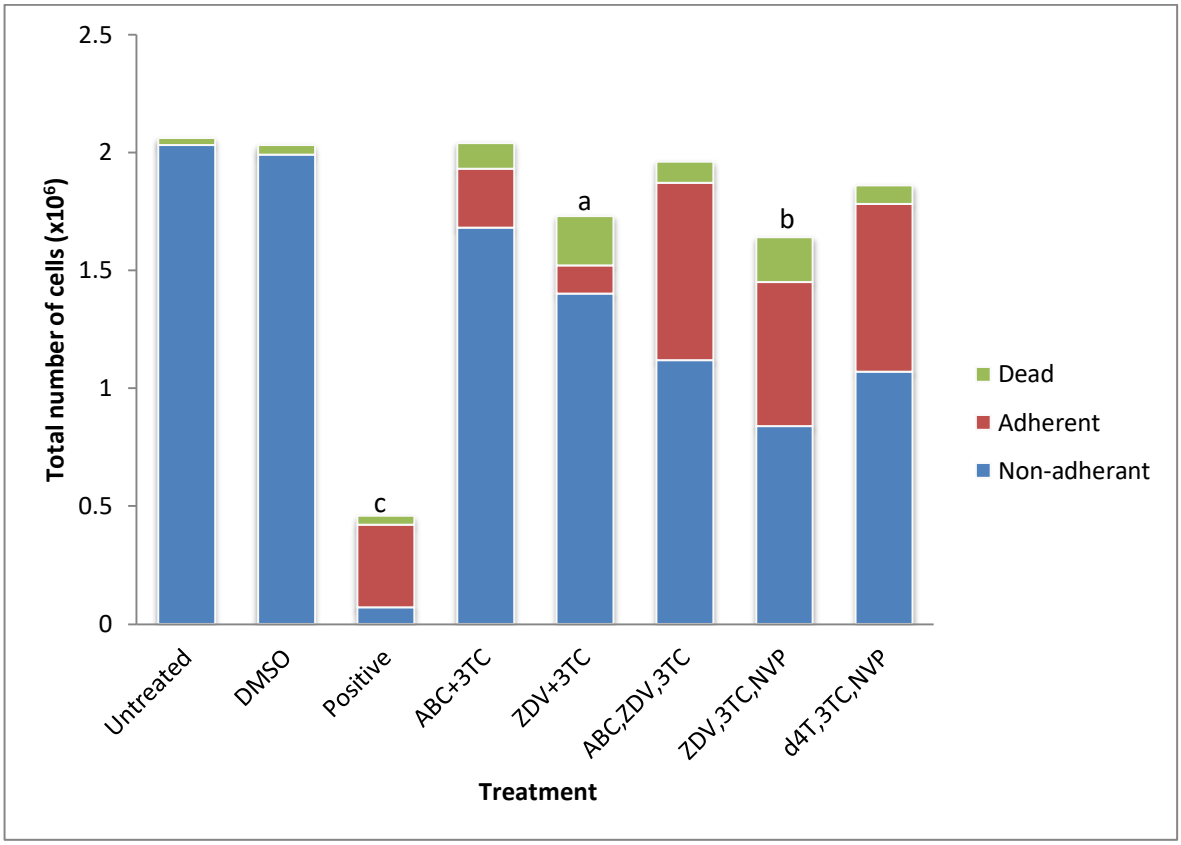

Fig 5.5: Differential conversion of U937 to adherent cells by combination antiretroviral treatment. U937 cells were treated with 10ng/ml PMA as the positive control (+ve), combination antiretroviral drugs, or left untreated (negative control) for 72 h. Vehicle cells were treated with 0.25% DMSO (w/v) for 72 h. The numbers represent the estimated cell count (mean ± SD, n=3). The total number of viable cells (adherent + non-adherent) for each treatment was compared to vehicle treated cells using the one-way ANOVA followed by the Dunnett's multiple comparison. Data that are statistically significant are indicated by a, b, and c where, (a<0.05, b<0.01, c<0.0001) (After: Mafuva and Morgan, 2016).

5.3.3 NBT reduction assay for the superoxide anion determination

The NBT reduction assay was carried out after 72 h of treating U937 cells with single and combination antiretrovirals and compared to vehicle treated (control) cells. Comparison of means between the negative control and different treatments was done using one-way ANOVA followed by Dunnet's multicomparison test (α=0.05). For single drug treatment, ZDV- and PMA-treated cells differed significantly compared to the negative control (Figure 5.6a). This demonstrates a corresponding increase in production of superoxide for ZDV-treated cells. Cognisant of the impact of differences in transformed cells, cell numbers were normalised and the relative absorbance compared per $1.0x10^5$ (100 000) cells. This was deduced from the adherent cell numbers shown in Table 5.1 compared to the the absorbances summarised in Figure 5:5a. Curiously, it was shown that ABC, 3TC and NVP had higher relative absorbances per $1.0x10^5$ cells compared to ZDV, despite ZDV inducing a higher percentage of transformed cells. The ability of ABC-treated cells to reduce NBT was even higher per $1.0x10^5$ cells compared to the positive control.

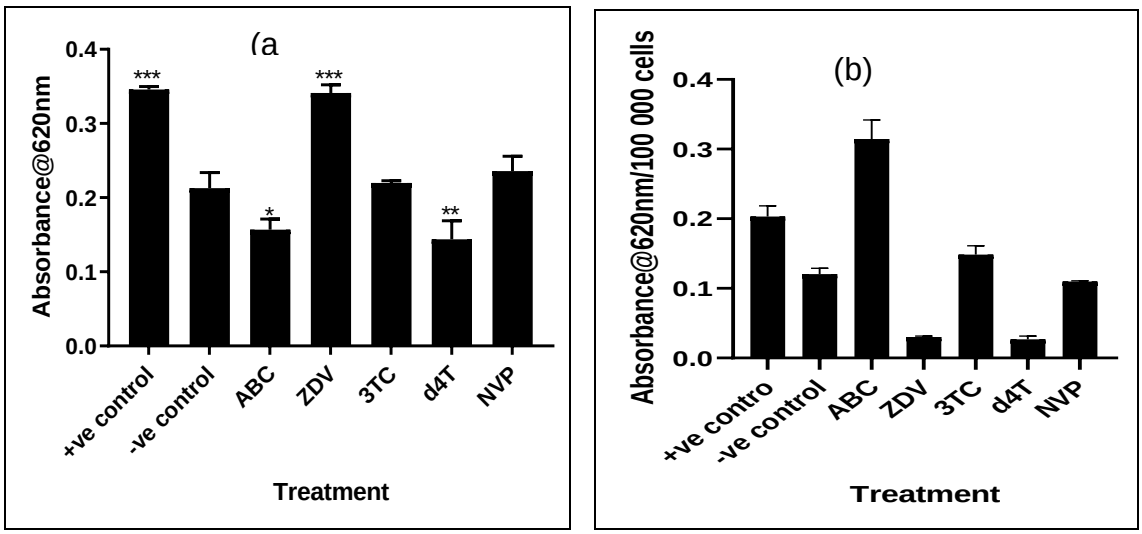

Fig 5.6: Effect of single antiretroviral treatment on U937 superoxide anion production. (a) Differential ability to reduce NBT of U937 cells treated with 50µg/ml of each single antiretroviral drug and left for 72 h. Data were analysed by the unpaired t-test (mean ± SD, n=3). Asterisks (*) indicate data that are significantly different compared to the negative control (*P<0.01, ** P< 0,001, **P<0.0001). (b) Absorbance of the single ARV-treated cells standardised to $1.0x10^5$ cells.

Following combination ARV treatment, significant differences from the control were observed for ABC+ZDV+3TC-, ZDV+3TC+NVP- and 4T+3TC+NVP-treated cells (Figure 5.6a), thus depicting increased $\bullet O_2^-$ production in treated cells. There were no differences obtained for ABC+3TC and ZDV+3TC cells and the negative control. The relative absorbances for the different combination-treated cells were standardised and compared per $1.0x10^5$ (100 000) cells. This was calculated from the adherent cell numbers shown in Table 5.2 against the absorbances shown in Figure 5:6a. It was shown that ABC+3TC and ZDV+3TC had higher relative absorbances per $1.0x10^5$ cells, compared to triple combinations, despite the triple combinations having an overall cumulative increase in transformed cells.

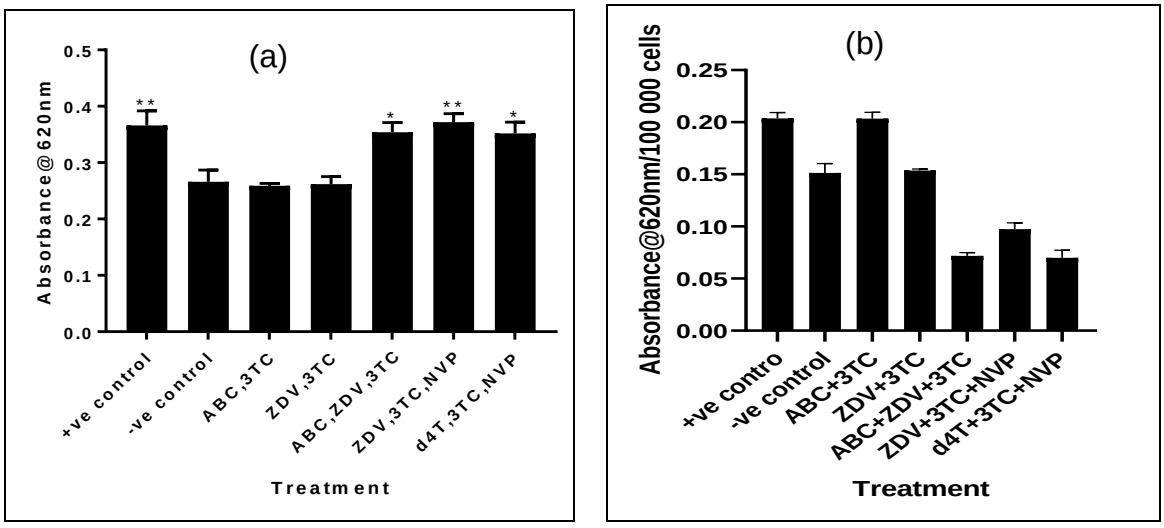

Fig 5.7: Effect of combination antiretroviral treatment on U937 superoxide anion production. (a) Differential ability to reduce NBT of U937 cells treated with 50µg/ml of each drug in combination for 72 h. The cells were treated with combination antiretroviral drugs and incubated at 5% CO_2 and 37°C. Data were analysed by one-way ANOVA followed by Dunnett's multiple comparison test (α=0.05). Data (means ± SD, n=3), indicate data that are significantly different compared to the negative control (*P≤0.001, ** P≤0,0001). (b) Absorbance of combination ARV-treated cells standardised to 1.0 x 10^5 cells.

5.4. Discussion

Hasegawa *et al.* (2009) demonstrated that peripheral monocyte turnover associated with tissue macrophage death correlates with AIDS progression.The present study demonstrates the varied transformation potential of single and combination antiretroviral drugs on U937 cells to adherent cells with 'macrophage-like' morphology. Furthermore, evaluation of the ability to reduce NBT demonstrates the discordance between transformation efficiency and $\bullet O_2^-$ generation. Although the pathogenesis of SNAEs remains obscure, antiretroviral toxicities have been suggested as causative among other factors. A reduced use of ZDV and d4T has previously been suggested cognisant of their recently perceived toxic effects (Housinkveld and van der Burg, 2011). As a result, the WHO guidelines of 2010 suggest a gradual withdrawal of d4T in combination regimens. Following 72 h treatment of cells with 10ng/ml of PMA, the cells were less sparsely distributed. $2x10^5$ cells were seeded in each well at the start of the experiment. For single drug treatment, 72 h post PMA treatment there were $3.7x10^5$ live cells (adherent and non-adherent), a number higher than the initially seeded cells. However, this cell count was far much lower than vehicle-treated cell count ($1.98x10^6$ cells). Cognisant of the observed granular morphology, this suggests lack of proliferation as the cells differentiate (Perihar *et al.,* 2009). The positve control thus miatains a low total cell count because the cells are predominantly differentiating rather than proliferating.

