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Effects of a synthetic indoline derivative on apoptosis in MID and late stage colon cancer cell lines

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EFFECTS OF A SYNTHETIC INDOLINE DERIVATIVE ON APOPTOSIS IN MID AND LATE STAGE COLON CANCER CELL LINES

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A dissertation submitted to the Faculty of Heath Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science

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Johannesburg – 2019

DECLARATION

I, **Vivash Naidoo** declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in Medicine, to the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

Student signature: _ _____

On the 7th of June, 2019

DEDICATION

Dedicated to the Memory of my loving Mother **Latchmy Naidoo** and Father **Abboyi Naidoo**

RESEARCH OUTPUTS

Presented at:

- Naidoo V, Kandhavelu J, Subramanian K, Kandhavelu M, Ruff P and Penny C. Wits Faculty of Health Sciences Research Day (2018), 6 - September, University of the Witwatersrand, Johannesburg. **Synthetic Indoline Derivatives as a potential drug for the treatment of colon cancer cell lines.**
- Naidoo V, Kandhavelu J, Subramanian K, Kandhavelu M, Ruff P and Penny C. Molecular Biosciences Research Thrust (2018), 29-November, Wits - Professional Development Hub, Johannesburg. **Indoline derivative as apoptotic inducer in mid and late stages of colorectal cell lines**
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- Naidoo V, Kandhavelu J, Subramanian K, Meenakshisundaram K, Ruff P and Penny C. Advanced Proteomics Workshop and Symposium, 20-22 August 2018, Wits Medical School.
- Naidoo V, Kandhavelu J, Subramanian K, Meenakshisundaram K, Ruff P and Penny C. 23-August 2018, Wits Medical School, **Symposium: Effect of Indoline derivatives on apoptosis genes of colon cancer cell lines.**

ABSTRACT

Colorectal cancer (CRC) is the fourth most common and the sixth most lethal cancer in South Africa. Although, there have been advances in anticancer drug development, there is nevertheless a need for the development of novel cost-effective drugs for treatment. Several reports have suggested that the indole ring contained in various natural and synthetic compounds, is an important chemical moiety with certain biological activities that confers anticancer properties against several cancers. Thus, the aim of this study was to evaluate the potential apoptotic effects of a synthesized Indoline molecule (N-(2-hydroxy-5-nitrophenyl (4-methylphenyl) methyl) (HNPMPI) on HT-29 (mid stage) and DLD-1 (late stage) colon cancer cell lines. In particular, the possible effects of HNPMPI on cell morphology and apoptosis (Annexin V) were assessed. After establishing the apoptotic profile of the drug treated cells, changes in the expression of key apoptotic proteins were evaluated using a focused human apoptosis protein array. Based on the identification of differentially regulated proteins identified from the array study, gene expressions of selected genes were analyzed using RT-PCR. Further, the subcellular localization of key proteins was determined using Confocal Microscopy. After treatment with HNPMPI, the Annexin V profiling demonstrated that HNPMPI more effectively induced apoptosis in HT-29 cells as compared to the DLD1 cell line. The protein profiler assay revealed differential expression of the apoptotic proteins and it showed that the extrinsic pathway of apoptosis is triggered in HT 29 cells; and Caspase mediated apoptosis was activated in DLD-1 cells, after the treatment. With regards to gene expression levels, the transcription levels of the pro-apoptotic genes BAD, BAX and p53 decreased in DLD-1 cells with HNPMPI treatment. In comparison in the HT-29 cells, while mRNA levels of BAX and p53 decreased, BAD levels were increased. The transcription of the two anti-apoptotic genes, Bcl-2 and CASP-3, had diverse responses to the drug treatment in the two cell lines CASP-3 transcription levels increased in both cell lines; while Bcl-2 levels were constant in the HT-29 cells, but decreased in the DLD-1 cells. In support of this, immunofluorescence confirmed that the intracellular protein expression of Bcl-2 and P53 reflected the results obtained from the protein array, after drug treatment.

The indole derivative HNPMPI showed effective apoptotic activity against both cell lines, representing mid and late stage colon cancer. A balance between an over-expression of pro-apoptotic genes and under expression of anti-apoptotic genes is responsible for carcinogenesis. Although gene expression of selected genes were altered, the protein expression results obtained here confirm that HNPMPI has promising pro-apoptotic properties acting to induce

both the intrinsic and extrinsic pathways of cell death in a stage specific manner in the two colon cancer cell lines.

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For a house to be built it takes some stones, cement, bricks and hands and that's how the Team Works to make a Dream Work.

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LIST OF ABBREVIATIONS

ABI	Applied Biosystems Informatics
AJCC	American Joint Committee on Cancer
AD	Anno Domini
ATCC	American Type Culture Collection
BAX	BCL2 Associated X
BCL2	B-cell lymphoma 2
BC	Before Christ
BAD	BCL2 associated agonist of cell death
BSA	Bovine serum albumin
CAT	Catalase
cFLIP	FLICE-like inhibitory protein
CRC	Colorectal cancer
cyto c	Cytochrome c
DAPI	4′,6-diamidino-2-phenylindole
DD	death domain
DIM 3	3'-diindolylmethane
DISC	Death-inducing signalling complex
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EA	Early Apoptosis
EGFR	Epidermal growth factor receptor
FADD	Fas-associated death domain
FAP	Familial adenomatous polyposis
FBS	Foetal Bovine Serum
FDA	Food and drug administration
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HIF-1α	Hypoxia inducible factor 1α
HNPCC	Hereditary nonpolyposis CRC syndrome
HNPMPI	N-(2-hydroxy-5-nitrophenyl (4'-methyl phenyl) methyl) indoline
HSP	Heat shock protein
I3C	Indole-3-carbinol
IAP	Inhibitor of apoptosis proteins