Although the total cell counts for ZDV, 3TC and d4T are lower than that for ABC and NVP. A lower number of dead cells for ZDV ($3.0x10^4$ cells), 3TC ($3.0x10^4$ cells) and d4T ($4.0x10^4$ cells) if compared to both ABC ($9.0x10^4$ cells) and NVP ($1.2x10^5$ cells) was observed. Interestingly, ZDV and 3TC dead cell counts estimates are similar to untreated cells control ($3.0x10^4$ cells) and 0.25% DMSO-treated cells ($3.0x10^4$ cells), hence suggesting that the lower cell count is predominantly due to differentiation rather than toxicity. Higher numbers of dead cells for ABC ($0.9x10^4$) and NVP ($1.2x10^5$) are suggestive of increased toxicity. Overall, triple combinations result in a lower number of dead cells 72 h post-treatment with triple ARV combinations relative to double combination-treated cells (Table 5.2). The exception to this phenomenal observation is ZDV+3TC+NVP, which results in higher dead cells relative to ZDV+3TC (Table 5). Coupled with morphological and higher percentages of adherent cells, these observations suggest that overall triple combination antiretroviral therapy has reduced cytotoxicity and higher transformation potential, compared to single and double ARV-treated cells.

Interestingly, the present results demonstrate the highest transformation of U937 cells upon ZDV treatment, compared to other single drugs. However the relative $\bullet O_2^-$ production per $1.0x10^5$ cells is higher for ABC, despite it having the least transforming potential. Similarly, triple combinations of drugs showed a higher transformation potential but less $\bullet O_2^-$ production per $1.0x10^5$ cells compared to ABC+3TC and ZDV+3TC. These observations suggest that toxicity is generally higher per cell for single- and double-combination-treated cells, despite the higher transformation potential into macrophage-like cells in triple combination-treated cells. The cumulative increased production of $\bullet O_2^-$ by the triple combination-treated U937 cells is of interest. This is not unusual for transformed U937 cells, as differential production of reactive oxygen species has been reported for PMA-transformed U937 cells (Choi *et al.*, 2006; Meshkini *et al*., 2009). The present results demonstrate an increased accumulation of superoxide anion in myoloid cancer cells. This increased $\bullet O_2^-$ generation suggests an inflammatory response, as ROS generation has been associated with removal of cancer cells and immune response (Hancock *et al*, 2001; Held, 2015; Choi *et al.*, 2006). M1 macrophages have been less associated with elimination of pathogens, virus-infected cells and cancer cells as well as generation of reactive oxygen species. On the contrary, M2 macrophages have been associated with tumour promotion (Housinkveld and van der Burg, 2011; Martinez and Gordon, 2014).

A number of factors have been reported to influence the transformation of U937 cells to adherent cells. Firstly, it has been established that transformation by PMA is dose-dependent (Garcìa *et al.*, 1999; Martinez & Gordon, 2014; Studzinski, 2001). Here, only concentrations of 50µg/ml were used to treat the cells, as this concentration falls within the EC_{15}-EC_{20} for each drug as optimised in Chapter 2 on HeLa cells. Secondly, monocyte transformation is also time-dependent (Deng *et al.*, 2014; Garcìa *et al.*, 1999). U937 transformation has been reported following 96 h or more treatment time with a variety of stimulants (Lin *et al.*, 2014). Similarly, variations of the cell culture medium with time may affect cell growth and differentiation (Meshkini *et al.*, 2009). While ZDV and dT4 show significant differences compared to untreated cells, the standard absorbance per $1.0x10^5$ cells is lower for ZDV compared to the negative control. This shows a reduced superoxide production per unit cell, thus demonstrating that the increased superoxide production is due to the cumulative effect from many cells. Interestingly, the percentage standardised absorbances for ABC, NVP and 3TC are higher or closer to the positive control, thus suggesting an increased superoxide production per unit cell, thus demonstrating that the increased superoxide production is due to the cumulative effect of all cells. The surface area on which cells adhere may be a limiting factor in rapidly

multiplying cells. As cells multiply and cover the whole surfaces of 24-well plates, some cells may become clustered as the surface becomes limiting, hence affecting adhesion of the cells.

Low dead cell counts associated with a reduced viable cell count have been associated with cell cycle arrest (Koczor *et al.*, 2009). The results in Fig 5.4 show an increased number of dead cells for ABC ($0.9x10^5$ cells) and NVP ($1.20x10^5$ cells) thus suggesting cytotoxic activity if compared to untreated cells ($0.4x10^5$ cells) and DMSO vehicle-treated cells ($0.3x10^5$) cells. On the contrary, ZDV ($0.3x10^5$ cells), 3TC ($0.3x10^5$ cells) and d4T ($0.4x10^5$) have fewer dead cells, although they present an overall decrease in cell count. This is suggestive of the cytotoxic effects of the drugs. Similarly, for combination drug treatment ABC+3TC ($1.1x10^5$ cells) ZDV+3TC ($2.1x10^5$ cells), ABC+ZDV+3TC ($0.9x10^5$ cells), ZDV+3TC+NVP ($1.9x10^5$ cells) and d4T+3TC+NVP ($0.8x10^5$ cells) are higher in total dead cell count, compared to the untreated control ($0.3x10^5$ cells) and NVP vehicle treated ($0.3x10^5$ cells). These observations suggest an antiproliferative effect resulting from combination ARV stimulation.

Overall, compared to other treatments, ZDV and triple combinations were shown to transform higher percentages of U937 monocytes to adherent cells with a corresponding high cumulative $\bullet O_2^-$ generation potential. In the present study, the transformed cells were not immunologically characterised with markers such as IL6 (a marker for tumour progression) IL10 (a marker for anticancer macrophage) and CD14 (a marker for monocyte transformation into macrophage). Such characterisation could help to determine whether each transformation is associated with either M1 or M2 macrophage-like cells. The present observations also demonstrate that antiretroviral drug treatment induces differential generation of reactive oxygen species in U937 cells. Cognisant of the anticancer and antiinflamatory responses associated with macrophages that generate reactive oxygen species, the present results suggest the anticancer activity of ZDV and triple combination antiretroviral-derived macrophages.

Previously in Chapter 3, time-dependent genotoxic DNA strand breakage was demonstrated in both CHO and HeLa cells following treatment with single NRTIs (ABC, 3TC, d4T and ZDV), as well as NVP. The present results demonstrate an increased superoxide anion production for standardised U937 cells after treatment with ABC,3TC and NVP (Figure 5.6b). This is suggestive of ROS and oxidative stress-dependent toxicity leading to the observed antiproliferative activity of these single drugs. ZDV treatment depicts a transformation-dependent antiproliferative effect, as demonstrated by the

accumulation of transformed (adherent) cells relative to non-adherent cells 72 h post-treatment (Figure 5.4). Interestingly, these ZDV-transformed cells cumulatively produce a significantly high amount of superoxide anion compared to the control (Figure 5.6a).

Interestingly, as depicted in Figure 5.7, HAART increases production of superoxide anion compared to the control. As depicted in Figure 5.5, ZDV+3TC+NVP (42.1%) suggest both cytotoxic and transformation-dependent antiproliferative activity, as shown by a high number of dead cells ($0.19x10^6$) and a high percentage of transformed cells. The high percentage of transformed cells observed with ABC+ZDV+3TC (40.11%) and d4T+3TC+NVP (39.89%) is suggestive of the therapeutic effect of HAART on haematopoietic malignancies. Apart from the triple HAART combinations, ABC+3TC and ZDV+3TC are double combinations which are also widely used for HIV treatment in low-resource settings in patients presenting with other medical conditions. The present results demonstrate a lower transformation potential (of U937 cells from non-adherent to adherent) of double ARV combination administration compared to HAART. The relative percentage of transformed cells for both double and triple combination cells is less than that for PMA-treated cells and this is coupled by a higher number of live cells relative to the latter. This suggests that the rate of transformation is lower in the combination ARV-treated cells relative to PMA treated cells. Lin *et al.* (2014) demonstrated that, depending on the substrate used for stimulation, U937 differentiation may take up to 96 h or more.

5.4.2 Conclusion

The present observations indicate the varied potential of ARVs to induce U937 cell transformation into adherent cells with 'macrophage-like' characteristics. Overall, our results demonstrate that highly active antiretroviral therapy can transform U937 cells into adherent cells whose cumulative production of superoxide anion significantly differs to the untreated control. Highly active antiretroviral treatment transformed premonocyte lymphoma cells (U937) to monocyte/macrophage-like morphology characterised by an increase in superoxide anion production. If replicated *in vivo*, this could go some way in explaining the SNAEs recognised with long-term HAART (Mafuva and Morgan, 2016). In the previous chapter, we demonstrated the beneficial effects of optimum apoptosis induction in human cervical cancer cells by triple antiretroviral combinations. In this chapter, it has been shown that triple ARV combination treatment of U937 cells results in microenvironmental increase in superoxide anion production; these joint observations suggest the beneficial effect of highly active antiretroviral therapy on apoptosis induction in cancer cells. MI macrophages attract cells of the adaptive immunity in response to

signals from bacteria and IFNγ (Housinkveld and van der Burg, 2011). These cells are associated with expression of reactive oxygen species (ROS), inducible nitric oxide synthatase (iNOS) and activation of Natural Killer cells, hence these are associated with inflammatory response (Martinez and Gordon, 2014). These observations further suggest endogenous stimulation of apoptosis in human cervical epithelial cells cells due to genotoxic effect of single antiretroviral treatment associated with intracellular oxidative stress (Elmore 2007; Rastogi and Sinha, 2009), hence resulting in programmed removal of damaged cells. This may suggest the further need to characterise the apoptotic pathways attributable to both single and combination treatments associated with antiretroviral drug-induced apoptosis in human cervical epethelial cancer cells.

CHAPTER 6

THESIS CONCLUSION AND FUTURE DIRECTIONS

6.1.0. Overall conclusion

AIDS-associated cancers present an emerging challenge in cancer epidemiology (Engels *et al*., 2006; Phatak *et al.,* 2010). The present results demonstrate the differential ability of antiretroviral drugs in induction of apoptosis and their effect on cervical epithelial cancer cell viability. This study has also shown the varied effects of contraceptive hormones (ethinylestradiol and levonorgestrel) on cervical cancer cell viability and apoptosis if co-administered with antiretroviral drugs. The main findings with reference to the research questions of the present study are summarised in Table 6.1. The themes of the summary tables are derived from the research questions that were outlined in chapter 1. Since combination antiretroviral treatment is the recommended method of treatment, the main findings on combinatorial effects are presented.

Table 6.1: Overall summary of major findings from the study

Antiproliferative effect of LNG on HeLa cells	1.0µg/ml	2.0µg/ml	4.0µg/ml
ABC+3TC+LNG	++	++	++
ZDV+3TC+LNG	+	+	+
ABC+ZDV+3TC+LNG	-	+	-
ZDV+3TC+NVP+LNG	++	++	-
d4T+3TC+NVP+LNG	+	++	+
Antiproliferative effect of EE on HeLa cells	**0.5µg/ml**	**1.0µg/ml**	**2.0µg/ml**
ABC+3TC+EE	-	-	+
ZDV+3TC+EE	-	-	+
ABC+ZDV+3TC+EE	-	-	-
ZDV+3TC+NVP+EE	-	-	-
d4T+3TC+NVP+EE	+	-	-
Genotoxic effect of ARVs on HeLa cells	**1 h**	**2 h**	**4 h**
ABC	+	+	++
ZDV	+	+	++
3TC	+	+	++
d4T	+	+	++
NVP	+	+	++
Effect of hormones on HeLa cell drug-induced apoptosis	**No hormone**	**Effect of levonorgestrel (4.0µg/ml)**	**Effect of ethinylestradiol (0.5µg/ml)**
ABC+3TC	+	++	++
ZDV+3TC	+	++	++
ABC+ZDV+3TC	+	++	++
ZDV+3TC+NVP	-	++	++
d4T+3TC+NVP	+	++	+
Transformation of U937 cells	**Adherence**	**Superoxide production**	**Macrophage-like morphology**
PMA treated	++	++	++
ABC+3TC	-	-	-
ZDV+3TC	-	-	+
ABC+ZDV+3TC	+	+	+
ZDV+3TC+NVP	+	++	+
d4T+3TC+NVP	+	+	+

Mild or non-significant changes are shown by (-), while (+) indicates significant changes. (++) indicates more drastic changes in the investigated parameter.

6.1.1. Genotoxic and apoptotic effect of antiretroviral treatment on human cervical epithelial cancer cells

The present study has demonstrated that 50µg/ml of the NRTIs (ABC, ZDV, 3TC and d4T) and NVP (a NNRTI) induce time-dependent DNA strand breakages in CHO and HeLa cells. Single ARD treatment of HeLa cells with these NRTIs and NVP drastically increase apoptosis, compared to untreated cells. This is suggestive of apoptotic activation response *via* the intrinsic stimuli of mitochondrial apoptotic pathway following genotoxic stress (Czabotar *et al*., 2014; Youle and Strasser, 2008). Co-administration with 4.0µg/ml LNG shows no effect on ABC-induced apoptosis. Co-administration with 0.5µg/ml EE gradually reduces ABC- and d4T-induced apoptosis while it has no effect on ZDV, 3TC and NVP-induced apoptosis. However, in all cases, the rate of apoptosis remains higher than untreated cells. This too-drastic apoptosis induction by single ARV treatment is indicative of a higher degree of toxicity. Single-drug antiretroviral therapy has been gradually replaced with HAART, due to the pathophysiological conditions resulting from emergence of resistant mutants (WHO, 2010; BHIVA, 2008). In the present study, single antiretroviral drug administration was thus carried out to understand the mechanistic differences when compared to combination antiretroviral therapy which is the recommended in present-day antiretroviral treatment.

In the absence of hormones, the regimen ZDV+3TC+NVP does not significantly change the rate of apoptosis, if compared to untreated control cells. However, the 72 h MTT assay for treatment with ZDV+3TC+NVP shows a decrease in HeLa cell viability. This suggests that this antiproliferative effect observed with ZDV+3TC+NVP treatment of HeLa cells is predominantly due to inhibition of growth relative to untreated cells, since decrease in reduction of MTT is an indicator of cell death and/or proliferation inhibition (Sieuwerts *et al*., 1995; Sylvester 2011; van Meerloo *et al*., 2011; Shi *et al.*, 2012). This *in vitro* observation concurs with some epidemiological observations, suggesting no effect of HAART on neoplastic progression (Ezechi *et al*., 2014; Shrestha *et al*., 2010; Sirera *et al*., 2008). ABC+ZDV+3TC and d4T+3TC+NVP significantly changed the rate of HeLa apoptosis compared to untreated control cells ($P \leq 0.05$), with the overall rate of apoptosis below 20%. This plausibly explains the epidemiological observations of a decrease in cervical neoplastic transformation and reduced cancer cell proliferation among some patients undergoing HAART (Adler *et al*., 2012; Firnhaber *et al*., 2012; Omar *et al.*, 2011; Chen *et al*., 2014; Minkoff *et al*., 2010). Similarly the double combinations ABC+3TC and

ZDV+3TC depict an increase in apoptosis relative to untreated cells thus suggesting antiproliferative activity on the HeLa cells.

6.1.2 Effect of combination ARV co-administration with contraceptive hormones on HeLa cell proliferation.

In this study, lone administration of combination antiretroviral drugs has been shown to induce optimal rates of apoptosis associated with decreased proliferation of the human cervical epithelial cancer cell. The contrary has been shown to occur upon co-administration of contraceptive hormones (0.5µg/ml EE and 4.0µg/ml LNG) which drastically increase the rate of apoptosis. This present study has, however, shown that drastic induction of apoptosis by EE in ABC+3TC, ZDV+3TC, ABC+ZDV+3TC and ZDV+3TC+NVP did not cause a significant alteration in the viability of cells compared to normal cells, as shown by the MTT assay. These results suggest cellular regeneration, since induction of apoptosis does not necessarily imply inhibition of remodelling (Shi *et al.*, 2012). In both normal and cervical cancer cells (HT3 and Caski), 17β-Oestradiol induces anti-apoptotic effects by attenuation of caspases-9 and terminal caspases-3 activity (Wang et *al.*, 2004). Here, ethinylestradiol (a derivative of 17β-oestradiol) has been shown not to alter cell viability in MTT assay on combination antiretroviral-treated cells, thus suggesting a cellular remodelling effect of EE.

4.0µg/ml LNG gives MTT results suggesting such cellular regeneration for the regimen ABC+ZDV+3TC only, while depicting antiproliferative activity for all other combination regimens (ABC+3TC, ZDV+3TC, d4T+3TC+NVP and ZDV+3TC+NVP). This therefore shows an antiproliferative effect of 4.0µg/ml LNG in human cervical epithelial cancer cells. While present results show too-drastic apoptosis with 4.0µg/ml LNG, the main focus in Public Health becomes to use optimum concentrations of LNG which induce optimal apoptosis rather than too drastic apoptosis. The levonorgestrel intra-uterine system (LNG-IUS) has since been shown to induce regression of complex endometrial hyperplasia (Wildemeersch *et al.*, 2015; Haoula *et al.*, 2011; Zhao *et al.*, 2015). LNG-IUS down-regulates expression of PRA, PRB, ER-α and ER-β (Vereide *et al.*, 2006; Gomes *et al.*, 2009). Complex atypical hyperplasia was also shown to decrease after progestin therapy (Gunderson *et al.*, 2014; Kudesia *et al.*, 2014). The present results depict the synergistic antiproliferative effect of 4.0µg/ml LNG on the HeLa cell in the presence of combination antiretroviral drugs. although the rate of apoptosis appears too drastic, thus suggestive of increased cell loss and possibly tissue damage.

HAART-induced apoptosis in HeLa cells is drastically increased with levonorgestrel and ethinylestradiol co-treatment for ABC+ZDV+3TC, ZDV+3TC+NVP and d4T+3TC+NVP treated cells. This increased toxicity upon co-administration of hormonal contraceptives is suggestive of increased tissue causality effect on cervical intraepithelial neoplasia progression or other serious non-AIDS events observed with some cohort studies correlating HAART to CIN progression (Lillo *et al.,* 2001; McKenzie *et al*., 2011). Overall, there is too-drastic apoptosis for single antiretroviral treatment, compared to combination antiretroviral treatment, thereby concurring with the concept that multi-drug therapy is less toxic in small combination doses while at the same time exhibiting a high therapeutic effect. One would envisage a need for further toxicity research on nucleoside reverse transcriptase inhibitor combinations with protease inhibitors, since such regimens have also found widespread use. This may help to identify less toxic, hence ideal, HAART regimens for hormonal contraceptive users.

Overall, the present results predominantly indicate the proliferative effect of 0.5µg/ml EE upon co-administering with combination antiretroviral drugs; the present results concur with other studies suggesting the cellular re-modelling effect of oestrogens on cancer cells. This is typified by Mosli *et al*. (2013) who demonstrated that *in vitro* exposure of non-transformed prostate epithelial cells (BPH-1) to catechol oestrogens (CEs) had a neoplastic effect. Further related studies have shown that oestrogen has proliferative effects in normal and MCF-7 breast cancer cells (Scaling *et al*., 2014; Pesiri *et al*., 2015). G protein-coupled estrogen receptor (GPER), previously known as GPR30, modulates breast cancer proliferation downwards. In MC7 10A cells, oestrogen stimulation in the presence of GPER-selective agonist G-1 was shown to increase the mitogenic index (Scaling *et al*., 2014).

On the other hand, the antiproliferative effect of LNG suggests the non-neoplastic effect of its co-administration with combination antiretroviral drugs. Such protective effect has also been substantiated by other studies on progestogen-based hormones. Zhao *et al*. (2015) have shown that LNG inhibits human endometrial stromal cell (HESC) and human endometrial glandular cell (HEGC) proliferation while inducing apoptosis in both cell lines. The levonorgestrel intra-uterine system (LNG-IUS) has since been shown to induce regression of complex endometrial hyperplasia (Wildemeersch *et al*., 2015; Haoula et *al*., 2011; Zhao *et al.,* 2015). Complex atypical hyperplasia was also shown to decrease after

progestin therapy (Gunderson *et al*., 2014; Kudesia *et al*., 2014). Women with complex atypical hyperplasia (CAH) and endometrial carcinoma had a response of about 65% upon receiving progestin-only treatment (Gunderson *et al*., 2014).

It is important to investigate the role of other different hormonal contraceptives on human cervical epithelial cancer cell proliferation. This is in view of contradictory findings depicting the antiproliferative effect of some oestrogens and the proliferative effect of progestins. For example, Mazzucco *et al* (2006) demonstrated that dual receptor activation of ERα and ERβ showed reduced cell proliferation, thus suggesting that ERα and ERβ modulate each other's activity. Two isoforms of progesterone receptor (PR), namely progesterone receptor-A (PRA) and progesterone receptor-B (PRB), are associated with endometrial differentiation (Vereide *et al.*, 2006). Deregulation of either of the genes has been associated with carcinogenesis of the endometrium and progestin therapy failure (Gunderson *et al*., 2014; Kudesia *et al*., 2014, Vereide *et al*., 2006). Whether or not antiretroviral drug induction of apoptosis is influenced by co-administration of different contraceptive hormones may be fascinating. Such investigative screening may provide a safe choice of contraception that is dependent on the HAART drug combination.

6.1.3 Apoptosis-induced cellular regeneration

In the present study, it was observed that, despite the drastic induction of apoptosis by 0.5µg/ml EE, ABC+3T, ZDV+3TC, ABC+ZDV+3TC and ZDV+3TC+NVP did not significantly differ to the untreated control in their ability to reduce MTT. This is suggestive of apoptosis-induced remodelling and possibly regenerative hyperplasia. LNG only depicted such non-proliferative difference upon co-administration only with the regimen ABC+ZDV+3TC. These results concur with the concept that that cell death by apoptosis does not necessarily imply inhibition of remodelling (Fan and Bergmann, 2008a; Ryoo *et al*., 2004; Ryoo and Bergmann, 2012; Shi *et al.*, 2012). Studies in *Drosophila* have shown two types of apoptosis related cellular regeneration. It has been shown that proapoptotic proteins can induce proliferation of adjacent cells through a process known as compensatory proliferation (CP) so as to regenerate cells (Ryoo and Bergmann, 2012; Fan and Bergmann, 2008b; Ryoo *et al.*, 2004). CP requires cell death-inducing genes, mainly coding for caspases. As such our present results suggest cellular remodelling, despite the induction of apoptosis.