IBD	Inflammatory bowel diseases
LA	Late Apoptosis
LS	Lynch syndrome
LV	Leucovorin
MAPK	Mitogen-activated protein kinases
MDM2	Mouse double minute 2 homolog
MKRN1	Makorin Ring Finger Protein 1
NCDB	National Cancer Data Base
NF-κB	Nuclear factor-κB
PBS	Phosphate buffered saline
PDGF	Platelet-Derived Growth Factor
PON2	Paraoxonase 2
pRB	Retinoblastoma protein
PSMB5	Proteasome subunit β5
PT	Permeability transitioning
ROS	Reactive oxygen species
SOD	Superoxide dismutase
STAT3	Signal transducer and activator of transcription 3
TGF-α	Tumour growth factor alpha
TNF	Tumour necrosis factor
TNFR	TNF/tumour necrosis factor receptor
TNM	Tumour, Node and Metastasis
TRADD	TNFR-associated death domain
TRADD	Tumour necrosis factor receptor type 1-associated DEATH domain
TRAIL	TNF-related apoptosis-inducing ligand
UC	Ulcerative colitis
UICC	International Union against Cancer
VDAC	Voltage-dependent anion channel porin
VEGF	Vascular Endothelial Growth Factor
5-FU	5-fluorouracil

CHAPTER 1: INTRODUCTION

1.1 Cancer

Cancer is a complex disease and is the result of genetic aberrations and epigenetic changes that lead to the cell escaping normal cellular controls. The word cancer originates from the Greek physician Hippocrates (460 - 370 BC) who is considered as the "Father of Medicine". He used the terms "*carcinos"* and "*carcinoma"* for the *non-ulcerative* and *ulcerative* tumours, respectively. In Greek, the words mean a "*crab"*, as most of the tumours that are likely to spread projections from them resemble the shape of a crab (Karpozilos and Pavlidis, 2004). Later these Greek terms were translated into Latin by the Roman physician, Celsus (28 - 50 BC). The Latin word for crab is *cancer*. In 130 - 200 AD, a Roman physician Galen used the word "*oncos"* to refer to cancer, and this word is still used as a part of the name of the cancer specialists "*Oncologists"* (Petrelli *et al.,* 2009, Hajdu, 2011).

A normal and healthy body contains approximately thirty trillion cells. These cells live as complex, interdependent entities and regulates proliferation of other cells and they reproduce when they are signalled to do so by other cells (Weinberg, 1996, Varmus *et al.,* 1993). This kind of collaboration ensures the tissue, organ and/or the body maintains its size and structure (Bianconi *et al.,* 2013). In contrast, tumour cells don't follow the instructions from these collaborating cells. They reproduce following their own internal signals and gain the ability to migrate from the site of origin. They can invade nearby tissue by rupturing the basal membrane and form tissues. They may also migrate to distant sites in the body through the lymphatic system and bloodstream. These malignant cells form tumours, which will become aggressive over time and disrupt the function of vital organs that are needed for the survival of the organism (Egeblad *et al.,* 2010).

The different stages of tumour development are depicted in Figure 1.1. The stimulatory and inhibitory pathways and their outcomes have been determined in recent years. These molecular pathways are active in the nucleus of the cells and are referred to as the cell cycle clock and function to control when a cell divides or not. In a normal cell, the cell cycle clock does this by integrating growth-regulating signals (Weinberg, 1996, Edmunds and Adams, 1981).

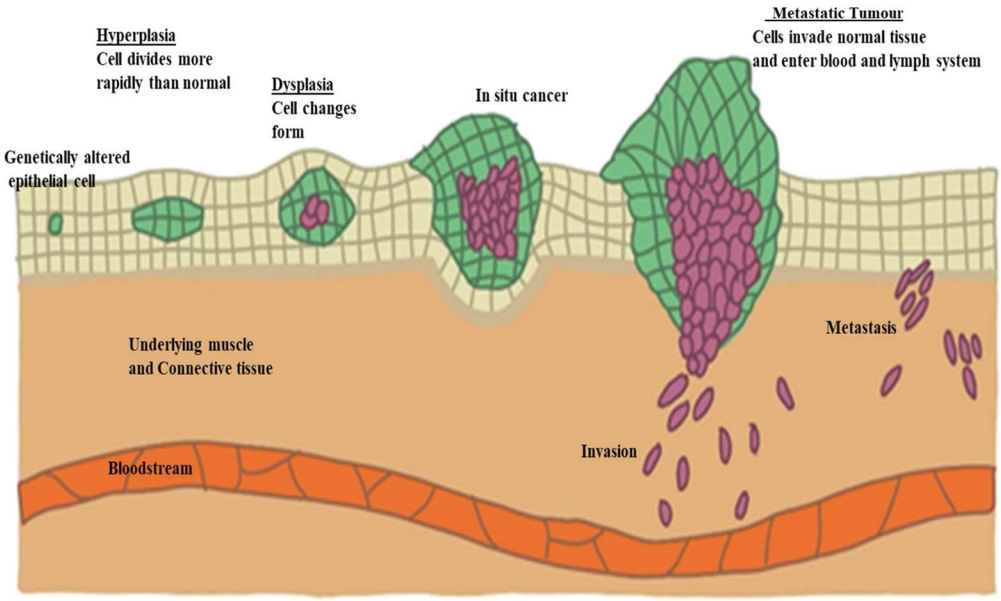


Figure 1.1 Stages of tumour development

The figure depicts the different stages in the development of cancer. From the initial cells where a mutation first arises, this leads to the loss of cell cycle control, regulation of apoptosis or one of the other "hallmarks" of cancer. The benign stages of tumour development then start with Hyperplasia or rapid cell division, followed by Dysplasia, where cell morphology changes; and following this a localised tumour form. This then develops into a malignant tumour that penetrates the basal cell membrane, enters the bloodstream or lymphatic system and spreads to other parts of the body (metastasis) (Adapted from Kanwal *et al*., 2013).

It is believed that a single abnormal cell with a mutated DNA sequence can eventually progress into cancer. This involves multiple-genes that eventually develop into cancer in a multi-step process (Vogelstein and Kinzler, 1993). This abnormal cell may acquire a second mutation, that may provide an advantage that allows uncontrolled proliferation. The proliferation of this cell propagates the mutation, giving rise to a tumour (Hejmadi, 2009).

1.2 Cancer Classification

Cancer can be classified into 5 types based on their site of origin (Hejmadi, 2009).

- Carcinomas - cancers of the epithelial cells of particular organs
- Sarcomas - muscles, bone, cartilage, and connective tissue cancers
- Myeloma - originates in the plasma cells of bone marrow
- Lymphomas - develops in the lymphatic system
- Leukaemias - occur in the haematopoietic system

1.3 Epidemiology of Cancer

Globally, cancer is a leading cause of morbidity and mortality. In 2012 there were 14 million new cases of cancer, resulting in approximately 8.2 million cancer-related deaths. Most countries show an increasing trend in the number of new cancer cases. The developing world accounts for 57% (8 million) of new cancer cases, 65% (5.3 million) of cancer-related deaths and only 48% (15.6 million) of the 5-year survival rate (Bray *et al.,* 2018). Nearly half of these new cases and more than half of the cancer-related deaths, occur in Asia (Jemal *et al.,* 2010). Estimates suggest that the cancer related death rate is higher in Asia and Africa, which have a higher proportion of cancer deaths (7.3% and 57.3%, respectively) in relation to their cancer incidence (5.8% and 48.4%, respectively). The most common cancer is lung cancer, which is also the leading cause of cancer-related death (Figure 1.2) (Bray *et al.,* 2018). This trend of higher mortality rates in developing countries is most likely caused by the types of cancer most commonly diagnosed in these countries as having a poor prognosis. This is compounded by a lack of facilities or resources to screen patients and provide early diagnosis and successful treatment (Bray *et al.,* 2018).

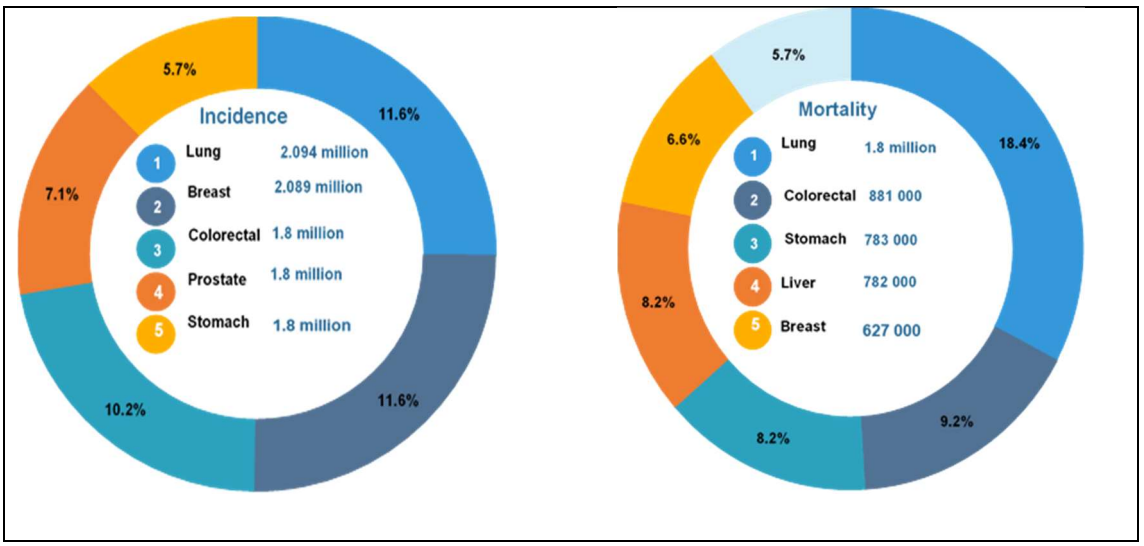


Figure 1.2 Incidence and mortality rates of different types of cancers in humans worldwide

A graphical representation of the incidence and mortality rates for colon cancer compared to the other most prevalent and deadly cancers, i.e. lung, breast, liver, stomach and prostate (Adapted from Bray *et al*., 2018).

Men show a higher cancer incidence rate than women, with rates of 205 per 100, 000 in men, compared to 165 per 100,000 in women. This is almost a 25% higher incidence rate in men. The different geographical regions of the world also show a larger gender variation in cancers,

almost five-fold higher in male cancer incidence rates than in women. In Western Africa the incidence rate is 79 per 100,000, while in Australia/New Zealand it is 365 per 100,000 (Siegel *et al.,* 2018). The variation in mortality between different geographical regions is much lower, with the mortality rate being lower in less developed regions compared to more developed regions. The mortality rate in developed regions is 15% higher in males and 8% higher in females. The regions with the highest mortality rates amongst men are in Central and Eastern Europe (173 per 100,000), while the region with the lowest mortality rate amongst men is Western Africa (69 per 100,000). Amongst women the regions with the highest mortality rates are Melanesia (119 per 100,000) and Eastern Africa (111 per 100,000) (Bray *et al.,* 2018).

1.4 Hallmarks of Cancer

In 2000, a seminal article by Hanahan and Weinberg defined "The Hallmarks of Cancer". These were common traits, or characteristics of cancer cells and helped to define the scientific community's "roadmap" for the study and treatment of cancer (Hanahan and Weinberg, 2000). This was expanded upon in 2010, with the addition of four new traits (Hanahan and Weinberg, 2011). All ten of these hallmarks of cancer (Figure 1.3) are discussed individually in the following sub-sections.

1.4.1 Sustaining proliferative signalling

For growth and division, normal cells have control mechanisms that govern growth and regulate cell division (homeostasis). These normal cells require external growth signals which are transmitted through receptors located on the plasma membrane. In the absence of growth signals, normal cells stop dividing (Gordon *et al.,* 2018). Cancer cells can bypass external growth signals, and some can generate their own. For example**,** glioblastomas can synthesise Platelet-Derived Growth Factor (PDGF) and sarcomas can synthesise Tumour Growth Factor alpha (TGF-α) (Raica and Cimpean, 2010). In some cases, mutated receptors can initiate signalling without the binding of a growth factor ligand (Hanahan and Weinberg, 2011).