Mosli *et al*. (2013) and Mazzucco *et al.* (2006) concur that, in addition to the genomic pathway, there is evidence that a non-genomic signalling pathway *via* ERs contributes to cell proliferation. This is evidenced by oestradiol-dependent cellular proliferation, which is mediated through ERα control of gene transcription and kinase activation (Pesiri *et al*., 2015; Scaling *et al*., 2014; Atawia *et al*., 2013). L^{429} and A^{430} are located within the ERα ligand-binding domain and contribute to E2-induced proliferation, although the mechanism of their action has not been fully elucidated (Pesiri *et al*., 2015). Likewise, other independent research has also evidenced that oestrogen has proliferative effects in normal and MCF-7 breast cancer cells (Scaling *et al*., 2014; Pesiri *et al*., 2015). The present *in vitro* experimental conditions could have interfered with the proliferative rates of ethinylestradiol-treated cells, since significant cellular proliferation was not observed. Instead, suggestive observations for cellular regeneration are of interest.

Apoptosis-induced cellular proliferation could plausibly explain the observed non-significant MTT difference of proliferative rates in 0.5µg/ml EE-treated HeLa cells compared to untreated cells. In *Drosophila*, the initiator caspase Dronc initiates compensatory proliferation following death signals, for example from irradiation. This process induces compensatory proliferation in neighbouring cells through secretion of mitogens (Ryoo and Bergmann, 2012). In these circumstances, apoptosis is initiated but subsequently inhibited by Hedgehog signaling, hence leading to arrest of cell death (Fan and Bergmann, 2008a; Ryoo and Bergmann, 2012). In higher-level organisms, the equivalents of Dronc are initiator caspases -8, -9 and -10 which mimic the former in their supposed action in CP (Fan and Bergmann, 2008; Ryoo *et al.*, 2004). This pathway is also known as the effector caspase-independent pathway (Ryoo and Bergmann, 2012). In the alternative pathway of CP, the effector caspase equivalent, DrICE triggers Hedgehog (Hh) signalling for compensatory proliferation. Alternatively, this is referred to as the effector caspase-dependent pathway of CP (Fan and Bergmann, 2008(a); Ryoo and Bergmann, 2012).

In higher level organisms, equivalents of DrICE are effector caspases -3, -6 and -7 (Fan and Bergmann, 2008a; Fan and Bergmann, 2008b; Ryoo *et al.*, 2004). It has since been demonstrated that 17β-oestradiol attenuates caspase-9 and terminal caspase-3 activity in its antiapoptotic effects (Wang *et al.*, 2004), a possible mechanism through which 0.5µg/ml EE also results in the currently observed regenerative proliferation. It has since been suggested that cervical cancer is a hormone-dependent tumour that may be stimulated by

oestrogens (Practice Committee of American Society for Reproductive Health, 2008; Jiang *et al.*, 2016). Regenerative hyperplasia has been associated with apoptosis dependent regeneration of lost cells (Ryoo and Bergmann, 2012). Co-administration of ABC+3T, ZDV+3TC, ABC+ZDV+3TC and ZDV+3TC+NVP with EE and co-administration of ABC+ZDV+3TC with LNG treated cells are thus suggestive of increased loss of cells and possibly tissue damage due to regenerative hyperplasia. Otherwise, if effector caspase-dependent apoptosis is within the optimal levels (not too drastic), it is beneficial in its role of eliminating damaged cells and replacing them with a healthy progeny (Ryoo and Bergmann, 2012).

6.1.4. Transformation of pre-monocyte U937 to adherent cells

Results from this study demonstrate that highly active antiretroviral therapy can transform U937 cells into adherent cells whose cumulative production of superoxide anion significantly differs to the untreated control. Highly active antiretroviral treatment transformed premonocyte lymphoma cells (U937) to monocyte/macrophage-like morphology characterised by an increase in superoxide anion production. MI macrophages attract cells of the adaptive immunity in response to signals from bacteria and IFNγ (Housinkveld and van der Burg, 2011). These cells are associated with expression of reactive oxygen species (ROS), inducible nitric oxide synthatase (iNOS) and activation of Natural Killer cells, hence these are associated with inflammation response (Martinez and Gordon, 2014). In previous chapters, it was demonstrated that these U937-transforming HAART regimens (ABC+3TC+NVP, ZDV+3TC+NVP and d4T+3TC+NVP) play a role in cervical cancer cell apoptosis. These joint observations demonstrate the anticancer activity of the triple antiretroviral combinations ABC+3TC+NVP and d4T+3TC+NVP. The antiapoptotic effect of ZDV+3TC+NVP suggests its contribution to serious non-AIDS events. Further classification of whether these transformed cells exhibit M1 or M2 macrophage characteristics could go a long way to explain the antiproliferative effect of some highly active antiretroviral regiments associated with cervical intraepithelial neoplasia regression observed among women initiating HAART.

6.1.5 Overall conclusion

Overall, it was observed that, despite the drastic induction of apoptosis by ethinylestradiol, its co-administration with ABC+3T, ZDV+3TC, ABC+ZDV+3TC and ZDV+3TC+NVP did not significantly differ to the untreated control ($P \leq 0.05$) in viability of cells, as measured by the MTT assay. This is suggestive of cellular remodelling effect of

ethinylestradiol. Treatment with 4.0µg/ml levonorgestrel demonstrates such non-proliferative difference upon co-administration with ABC+ZDV+3TC and ZDV+3TC+NVP. This suggests that, the observed levonorgestrel cytotoxicity with other combinations (ABC+3T, ZDV+3TCand d4T+3TC+NVP) does not positively impact on tissue regeneration through proliferation or compensatory hyperplasia but it rather results in the beneficial increased human cervical cancer cell loss. Co-administering 2.0µg/ml LNG shows antiproliferative activity with all combination antiretroviral treatments on HeLa cells. Subject to further clinical research, levonorgestrel-only contraceptives may prove a better option for people on highly active antiretroviral treatment. The transformation of U937 cells to 'macrophage-like' cells by ZDV+3TC+NVP is of interest, in view of the antiapoptotic effect of this regimen. This suggests a possibility of precipitating serious non-AIDS events. If replicated *in vivo*, these results could go some way to explaining the SNAEs seen with long-term therapy (Mafuva and Morgan, 2016).

6.2.0. Directions for further research

The present study highlights some preliminary aspects of antiretroviral drug effects on cancer cell proliferation. However, limitations in time and financial resources prevented a possibility of further interrelated explorations in this seemingly vast reproductive health and cancer research area. This section highlights further envisaged future directions emanating from the present observations.

6.2.1. Apoptosis and regenerative hyperplasia pathways

Whether the apoptotic pathway in human cervical cancer epithelial cells is *via* the intrinsic or extrinsic pathways can be evaluated. As explained earlier, the extrinsic pathway is signalled through cell-membrane receptors by death factors such as FAS and TNF receptors that result in a cascade leading to activation of caspases -3, -6 and -7 which are executors of apoptosis (Matt and Hoffman, 2016; Kang *et al.*, 2012). On the contrary, the intrinsic pathway for apoptosis is signalled *via* an intracellular domain that results in DNA digestion (Matt and Hofmann, 2016). The protein Apaf-1 is activated by cytochrome c, thus activating caspase-9 which, in turn, activates executioner caspases (Kang *et al*., 2012). Using flow cytometry, determination of whether or not each class of caspases is regulated will go a long way in establishing the mechanisms of toxicity and/or the pathway by which apoptosis is attained. While the present observations depict proliferative effect of EE, the exact pathways through which this occurs was not evaluated. As described earlier, the two pathways of CP are the effector caspase-independent and the effector caspase-dependent

pathways (Fan and Bergmann, 2008a; Ryoo *et al.*, 2004, Ryoo and Bergmann, 2012). One might envisage an experimental design in which the *Drosophila* is exposed to combination antiretroviral drugs mixed through their liquid food supply. After a certain exposure time, expression of Dronc, DrICE and p35 can then be determined to evaluate the CP pathways that are affected by exposure to antiretroviral drugs with or without hormonal contraceptives.

6.2.3 Classification of macrophage-like cells

The present research has demonstrated that combination ARVs have the varied potential to differentiate U937 cells into adherent cells. Cells from the triple combinations ABC+3TC+NVP, ZDV+3TC+NVP and d4T+3TC+NVP transformed U937 to cells with macrophage-like morphology. These transformed cells were able to generate the superoxide anion. Further characterisation assays may be carried out on these adherent cells. To investigate phagocytic activity, the transformed cells can be assessed for the ability to phagocytose protein-coated latex particles which are commercially marketed as pregnancy test kit (Meshkini *et al.,* 2009). Macrophages display CD11b and CD14 surface markers. To further assess macrophage-like anatomy, the adherent cells can be incubated with anti-CD11b and anti-CD14 antibody and the cells analysed by flow cytometry (Lin *et al.,* 2014; Meshkini *et al.,* 2009). Time and financial constraints did not allow continuation with such immunological characterisation.

M1 macrophages eliminate pathogens, virus-infected cells and cancer cells. These are associated with intracellular reactive oxygen species (ROS) (Housinkveld and van der Burg 2011; Martinez and Gordon, 2014). M2 macrophages have been associated with tumour promotion. M1 and M2 macrophages are also known as classical and alternative macrophages, respectively (Martinez and Gordon, 2014). Overall, M1 macrophages are stimulated by IFN-γ, tumour necrotic factor (TNF) or lipopolysaccharide (LPS). M2 macrophages have different stimuli namely interleukin (IL-4)and IL-13 for M2a macrophage, IC and TLR/IL-1R ligands for M2b macrophage and IL-10 forM2c macrophage (Martinez and Gordon, 2014). M1 macrophages generate NO whereas M2 macrophages make trophic polyamines (Martinez and Gordon, 2014). These and other properties can be used to assess macrophage characteristics.

6.2.4. Cohort and case-oriented designs

While *in vitro* studies cannot be conclusive on their own, the observed differential potential of antiretroviral treatment on apoptosis induction with or without contraceptive hormones (levonorgestrel and ethinylestradiol) is of interest. Cohort (follow up) and case-oriented (retrospective) designs incorporating exploration on antiretroviral use and history of contraception may provide further insight to corroborate these *in vitro* observations. Figure 6.1 summarises a typical cohort study design envisaged from these results. A mixed-method approach during such a follow-up study helps the practitioners to infer into the service user's lived experience. Such a phenomenological approach makes it easy to evaluate the contraceptives used through questionnaires. In addition, cytological screening for n1, n2 and n3 depicted in Figure 6.1 enables classification of cervical intraepithelial neoplasia and cancer. The main advantage of cohort designs is that they can be reliable as they pick up the cause-and-effect relationship through observations over a period of time. Observations from these studies can be evaluated in view of the present preliminary *in vitro* observations.

A number of limitations affect cohort studies. Firstly, the design is time-consuming as participants need to be followed over long periods, typically 2 years and above. Secondly, follow up studies maybe expensive especially in low-resource areas where participants may need funding for sundry expenses, such as transport costs to attend their appointments. Accounting for confounders related to lifestyle may present challenges on its own. Finally, attrition is another problem, whereby participants are lost during the course of the research. This could be due to death, truancy, voluntary withdrawal and any other unforeseen factors.

The case-oriented design (retrospective study) is an alternative epidemiological design which can be used to investigate use of contraceptives among HAART patients. This is dependent on sampling patients with their consent as they present to the HAART clinic. Here the practitioners work 'retrospectively'. Consenting patients are asked to provide information on their previous use of hormonal contraceptives, how long they have been on HAART and questions on any other confounding factors such as parity and smoking history. To complement the retrospective questions, Papanicolaou (Pap) smear evaluation correlates contraceptive and HAART use to CIN progression. This mixed method approach can also augment the discourse for our present *in vitro* observations. The main advantage of such an approach is that it is less time-consuming and less expensive in comparison to

the former and incorporates user experience. The major disadvantage is its dependency on patients' memory, as some may not be good in remembering dates on which they initiated taking specific contraceptives or changed from one form of contraception to another.

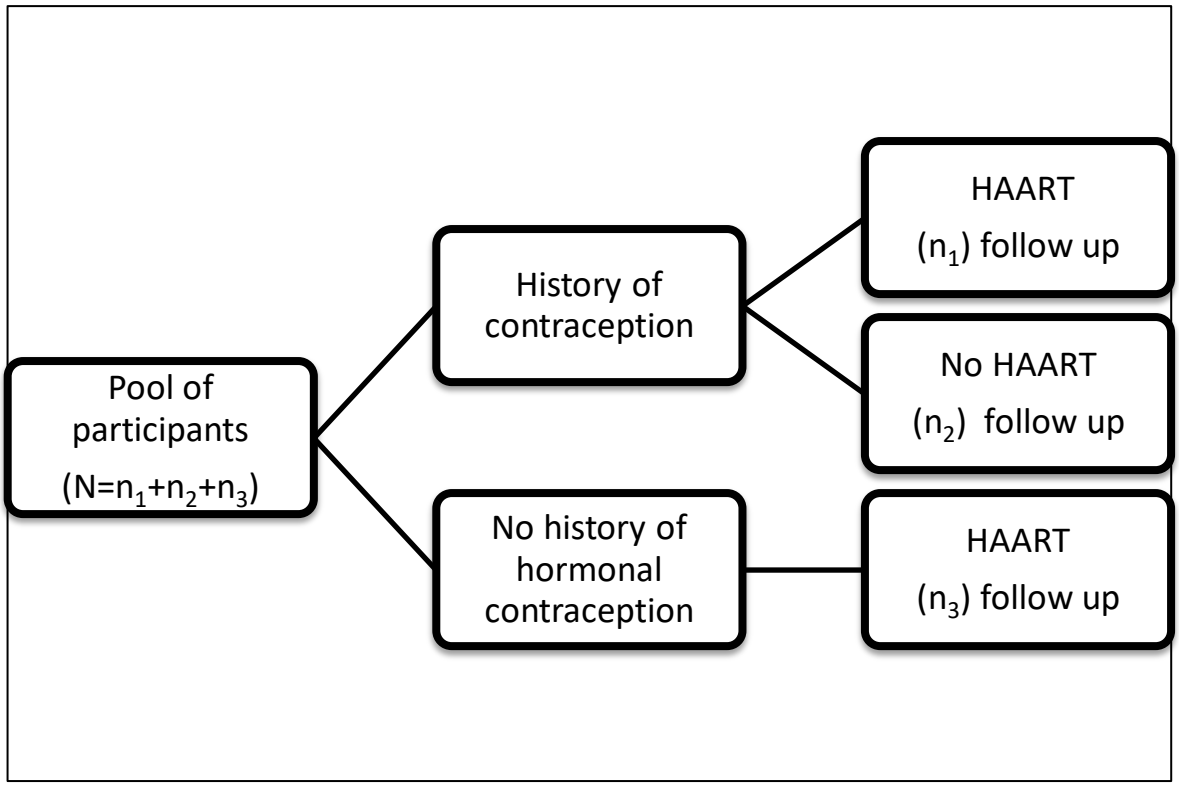

Fig 6.1: Outline of participant categories in a HAART and contraception cohort study. The participants are selected from people attending HAART treatment at a local reproductive health clinic. The participants fall into those with a history of contraception use and those with no history of contraception use. The participants are then further sub-categorised as those exposed to HAART and those not on HAART as they are followed over a period and parameters such as Papanicolaou (Pap) smear detected to evaluate cervical dysplasia. N represents the total number of participants while n_1, n_2 and n_3 represent number of participants in each group.

6.3.0. Implications for reproductive health practice

The present research demonstrates the variable effects of ethinylestradiol and levonorgestrel on combination antiretroviral-induced apoptosis in human cervical cancer cells. Hormonal contraceptives have been suggested as co-factors for proliferation of HPV-infected human cervical cancer cells (Webster *et al.,* 2001). There are limited *in vivo* studies on causality and effect of hormonal contraceptives on cervical cancer progression among women on HAART. This section explores the potential of utilising reproductive services to enquire into the benefits of evidence-based practice in evaluating neoplasia and cervical cancer aetiology among HAART patients.

6.3.1 Reproductive health clinics as research resource centres

Cognisant of improved cervical cancer screening programs globally, reproductive health service providers have generally escalated cervical cancer screening among women. With the current increased incidence and prevalence of HIV, these reproductive health services may work in conjunction with HIV clinics in monitoring and evaluating the effects of contraceptive hormones among HAART patients. Such a phenomenological approach enables Public Health practitioners to study the contraception choices and use by women considered for cohort or case-oriented studies. While the present study was limited to combinations of NRTIs and NVP, an exploration based on family planning clinics would provide a holistic approach incorporating all antiretroviral and contraceptive choices among different communities. *In vitro* conditions are not necessarily similar to *in vivo* conditions. *In vivo*, cohort- and case-oriented designs can be used to evaluate the cause-and effect relationship between co-administration of hormonal contraceptives with antiretroviral drugs and cervical cancer progression. This allows evaluation of different permutations and combinations to be feasibly evaluated against cervical cancer development or progression.

6.3.2 Use of voluntary testing and counselling centres

There are a number of HIV testing and counselling centres in various countries that are used as a surveillance strategy. Participants at these centres may be ethically recruited and case oriented studies used to explore ever-use of contraceptives. Cohort studies may then be used to investigate the effect of HAART among patients using different hormonal contraceptives. Through monitoring and evaluation such an approach will enable recommendations for safer contraceptive options dependent on the regimen of HAART one is on.

6.3.3 Further scientific research on other drugs

While this research focussed on NRTIs and NVP (an NNRTI), in practice, there are number of contraceptives alternatives including those tailor-made for some co-existing medical conditions among patients receiving HAART. As described in the first chapter classes of antiretroviral drugs include NRTIs, protease inhibitors, integrase inhibitors and NRRTs. Similarly, there exists a variety of hormonal contraceptives in addition to the ethinylestradiol and levonorgestrel COCs discussed in this study. These include but are not limited to drospirenone-containing, levonorgestrel-containing and other progestogen-containing COCs, levonorgestrel-only contraceptives, injectable norethisterone enanthate and depot medroxyprogesterone acetate (DMPA). Human epithelial cancer cell toxicological studies involving various permutations of these contraceptives and HAART co-administration may inform increased safety in contraception options among HAART patients.

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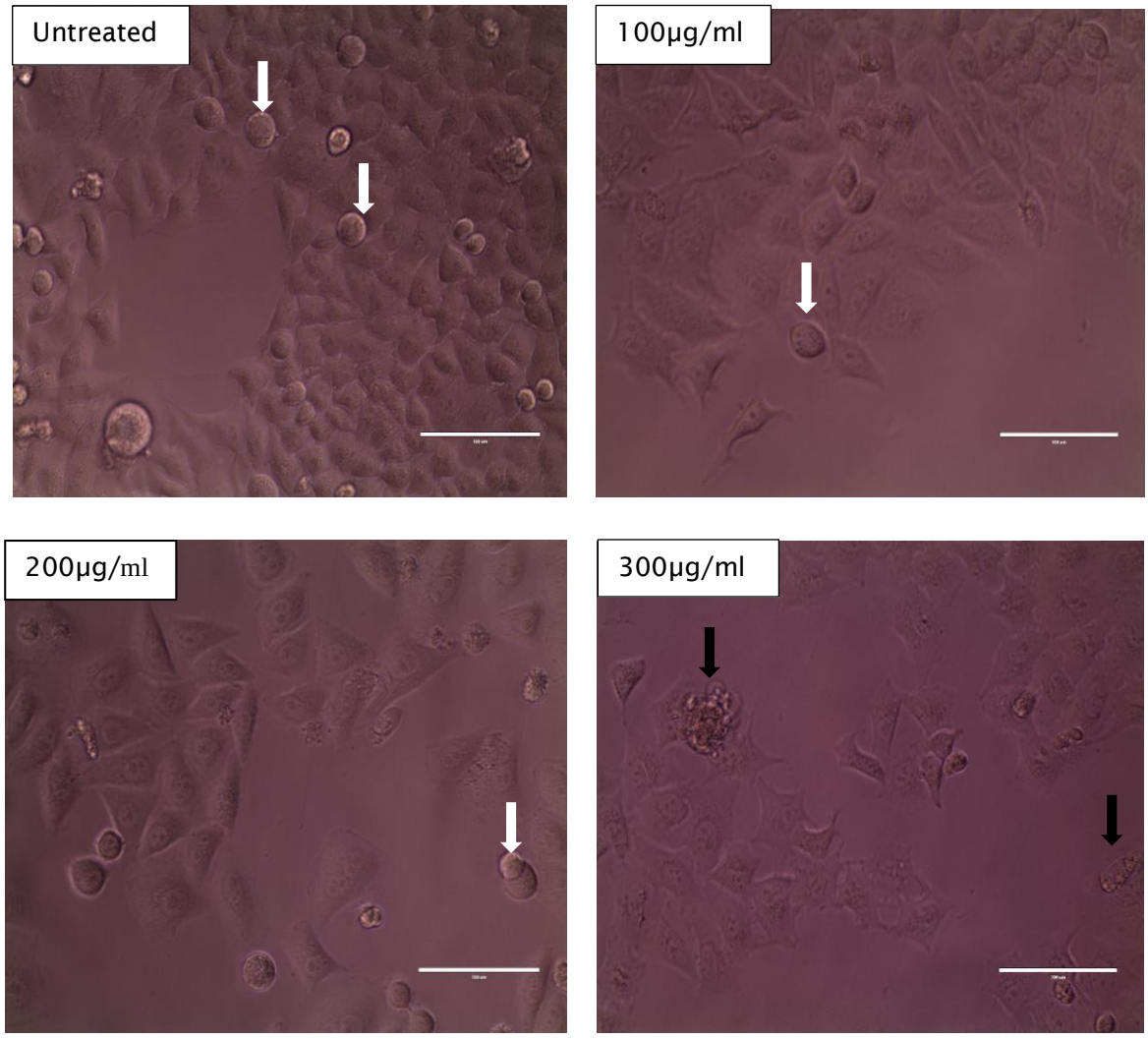
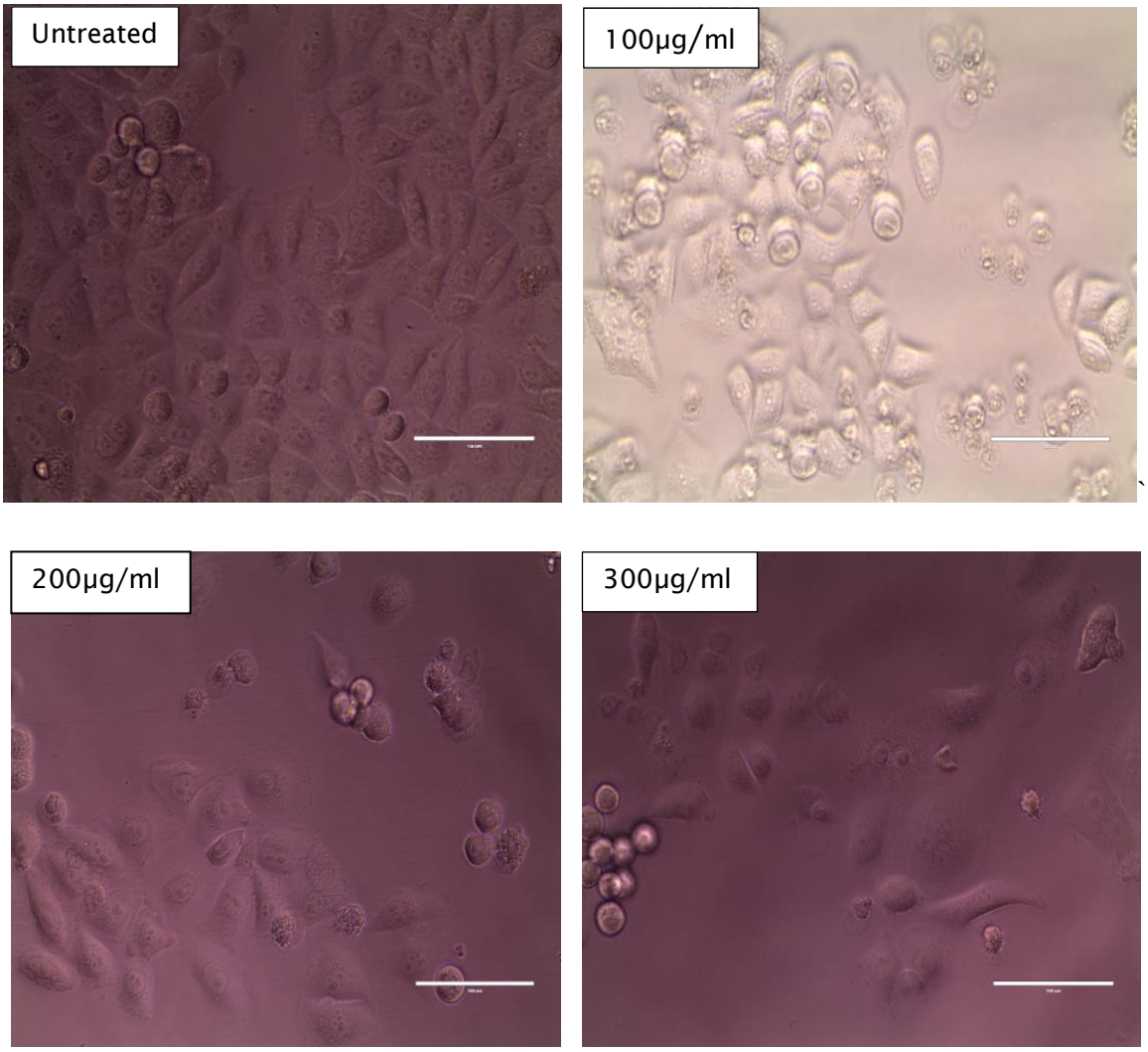
APPENDIX 1