1.4.2 Evasion of growth suppressors

Cell proliferation in normal cells is a tightly controlled process where the proliferation and anti-proliferation signals co-ordinate their activities in order to properly regulate the cell cycle. The G_1 phase of the cell cycle is a particularly vital checkpoint where the antigrowth signals exert their influence to block cell proliferation. The growth inhibitors whose primary function is to keep the growth of normal cells under control are generally ineffective on cancer cells (Calcinotto *et al.,* 2019). In such cells, mitosis could not be interrupted by the inhibitors during

interphase and cells continue proliferating, for example Retinoblastoma protein (pRB) normally negatively regulates E2F target genes that are required for the cell to enter S phase. In cancer, pRB is downregulated and the cell continues proliferation and the loss of p53 allows for cell cycle progression despite DNA damage and cellular stresses (Duronio and Xiong, 2013).



Figure 1.3 Hallmarks of Cancer

Hallmarks of cancer are biological traits that define the transition from normal cells to the cancer cell. These underlying traits are common to all cancers and may be used to target cancer cells during treatment (Adapted from Hanahan and Weinberg, 2011).

1.4.3 Resistance to cell death

In the event of cell damage, the cells are programmed to die through a mechanism called '*apoptosis'*. Unfortunately, cancer cells possess the ability to bypass this mechanism and grow uncontrollably by silencing pro-apoptotic proteins and overexpressing anti-apoptotic proteins (Hanahan and Weinberg, 2011).

1.4.4 Enabling replicative immortality

Cancer cells surpass the limit of the number of divisions a cell is normally limited to leading to indefinite growth resulting in immortality. However, they possess damaged chromosomes and hence become cancerous. This limit to the number of times a cell can divide is known as the

Hayflick limit. The Hayflick limit of mammalian cells is about 60-70 doublings prior to senescence (Shay and Wright, 2000). The limits are surpassed by disabling the expression of pRB and P53 tumour suppressor proteins, leading to a stage called the *crisis*. Eventually, they will become immortal and can double without limit. Associated with this in most cancer cells is the upregulation of the enzyme telomerase which maintains telomeres, the counting device for cell doublings (Hanahan and Weinberg, 2011).

1.4.5 Activation of invasion and metastasis

Metastasis and tissue invasion are the means whereby tumour cells spread to distant organs from the primary site (site of origin) (Zeeshan and Mutahir, 2017). Metastasis includes local tissue invasion, intravasation, transition through the blood and lymphatic system and colonization of foreign tissue, e.g. altered N-CAM allows metastases in Wilm's tumour, neuroblastoma and small cell lung cancer (Hanahan and Weinberg, 2011, Kanada *et al.,* 2016).

1.4.6 Genome instability and mutation

If cell division is altered in normal cells, caretaker genes are activated, allowing for the detection of DNA damage. These genes are then able to either directly activate DNA repair or inactivate the mutagenic substances (Vogelstein *et al.,* 2013). However, in cancer cells, the activity of mutagenic agents increase or DNA repair mechanisms shut down leading to genome instability and mutation, for example, P53 or BRCA1 (Hanahan and Weinberg, 2011, Liang *et al.,* 2009).

1.4.7 Promotion of inflammation by tumours

Tumours and the areas surrounding them can be infiltrated by immune system cells (both adaptive and innate immune cells). The release of pro-inflammatory cytokines into the environment of the tumour by immune cells aids in the growth of the tumour. This can be interpreted as a means whereby tumours mimic inflammatory conditions seen in normal tissues. Chemokines released during the inflammatory response aid in angiogenesis and metastasis, by assisting the breakdown of the extracellular matrix (ECM). These factors may also lead to genetic mutations that enable and accelerate the formation of a tumour (Jinushi, 2014).

1.4.8 Reprogramming energy metabolism

In order to sustain proliferation, cancer cells need to utilize energy resources not available to other cells. This can be done by altering their metabolic processes. Normal cells convert glucose into pyruvate via glycolysis. This occurs in aerobic conditions in the cytosol and the pyruvate then enters the citric acid and oxidative phosphorylation pathways to produce energy. Cancer

cells convert glucose to lactate, under anaerobic conditions, favouring glycolysis over subsequent energy pathways, with relatively little pyruvate being dispatched to the oxygen-consuming mitochondria (Warburg, 1956, Hanahan and Weinberg, 2011, Hay, 2016).

1.5 Colorectal Cancer

The cancer of the large intestine, the final part of the digestive system, is known as bowel cancer, colon cancer, rectal cancer or colorectal cancer (CRC), depending on the site of origin (Marmol *et al.,* 2017). It commonly emerges as a benign polyp in the lining of colon or rectum and multiplies into the walls of the colon to form a tumour. The changes occurring at the DNA level such as the activation of oncogenes and or loss of tumour suppressor genes will lead to the tumour becoming malignant (Brody, 2015).

CRC is a common malignancy and was reported to be the third most commonly diagnosed cancer. Globally, it is the fourth deadliest of all cancers in men and third in women. It is estimated to claim the lives of 320,600 men and 288,100 women annually. Worldwide, between 1.2 and 1.4 million new cases are reported every year (Jemal *et al.,* 2011, Favoriti *et al.,* 2016, Ferlay *et al.,* 2013).