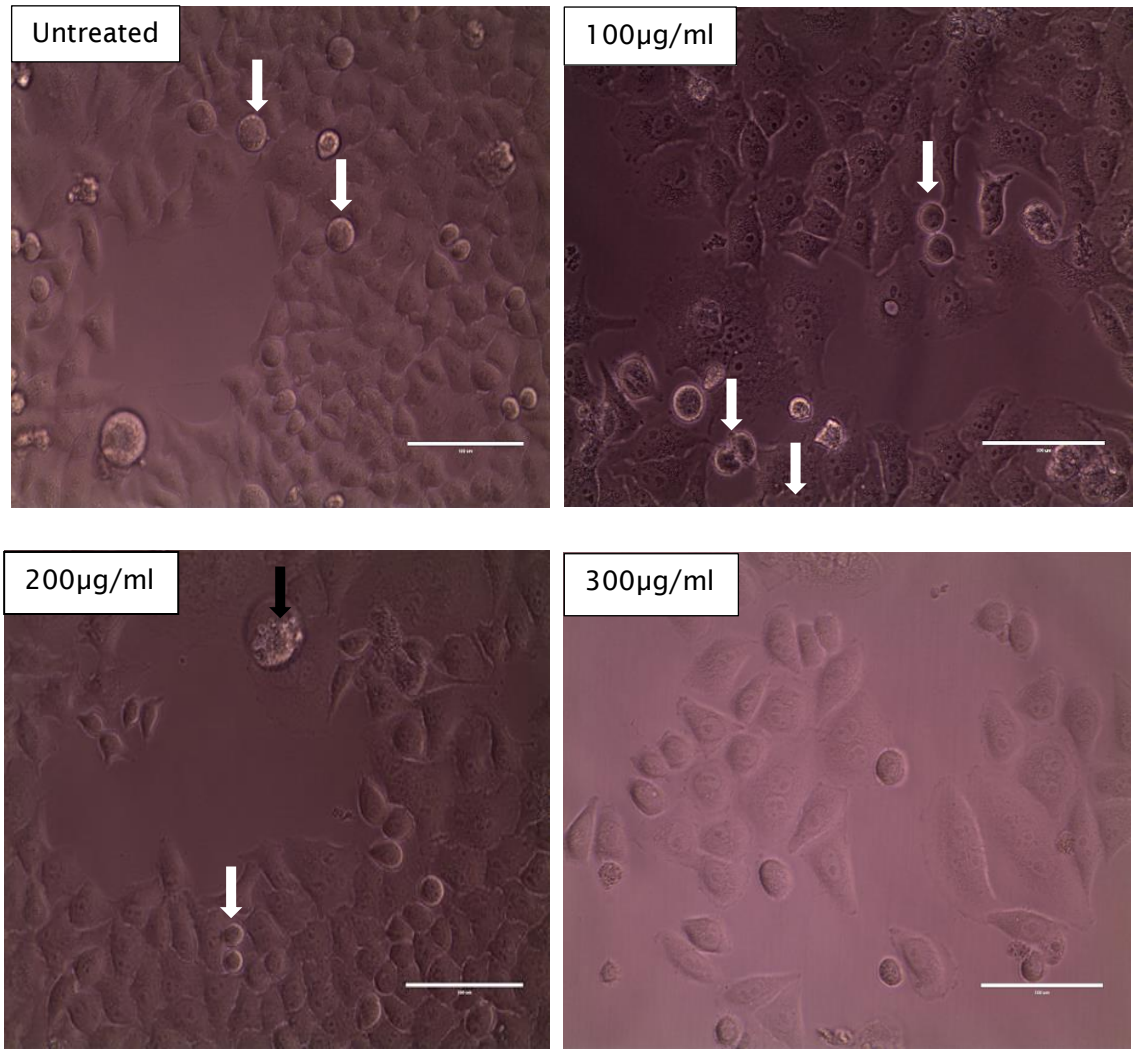
MORPHOLOGY OF HeLa CELLS ON 24 HOUR PRELIMINARY TREATMENT WITH ANTIRETROVIRAL DRUGS



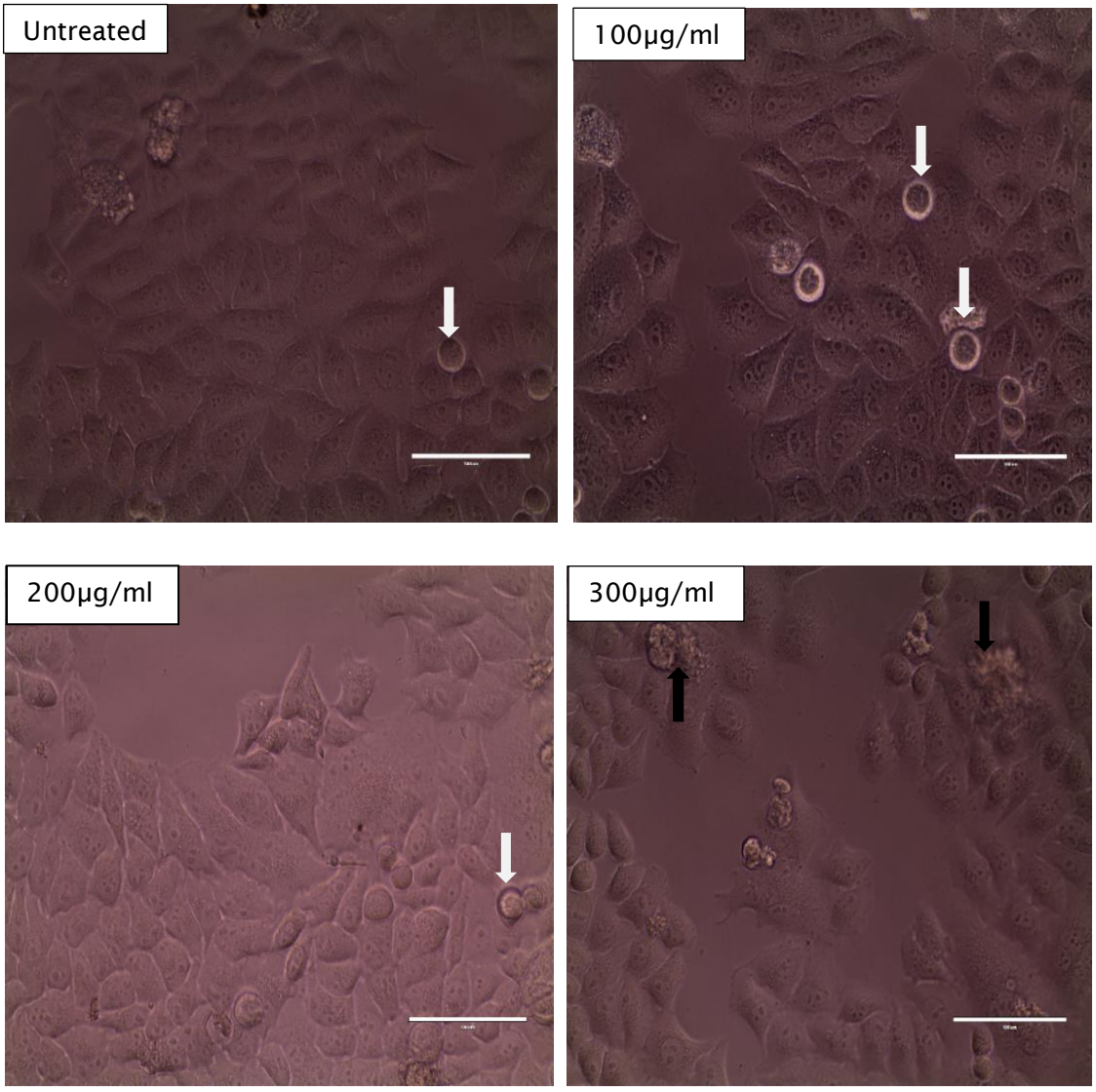
(a) Microscopic appearance of HeLa cells after 24 h treatment with abacavir sulfate. At 100ug/ml and 200µg/ml there is no microscopically obvious morphological change compared to untreated cells and there are some dividing cells as evidenced by large nuclei. Large nuclei of dividing cells are apparent as depicted by white arrows. As the concentration increases to 300µg/ml, the cells become spindle- and star-shaped while there is a decrease in cell density. At 300µg/ml, black arrows indicate cells with protruded membrane and apoptotic bodies. The scale bar indicated on the right bottom of each picture represents 100µm.



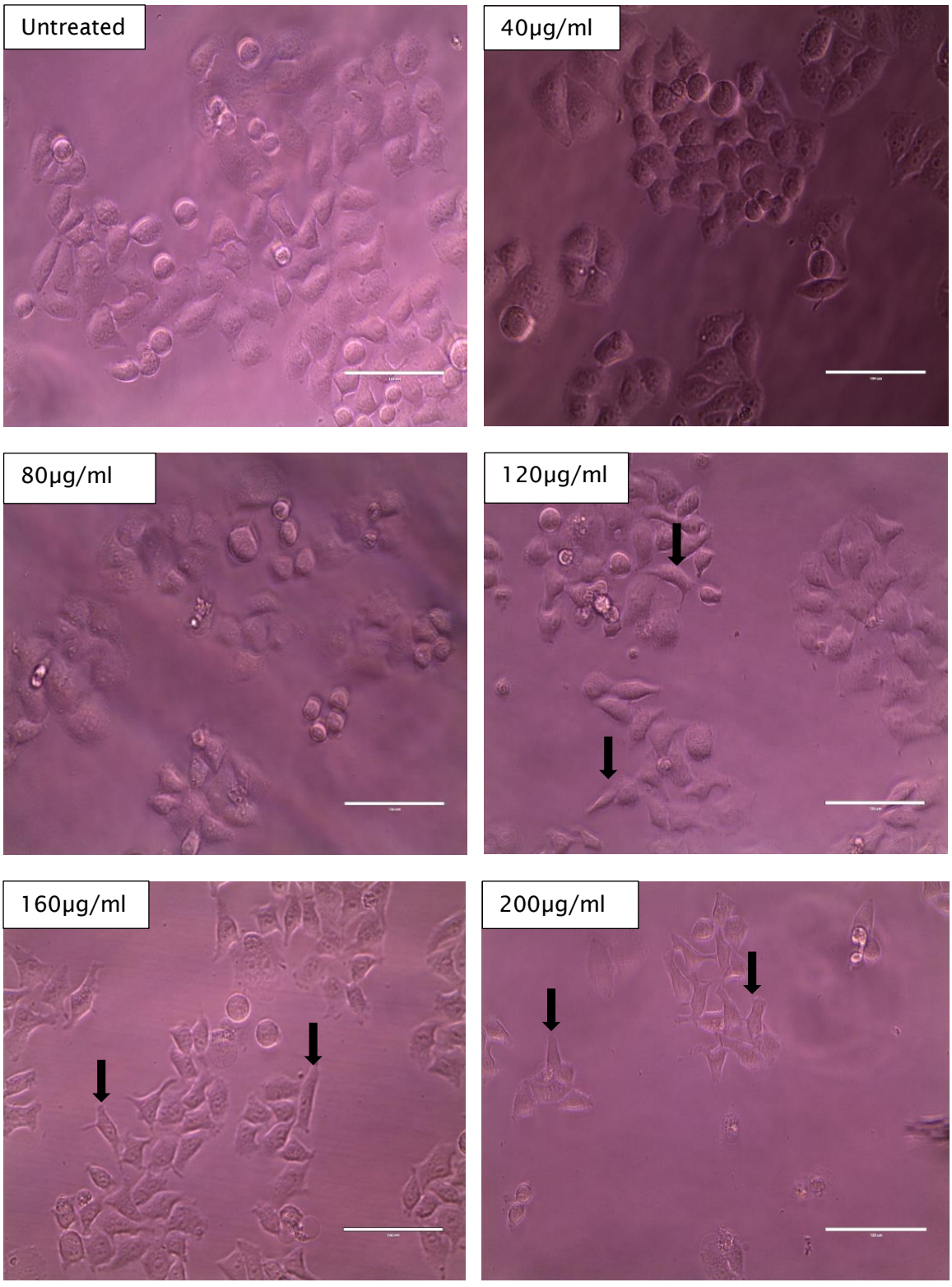
(b) Microscopic appearance of HeLa cells after 24 h treatment with zidovudine. Cells treated with 100µg/ml still exhibit the generic HeLa morphological characteristics. Increasing the concentration to 200µg/ml and 300µg/ml produces cells that are heterogenous in morphology. Large nuclei of dividing cells are apparent as depicted by white arrows. There is a gradual decrease in cell density with increasing concentration.



(c) Microscopic appearance of HeLa cells after 24 h treatment with lamivudine. At 100ug/ml the typical HeLa morphology is mantained. At 200ug/ml and 300µg/ml the cells are still viable and actively dividing with no obvious morphological changes, although the cells appear less dense with 300µg/ml. Large nuclei of dividing cells are aparent as depicted by white arrows. At 200µg/ml, the black arrow indicates cells with protruded membrane and apoptotic bodies. The scale bar indicated on the right bottom of each picture represents 100µm.



(d) Microscopic appearance of HeLa cells after 24 h treatment stavudine. At 100µg/ml and 200µg/ml cells have semblance to untreated cells regarding the nuclear/cytoplasmic ratio. Large nuclei of dividing cells are apparent as shown by white arrows. At 300µg/ml, the cells are still dividing as depicted by the spherical nuclei although the cells seem to be varied as depicted by a mixture of some anvil, spindle and normal shaped cells. Black arrows at 300µg/ml indicate cells with protruded membrane and apoptotic bodies. The scale bar indicated on the right bottom of each picture represents 100µm.



(e)Structure of HeLa cells after 24 h treatment with varying concentraions of nevirapine. As shown in the diagram, most cells exhibit morphological features of viable cells at all concentrations up to 80µg/ml. However, from 120µg/ml to 200µg/ml the cells are increasingly becoming spindle-shaped as shown by the arrows. Cells also become less dense with increasing concentration. The scale bar represents 100µm.

APPENDIX 2

Opinion article in the Journal of Public Health in Africa

Full article is available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5345434/

Journal of Public Health in Africa 2013; volume 4:e16

Hormonal contraception and HIV/AIDS transmission: challenges for Zimbabwe's reproductive health service providers in promoting informed contraception choices

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Abstract

None-barrier methods are the most predominant contraceptive methods of choice among Zimbabwean women, with the contraceptive pill being the most popular. The spread of HIV/AIDS is most prevalent in sub-Saharan African countries, Zimbabwe included. The prevalent mode of transmission is unprotected heterosexual sex. Although Zimbabwe boasts of a high literacy rate some women may still be vulnerable like in other parts of the world, as they may not understand the role of the Zimbabwe National Family Planning Council (ZNFPC) and other reproductive health service providers. This is because some women at risk may expose themselves to unprotected sex while they are on hormonal contraceptives. This paper seeks to infer into pros and cons of hormonal contraceptive use among Zimbabwean women. There is also need to discuss the effectiveness of providers (ZNFPC clinics and the Ministry of Health) in educating women about the risk of HIV transmission, which may be associated with some non-barrier methods of contraception. An understanding of women's attitudes towards the different forms of contraception is of paramount importance as is that of the factors that could contribute to women in different social settings resorting to uninformed contraceptive choices.

Introduction

It has since been established that unprotected heterosexual sex is the predominant mode of HIV transmission among Zimbabweans. The Zimbabwe National Family Planning Council (ZNFPC) works in conjunction with the Ministry of Health clinics in distributing different types of contraceptives depending on the client's preferences. Previous research has noted that the contraceptive pill is the most popular method of family planning in Zimbabwe.[1,2] In Bolivia, Nicaragua and Zimbabwe, between 50% and 60% of sexually active unmarried women have used the pill whereas 80% of married women have used the pill in Brazil, as have two thirds of married Zimbabwean women.[2] Use of the injectable depot is an alternative choice although it is not as popular among Zimbabwean women.[2]

While a number of studies found no significant association between hormonal contraception and HIV acquisition, a study of HIV-discordant couples in 7 African countries demonstrated a double risk of HIV transmission and acquisition associated with hormonal contraception. In this regard, it is argued that women at risk of HIV acquisition should be counseled about the potentially high risk of HIV acquisition with use of hormonal contraceptives.[3] These findings relate well with another study carried out in Uganda, Zimbabwe and Thailand in which both combined oral contraceptive and depot-medroxyprogesterone (DPMA) users had an increased risk of HIV acquisition compared with non-hormonal group.[4] These observations were statistically significant for DPMA while there was a weak correlation for combined oral contraceptives. Meanwhile research in South Africa does not rule out moderate increases in HIV risk transmission among DPMA users although there could be need to carry out further research.[5,6] The World Health Organization's technical statement on hormonal contraception and HIV maintains that because of the inconclusive nature of the causality and effect relationship, women can continue to use all existing hormonal contraceptives without restriction. It also encourages that women using hormonal contraceptives and at risk of HIV acquisition should be advised to always use condoms and other preventive measures.[7] There is need to further explore the synergism between hormonal contraceptive use and unprotected sex among Zimbabwean women. Nanda *et al.*[8] undertook a study in Uganda, Zimbabwe and Thailand in which they found out that following HIV diagnosis, 135 (98.5%) of 137 hormonal users continued hormonal contraceptives (HC) and 14 (25%) of 57 nonusers began using HC. It is therefore important to infer into the behaviors of such HIV positive women on HC as this could pose a risk should they engage in indiscriminate and unprotected sex with multiple partners. Such an inference would also help to establish whether it maybe necessary for family planning clinics and other distributors of hormonal contraceptives to provide voluntary screening for HIV infection so that users receive counseling and are ultimately encouraged to use alternative forms of contraception such as barrier methods typified by the condom to avoid infection and re-infection with different strains of the virus. This helps to empower HIV positive women to make informed choices relating to their reproductive lives, free of cohesion.[9]

Media reports have highlighted that sex workers in Zimbabwe are yearning for alternative means of earning money, as they believe that such initiatives would go a long way in helping reduce the spread of HIV/AIDS. Some of these women have even raised concern that they believe most of their clients' wives will be infected despite their innocence as some men desist from using barrier methods such as condoms.[10] There is thus need to embrace sex education as a tool to empower vulnerable women using HCs as they may be at risk of contracting HIV through unprotected sex.

Research by Kiddugavu *et al.*[11] in Uganda found a weak association between use of hormonal contraceptives and HIV acquisition and this had no statistical significance following adjustment for behavioral confounding factors hence this cohort study allowed women to continue using their voluntarily chosen contraceptive. On the contrary, Mantel *et al.*[12] and Nanda *et al.*[8] clearly emphasize the need to use barrier methods such as the condoms in conjunction with HCs in order to reduce the risk of sexually contacting HIV and other sexually transmitted infections among women at risk.

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Key words: hormonal, contraception, HIV/AIDS, Zimbabwe.

Acknowledgements: the authors would like to express their sincere gratitude to Dr Alan Buckingham from the Department for Health (University of Bath) for his invaluable guidance on approaches of appraising literature in line with research based practice and management in health services research.

Conflict of interests: the authors declare no potential conflict of interests.

Received for publication: 24 July 2013.
Revision received: 11 October 2013.
Accepted for publication: 12 October 2013.

Licensee PAGEPress, Italy
Journal of Public Health in Africa 2013; 4:e16
doi:10.4081/jphia.2013.e16

APPENDIX 3

Abstract accepted for the British 'Pharmacology 2016' annual conference

TRANSFORMATION OF MONOCYTES TO MACROPHAGES BY COMBINATION ANTIRETROVIRAL TREATMENT

Introduction: The increased use of highly active antiretroviral therapy (HAART) has resulted in a dramatic increased survival of HIV-infected individuals. A downside of the success of antiretroviral therapy (ART) is the premature onset and increased risk of certain inflammatory age-related diseases associated with low levels of chronic immune activation. Such activation is thought to contribute to what is described as serious non-AIDS events (SNAEs) [1]. HAART entails combining two nucleoside reverse transcriptase inhibitors (NRTIs) with either one or two protease inhibitors (PIs) or non-nucleoside reverse transcriptase inhibitors (NNRTIs), in this study usually nevirapine (NVP) [2].