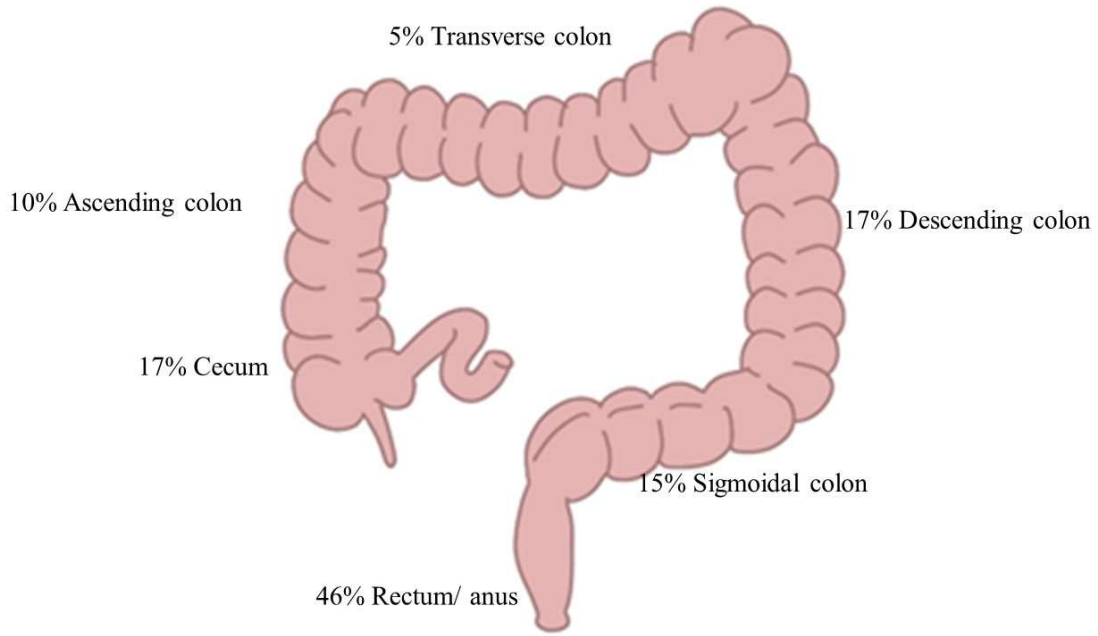


Figure 1.4 Distribution of colorectal cancer by site

Diagrammatic representation of the distribution of colon cancer cases by anatomical site throughout the colon or large intestine (Adapted from Brule *et al.*, 2015)

There are reports suggesting a significant increase in the incidence of CRC, particularly in terms of the number of new cases being diagnosed from 783,000 in 1990 to 1,361,000 in 2012 (Ferlay *et al.,* 2015, Rafiemanesh *et al.,* 2016, Arnold *et al.,* 2017).

Clinical variation in CRCs can be related to the location of the original tumour in the colon or rectum (e.g. proximal or distal), the pathology/histology of the cancer (e.g. adenocarcinoma/serrated adenocarcinoma) and invasiveness/metastatic behaviour of the cancer (e.g. loco-regional or distant organ site) (Hamilton and Aaltonen, 2000). Adenocarcinomas originating from the epithelial lining of the colon, are found in 90 % of CRC patients. The remaining 10% include neuroendocrine, adenosquamous, signet ring cell, squamous cell, spindle cell and undifferentiated carcinomas (Fleming *et al.,* 2012). Almost 46 % of all CRC cases are located in the rectum and the remaining 54% of the cases are found in the colon (Marmol *et al.,* 2017) (Figure 1.4).

1.6 Epidemiology of CRC in South Africa

The incidence of CRC is higher among high-income countries compared to low and middle-income countries; however, the death rate from CRC in advanced countries seems to be decreasing with time as a result of early-stage screening and detection (Haggar and Boushey, 2009).

Table 1.1 South African Colorectal Cancer Incidence Statistics (South African National Cancer Registry, 2014)

Population	**Age-standardised incidence rates per 100, 000**	**Percentage of all cancers**
All females	6.60	4.29
All males	11.28	5.28
Black females	2.68	2.89
Black Males	3.76	4.38
White females	18.57	5.09
White males	27.57	4.94

Of 19 Sub-Saharan African countries whose colon cancer rates were studied, South Africa and Zimbabwe recorded the highest incidence of CRC (Graham *et al.,* 2012). The incidence was reported as highest among white population, followed by Asians and then coloured populations, with the black population having the lowest reported incidence. However, in Sub-Saharan Africa, there now seems to be an increasing incidence of CRC in black populations. Moreover, it would also seem that within the black population, the condition develops much earlier than in their white counterparts (Angelo and Dreyer, 2001) and South Africa mirrors this trend (Cronje *et al.,* 2009). Represented below are CRC statistics from different South African population groups in 2014 (Table 1.1) (South African National Cancer Registry, 2014).

1.6.1 Colorectal carcinogenesis

Characteristically, cancer is thought to arise due to mutations in DNA base pairs. Most often these mutations in the genetic code are inconsequential and do not alter the cell functions (Vogelstein *et al.,* 2013). However, sometimes DNA repair machinery cannot effect repairs, resulting in either an increase in the expression of proteins encoded by oncogenes and/or a decrease in the expression of proteins encoded by tumour suppressor genes, leading to a cancerous phenotype (Nixdorf, 2009).

CRC is a heterogeneous disease, which manifests through variable clinical circumstances and divergences in response to therapeutic treatments (Blanco-Calvo *et al.,* 2015). In the 1980's, a breakthrough was achieved when it was first hypothesized that CRC develops through a multi-step process, which is accompanied by the sequential accumulation of genetic mutations (Figure 1.5) (Fearon, 2011), leading towards transformation of normal colonic mucosa into invasive cancer over many years (Lengauer *et al.,* 1998). CRC can be inherited, acquired or arise due to environmental factors. Inherited CRC accounts for 20% of total cases, which is classified mainly as two types:

i) Hereditary nonpolyposis CRC syndrome (HNPCC) or Lynch Syndrome and
ii) Familial adenomatous polyposis (FAP) (Marmol *et al.,* 2017).

The most prevalent type of polyp is the adenomatous polyp or adenoma, which arises from glandular cells. Inflammatory bowel diseases (IBD) such as ulcerative colitis (UC) is of special mention because while it does not include adenomas or polyps, it carries a high risk for colitis-associated CRC. Individuals may not have the inherited defects or ulcerative colitis but may be inherently susceptible, exposed to specific agents or exposed to growth-promoting metabolites,

which can also increase the likelihood for the development of polyps or dysplasia (Kulaylat *et al.,* 1995).

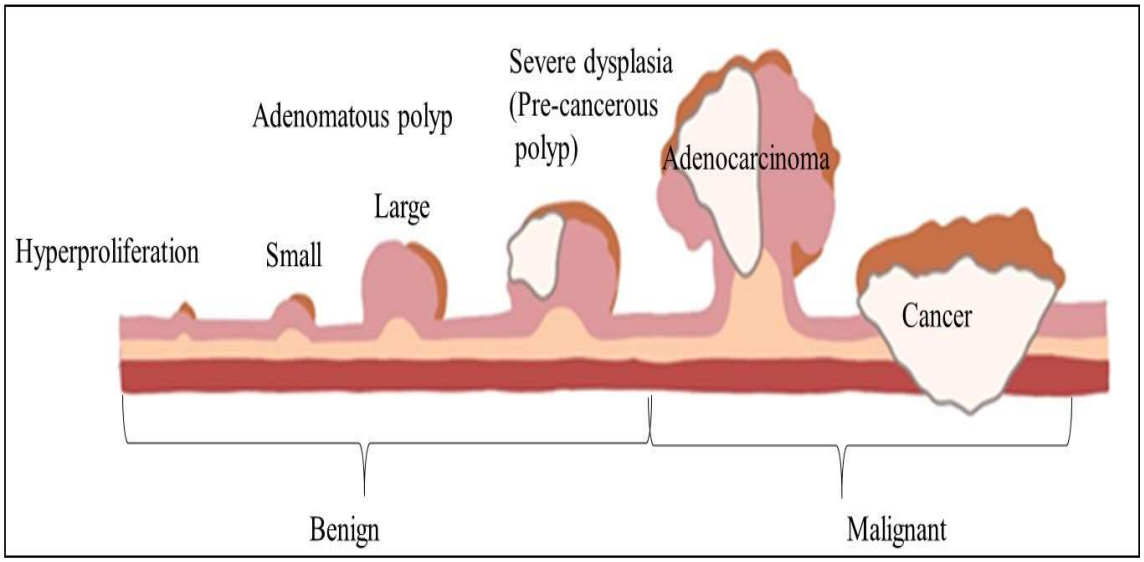


Figure 1.5 Multistep model of colorectal carcinogenesis

A diagrammatic representation depicting the stepwise progression of colorectal cancer. It shows the development of adenomatous polyp due to hyperproliferation, which is followed by the progression from colorectal polyp to CRC (Adapted from Fearon, 2011).

1.6.2 Pathology

The initiation, development and progression of CRC in terms of histology and molecular genetic changes have been studied extensively resulting in the development of several staging systems for CRC. The older classification systems, the Dukes' and Astler-Coller staging systems, are based on the degree of invasion and metastasis and are no longer in common use. The newer TNM (Tumour, Node and Metastasis) system was introduced by the American Joint Committee on Cancer (AJCC) and the International Union against Cancer (UICC) (Fleming *et al.,* 1997).

Accurately determining the stage of colon cancer is important for a number of reasons. Firstly, the stage is the most reliable predictor of CRC patient survival. Secondly, the stage of the cancer also dictates what is the most appropriate strategy for the treatment of the patient. Lastly, the accurate determination of the stage of CRC allows for meaningful clinical research (Compton and Greene, 2004). The AJCC and the UICC have created a standardized worldwide staging system that has been used extensively to quantify tumour burden in CRC patients. With the release of each new edition of the *AJCC Cancer Staging Manual,* new refinements to this

staging system are included. Alterations to the staging system are based on data gathered from the National Cancer Data Base (NCDB) (Greene *et al.,* 2002, Compton and Greene, 2004).

1.7. Treatments

CRC patients are treated according to the stage of disease and their condition (Binefa *et al.,* 2014). For CRC at early stages, patients are frequently subjected to active surveillance, polypectomy i.e. removal of a polyp or local excision through the colonoscopy (Hurlstone *et al.,* 2007). As the disease progresses, patients will be treated with surgical resection followed by chemotherapy, radiotherapy and biologically targeted therapies, which may be given alone or in combination to relieve symptoms and prolong survival (Hacker *et al.,* 2010). Chemotherapy, radiation and biologically targeted therapies usually act as adjuvant therapy after surgery (Castaneda *et al.,* 2018, Gallois *et al.,* 2018).

1.7.1. Surgery

Surgery, also known as surgical resection, is the most common treatment regimen for early stage CRC patients. The type of surgical resection used is based on the stage of cancer and the location of the tumour. Stage 0 and early stage 1 CRCs and most polyps can be removed during colonoscopy. In polypectomy, the cancerous region is removed from a polyp (Moss and Nalankilli, 2017). While a local excision is performed on tumours with some healthy tissue surrounding them, here the tissue along with lymph nodes is removed. Colectomy is the removal of the whole colon (Brody, 2015).

1.7.2. Radiotherapy

Radiotherapy is the centralised treatment for CRC patients. For advanced disease conditions, it can be used as a palliative treatment. Several reports have shown that it decreases the risk of local recurrence if given pre-operatively to shrink the size of a tumour so that it is easier to remove or post-operatively to destroy any residual cancerous cells (Påhlman and Glimelius, 1992). Sometimes, to increase the effectiveness a chemotherapy is often given along with radiotherapy, which is known as radio-chemotherapy/chemoradiation (Gallo *et al.,* 2018).

1.7.3. Chemotherapy

Chemotherapy involves the use of drugs to kill the cancerous cells when they are dividing (de Gramont *et al.,* 2011). Chemotherapy can be administered in different ways. Systemic administration involves administration by mouth or directly injected into a vein. Here drugs

enter the bloodstream and reach the cancerous cells throughout the body (de Gramont *et al.,* 2011). Regional chemotherapy uses a localised administration of the drug by placing it directly into the cerebrospinal fluid, organ or body cavity. These drugs mainly affect cancerous cells in the areas where the drugs are administered (Meyers *et al.,* 2017).

1.7.4 Targeted therapy

Researchers are turning towards targeted therapies after understanding more about gene and protein changes responsible for CRC. These newer drugs specifically target cancerous cells having such changes (de Gramont *et al.,* 2011). Some of the drugs approved by the Food and Drug Administration (FDA) for targeted therapy are listed below: (Brown and Solomon, 2018, Angarita *et al.,* 2018).