Method: In an attempt to better understand the impact of HAART on the immune system, U937 cells (a histolytic lymphoma monocyte line) were exposed to a range of drugs used for HAART therapy and differentiation to macrophage morphology assessed. In 24 well plates, cells were treated with single and combination drugs at a final concentration of 50µg/ml of each drug and incubated at 37^{o}C and 5% CO_2 for 72hrs (n=3 in all treatments). Single drug antiretroviral treatment was with abacavir sulfate (ABC), zidovudine (ZDV), lamivudine (3TC), stavudine (dT4) and nevirapine (NVP). The following antiretroviral combinations were considered: ABC+3TC, ZDV+3TC, ABC+ZDV+3TC, ZDV+3TC+NVP and d4T+3TC+NVP. Following 72 hours treatment with antiretroviral drugs, the percentage of adherent cells as a fraction of total live cell count was assessed.

Results: Adherence of single drug treatment was as follows: 3.25% for ABC, 86.03% for zidovudine ZDV, 21.05% for 3TC, 47.22% for stavudine dT4 and 12.50% for nevirapine NVP. The effect of treatment with combination antiretroviral drugs was as follows: 13.51% for ABC+3TC, 7.89% for ZDV+3TC, 40.11% for ABC+ZDV+3TC, 42.07% for ZDV+3TC+NVP and 39.89% d4T+3TC+NVP. Distinct morphological changes including amorphous and granular structure coupled with increased cell size and adherence were predominantly observed for ZDV and triple antiretroviral drug combination treated cells.

Conclusion: These initial observations indicate that HAART is capable of stimulating the differentiation of monocyte (pre-macrophage) into cells with "macrophages-like" morphological characteristics. If replicated in vivo this could go some way to explaining the SNAEs seen with long term highly active antiretroviral treatment.

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TRANSFORMATION OF MONOCYTES TO MACROPHAGES BY COMBINATION ANTIRETROVIRAL TREATMENT

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1. INTRODUCTION

- The increased use of highly active antiretroviral therapy (HAART) has resulted in a dramatic increase survival of HIV-infected individuals. A downside of the success of anti-retroviral therapy (ART) is the premature onset and increased risk of certain inflammatory age-related diseases associated with low levels of chronic immune activation. Such activation is thought to contribute to what is described to as serious non-AIDS events (SNAEs)(1). HAART entails combining two nucleoside reverse transcriptase inhibitors (NRTIs) with either one or two protease inhibitors (PIs) or non-nucleoside reverse transcriptase inhibitors (NNRTIs), in this study usually nevirapine (NVP). In the present study the impact of different combinations of anti-retroviral drugs (ARDs) was assessed on monocyte cells.

2. METHODOLOGY: U937 cells (a histolytic lymphoma monocyte line) were exposed to a range of drugs used for HAART therapy and differentiation to macrophage morphology assessed for 72 hours in 24 well plates. Cells were assessed for viability, adherence to the place and differentiation to macrophage morphology.

3. RESULTS

Individual and combinations of ARD reduced the viability of U937 cells and also increased the numbers of cells with macrophage morphology (Figure 1). Table1 and Table2 depict the number of cells transformed to macrophage by single and combination antiretroviral treatment respectively while Figure 2 and Figure 3 summarize the total numbers of viable and transformed cells.

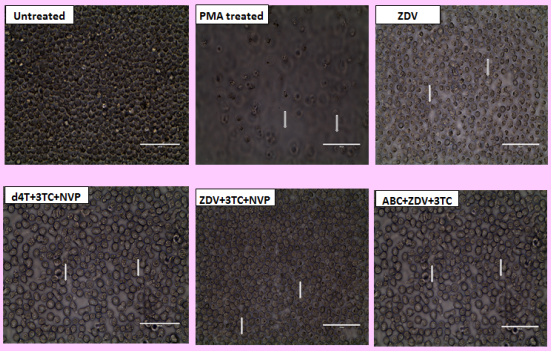

Fig 1: Morphological appearance of cells after 72hr treatment with ZDV and combination antiretroviral treatment. Cells were treated with ZDV or combination drugs at a final concentration of 50µg/ml for each drug and incubated at 37^{O}C and 5% CO2 for 72hrs. A high degree of granulation was observed for ZDV, d4T+3TC+NVP and ZDV+3TC+NVP treated cells. ABC+ZDV+3TC treatment resulted in a mixture of granular and non-granular cells. Arrows indicate typical transformed cells with some distinct granulated morphology.

Table 1: Descriptive statistics for U937 cells pre-treated with single drugs

Cell ($x10^6$)	-ve	+ve	ABC	ZDV	3TC	d4T	NVP
Non-adherent	1.96 ± 0.17	0.04 ± 0.04	1.49 ± 0.08	0.19 ± 0.18	0.75 ± 0.08	0.57± 0.06	1.12 ± 0.08
Adherent	0.00 ± 0.01	0.33 ± 0.06	0.05 ± 0.02	1.17 ± 0.06	0.20 ± 0.04	0.51 ± 0.08	0.16 ± 0.04
Dead	0.04 ± 0.02	0.03 ± 0.07	0.09 ± 0.02	0.03 ± 0.02	0.03 ± 0.02	0.04 ± 0.04	0.12 ± 0.04

U937 cells were treated with 10ng/ml PMA as the positive control (+ve) or treated with single antiretroviral drugs or left untreated (-ve) for 72hrs. The numbers represent the estimated total cell count ($x10^6$ cells) ± SD for three independent experiments.

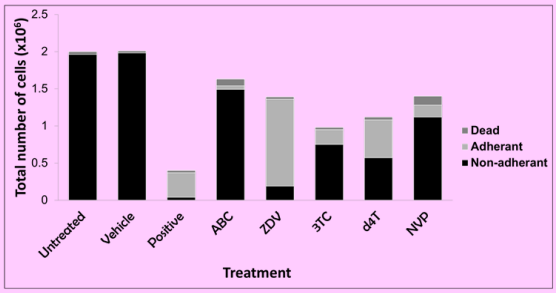

Fig 1: Differential conversion of U937 monocytes to adherent cells by single antiretroviral treatment. U937 cells were treated with 10ng/ml PMA as the positive control (+ve) or treated with single antiretroviral drugs or left untreated (-ve) for 72hrs. The numbers shown represent the estimated total cell count ($x10^6$ cells) for three independent experiments.

Table 2: Descriptive statistics for U937 cells pre-treated with combination antiretroviral drugs (n=3, mean ± SD)

Cell type ($x10^6$)	-ve	+ve	ABC+ 3TC	ZDV+ 3TC	ABC+ZDV +3TC	ZDV+3TC +NVP	d4t+3TC +NVP
Non-adherent	2.03 ± 0.12	0.07 ± 0.06	1.60 ± 0.08	1.40 ± 0.12	1.12 ± 0.08	0.84 ± 0.12	1.07 ± 0.14
Adherent	0.00 ± 0.00	0.28 ± 0.04	0.25 ± 0.06	0.12 ± 0.04	0.75 ± 0.06	0.61 ± 0.08	0.71 ± 0.17
Dead	0.04 ± 0.02	0.04 ± 0.04	0.11 ± 0.02	0.21 ± 0.16	0.09 ± 0.02	0.19 ± 0.06	0.08 ± 0.04

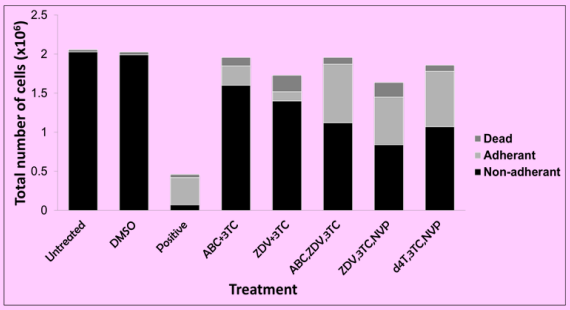

Fig 3: Differential conversion of U937 cells to adherent cells by combination antiretroviral treatment. U937 cells were treated with 10ng/ml PMA as the positive control (+ve) or treated with combination antiretroviral drugs or left untreated (-ve) for 72hrs. The numbers represent the estimated total cell count ($x10^6$ cells) for three independent experiments.

Conclusion

Overall, adherence of single drug treatment was as follows: 3.25% for a ABC, 86.03% for ZDV, 21.05% for 3TC, 47.22% for dT4 and 12.50% for NVP. The effect of treatment with combination antiretroviral was as follows: 13.51% for ABC+3TC, 7.89% for ZDV+3TC, 40.11% for ABC+ZDV+3TC, 42.07% for ZDV+3TC+NVP and 39.89% d4T+3TC+NVP. Morphological changes including an increase in the cell size were noted predominantly for ZDV and triple antiretroviral drug combination stimulated cells. These initial observations suggest that HAART drugs are capable of stimulating the differentiation of monocyte (pre-macrophage) into cell with some characteristics of differentiated macrophages. If replicated in vivo could go some way to explaining the SNAEs seen with long term HARRT therapy.

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APPENDIX 6

PROCEEDINGS OF THE 13th WORLD CONFERENCE ON INFLAMMATION

Role of contraceptive hormones on combination antiretroviral treatment induced apoptosis in human cervical cancer cells

Available from:
http://static.springer.com/sgw/documents/1614767/application/pdf/13.+WCI+London+2017+Abstract+Book+-+Springer+Portal.pdf pp101-102.

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INTRODUCTION

Combination of nucleoside reverse transcriptase inhibitors (NRTIs) is the predominant method for HIV treatment. Highly active antiretroviral treatment (HAART) entailing two or more NRTIs and nevirapine (NVP) has been the method of choice in most resource limiting countries. Abacavir (ABC), lamivudine (3TC), zidovudine (ZDV) and stavudine (d4T) have been in use in these settings. Some double combinations have been of choice in patients presenting with co-infections or other clinical conditions. We previously evaluated the effect of levonorgestrel (LNG) and ethinyl estradiol (EE) [constituents of combined oral contraceptives] on ZDV+3TC+NVP induced apoptosis in human cervical epithelial cells. Here, we further evaluate the effect of (LNG) and (EE) on ABC+3TC, ZDV+3TC, ABC+ZDV+3TC and d4T+3TC+NVP induced apoptosis.

METHOD

HeLa cells (a human cervical epithelial cancer cell line) were treated with combination antiretroviral drugs (ARDs) at final concentrations of within the 20-25% inhibitory concentration of each drug. The cells were treated with antiretroviral drugs alone for 24hrs and co-treated with varying EE and LNG for a further 48hrs. Untreated cells saved as control. MTT cell proliferation rate was analysed by one-way ANOVA followed by Dunnett's multiple comparison test ($n=3$, $P<0.05$). Further, HeLa cells were treated with ARDs (in the presence or absence of EE or LNG) over 48hrs and apoptosis measured by four-way dot plots. Data was analysed by the one-way ANOVA followed by Tukey's test.

RESULTS

Overall, combination ARDs increase the rate of apoptosis compared to untreated cells ($P<0.05$). Co-administration with contraceptive hormones further drastically increases the rate of combination ARD induced apoptosis compared to cells treated with ARDs only.

CONCLUSION

Combination ARD induced apoptosis is suggestive of decreased cervical neoplastic transformation as observed in some studies (1,2). Combination ARD co-administration with contraceptive hormones is suggestive of increased cell loss and possibly tissue damage as depicted by some epidemiological studies suggesting cervical neoplasia progression among HAART patients (3). Further prospective and retrospective clinical studies maybe of interest.

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Keywords: apoptosis, antiretroviral, contraceptives