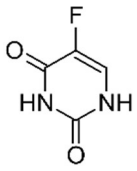
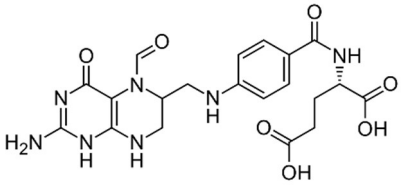
- *Drugs that target angiogenesis*
    - Vascular endothelial growth factor (VEGF) is a protein which helps tumours to form new blood vessels i.e. angiogenesis. The following drugs are used to treat CRC by inhibiting VEGF: Bevacizumab (Avastin), Ziv-aflibercept (Zaltrap), Ramucirumab (Cyramza) (Lopez *et al.,* 2019).
- *Drugs that target cells with EGFR changes*
    - Epidermal growth factor receptor (*EGFR)* is a protein on the surface of cancerous cells which help them to grow and metastasize. Examples of drugs targeting EGFR include: Cetuximab (Erbitux), Panitumumab (Vectibix) (Zaniboni and Formica, 2016).
- *Other targeted therapy drugs*
    - Regorafenib (Stivarga) - Is a type of targeted therapy known as kinase inhibitors. Kinases are proteins on the cell surface, which transduce extracellular signals required for nuclear signalling. This drug blocks several kinases that help cancerous cells to grow or that promote angiogenesis, resulting in the growth arrest of cancerous cells (Brown and Solomon, 2018, Angarita *et al.,* 2018).

1.7.5. Chemotherapeutic drugs for CRC

The standard chemotherapeutic treatment regimen of CRC includes 5-fluorouracil (5-FU) or capecitabine combined with leucovorin (LV) for a period of 6-12 months. The mainstay chemotherapeutic drug for CRC is 5-FU, showing the highest clinical efficacy. Despite this,

around 80% of CRC patients don't get the advantage of this treatment due to various reasons. Table 1.2 illustrates the characteristics of 5-FU and LV (Meyers *et al.,* 2017).

Table 1.2 Characteristics of 5-Fluorouracil and leucovorin (Reddy, 2004, Sanz - Altamira *et al*., 1998)

	5-Fluorouracil	**Leucovorin**
Structure		
Description	Antineoplastic and an antimetabolite. Pyrimidine analogue. Blocks the thymidylate synthetase conversion of deoxyuridylic acid to thymidylic acid thereby interfering with DNA synthesis.	Leucovorin, Folinic Acid or 5-formyl tetrahydrofolic acid is the 5-formyl derivative of tetrahydrofolic acid.
Categories	Antimetabolites, Immunosuppressive, Antineoplastic	Antidote to folic acid antagonists
Chemical formula	$C_4H_3FN_2O_2$	$C_{20}H_{23}N_7O_7$
Pharmacology		
Absorption	28-100%	l-isomer ~ 100% d-isomer – 20%
Metabolism	Hepatic	Hepatic and intestinal mucosa
Half-life	10-20 min	6.2 hrs

Drug resistance remains a major obstacle for the effectiveness of chemotherapy and includes two broad categories: intrinsic and acquired (Zahreddine and Borden, 2013). Acquired drug resistance means that tumour cells that were initially sensitive to drugs become resistant due to mutations and various adaptive responses during treatment (Tsuruo *et al.,* 2003). Tumour cells are constantly selected for survival and proliferation. In general, cancer cells develop multi-drug resistance due to the mechanisms outlined below in figure 1.6 (Kozovska *et al.,* 2014).

1.7.5.1 Resistance to chemotherapeutic drugs



Figure 1.6 Possible multi-drug resistance mechanisms developed in cancer cells (Adapted from Kozovska *et al*., 2014)

1.7.5.2 Adaptive response

After a drug has accumulated and inhibited its target(s), the effectiveness of treatment depends on how the cancer cell responds. Many anticancer drugs act by inducing DNA damage, directly or indirectly (Gottesman, 2002). The cell may respond to DNA damage in two ways: by repair or cell death. The response of cancer cells to DNA damage is a major factor determining the effectiveness of DNA-damaging drugs. Examples are deficiencies or mutations in mismatch repair (MMR) genes or/and P53, which may confer resistance to DNA damaging drugs (Kozovska *et al.,* 2014).

1.7.5.3 Deregulation of apoptosis

Mutations, amplifications, chromosomal transactions and overexpression of anti-apoptotic proteins, such as anti-apoptotic BCL-2 family members, an inhibitor of apoptosis proteins (IAPs) and the caspase 8 inhibitor FLIP, are also associated with resistance to chemotherapy (Zhang and Yu, 2013). Moreover, these genes may also be targets for pro-survival transcription

factors. For example, nuclear factor-κB (NF-κB) and signal transducer and activator of transcription 3 (STAT3), the expressions of which are frequently activated during tumorigenesis (Zhang and Yu, 2013).

1.8 Apoptosis and colon cancer

Cell death can occur in different forms such as apoptosis, necrosis and autophagic cell death. In 1972, apoptosis or programmed cell death was defined as a "distinct physiological mode of cellular suicide" which occurred under various physiological and pathological conditions (Debatin and Krammer, 2004, Hsu *et al.,* 2004, Scoltock and Cidlowski, 2004). Apoptosis is induced in the cells through activating specific cell death signalling pathways (Huang *et al.,* 2004, Pulido and Parrish, 2003).

Apoptosis plays an essential role in embryogenesis, development, immune response, morphogenesis, pathophysiology and normal tissue homeostasis; thus any disruption in this process may have severe consequences (Scoltock and Cidlowski, 2004, Pulido and Parrish, 2003). The discovery that resistance to apoptosis is an important hallmark of cancer has paved the way for development of novel drugs that induce apoptosis. These drugs can be used to treat cancer and other diseases (Hsu *et al.,* 2004). There are positive and negative regulatory pathways of apoptosis and a balance between these pathways determines cell fate (Pulido and Parrish, 2003). The morphological and biochemical features of apoptosis are distinct (Debatin and Krammer, 2004, Huang *et al.,* 2004). These include depolarisation of the plasma membrane, phosphatidylserine translocation to the outer membrane surface, membrane blebbing, cell shrinkage, alterations in intracellular ion concentrations, mitochondrial membrane depolarization and disruption in mitochondrial membrane integrity, nuclear condensation, chromatin aggregation, degradation of DNA and apoptotic body formation prior to cell lysis (Figure 1.8) (Debatin and Krammer, 2004, Hsu *et al.,* 2004, Scoltock and Cidlowski, 2004, Pulido and Parrish, 2003).

Apoptosis occurs in many organelles including the mitochondria, endoplasmic reticulum, Golgi apparatus and lysosomes (Pulido and Parrish, 2003). However, most research has focused on the role of mitochondria in apoptosis, which is considered to be the regulatory centre of the apoptotic process and plays an important role in cellular homeostasis (Pulido and Parrish, 2003, Strohmeier *et al.,* 2002). The biochemical and molecular factors regulating apoptosis are complex and not completely understood. However, some regulatory mechanisms that have been identified including death receptors, cysteinyl aspartate-specific proteases (caspases), the

mitochondria, the Bcl-2 family of proteins and tumour suppressor genes (Pulido and Parrish, 2003).

Tumour suppressors balance the effect of oncogenes, and essentially function to modulate the cell cycle, providing a fine balance between cell proliferation and apoptosis. Oncogenes promote tumour cell proliferation resulting in undesirable conditions as manifested in cancers.

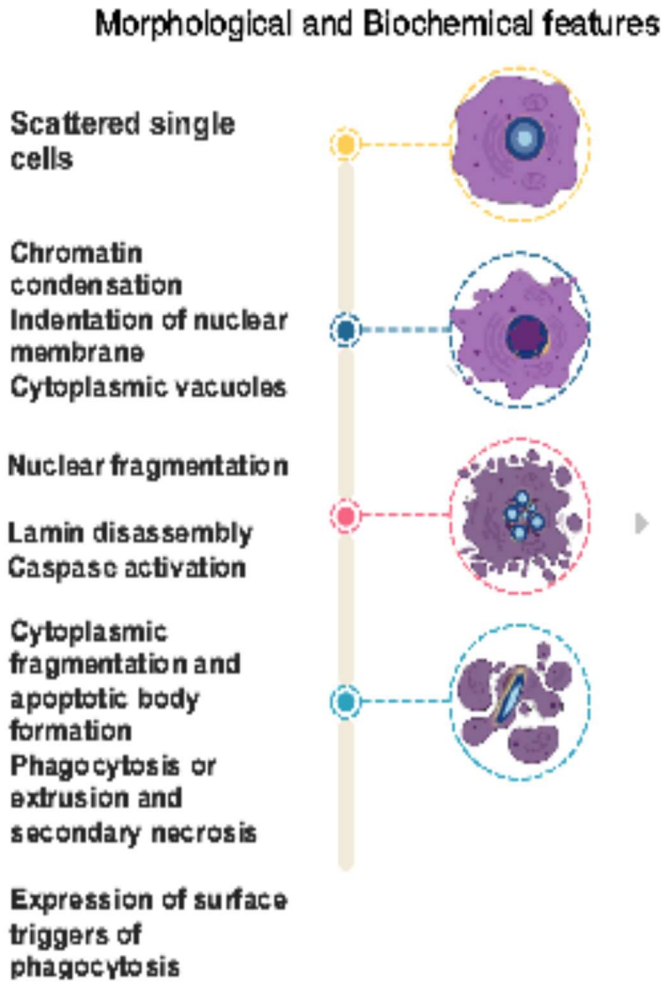


Figure 1.7 Morphological and Biochemical features of Apoptosis (Adapted from Hardy, 1999)

Conversely, tumour suppressors suppress cell growth when conditions are undesirable; for example, when DNA is damaged or when there are insufficient growth factors present, or if the cell division apparatus is faulty (Chatterjee *et al.,* 2010, Coppé *et al.,* 2008, Enari *et al.,* 2006, Yuan *et al.,* 2005).

1.9 Mechanisms of Apoptosis

The mechanisms of apoptosis include various energy-dependent cascades of events, which are sophisticated and highly complex (Elmore, 2007). Thus far, research indicates that two main pathways can trigger apoptosis: firstly, the intrinsic pathway (mitochondrial pathway), which primarily involves the release of cytochrome c (cyto c) from mitochondria, results in the activation of different caspases as downstream signals; and secondly, the extrinsic pathway (Death receptor pathway) which involves the binding of ligands to death receptors on the cell membrane (such as the Fas death receptor). These receptors are stimulated by external cellular signals (Kiraz *et al.,* 2016, Igney and Krammer, 2002) (Fig 1.8). In addition to these pathways, an additional pathway known as the perforin/granzyme pathway exists which involves T-cell mediated cytotoxicity (Trapani and Smyth, 2002). The activation of all of these pathways leads to different signalling cascades, involving different intermediate molecules. Both the intrinsic and extrinsic pathways commonly initiate the cleavage of Pro-caspase-3. This is known as an executioner of apoptosis; and then Caspase-2 then cleaves downstream targets, resulting in DNA fragmentation, cross-linking of proteins, expression of ligands for phagocytic cell receptors, degradation of cytoskeletal and nuclear proteins, formation of apoptotic bodies and finally uptake by phagocytic cells (Elmore, 2007). In contrast, the caspase-independent cell death pathway is activated by the granzyme A pathway via single-stranded DNA damage (Martinvalet *et al.,* 2005, Ghobrial *et al.,* 2005 Lowe and Lin, 2000, Ghobrial *et al*., 2005, Hsu *et al*., 1995, Wajant, 2002).

1.9.1. Intrinsic Pathway

The intrinsic signaling apoptosis pathway triggers some non-receptor-mediated stimuli, which in turn produce intracellular signals to activate targets within the cell. These signals and molecules act on the mitochondria, leading to Cyto C efflux from the mitochondria (Elmore, 2007). The absence of certain growth factors, hormones and cytokines that normally inhibit apoptosis result in the initiation of pro-apoptotic signaling. This pathway can also be triggered by radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals (Peña-Blanco and García-Sáez, 2018).

Members of the Bcl-2 (BH3 domain containing) family of proteins regulate the release of Cyto C from the mitochondria (Cory and Adams, 2002). These Bcl-2 proteins are themselves regulated through the activity of the tumour suppressor protein P53 (Schuler and Green, 2001). The Bcl-2 family of proteins influences the permeability of the mitochondrial membrane. Some

members are pro-apoptotic (Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, and Blkb) and others are anti-apoptotic (Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w, BAG). These proteins are encoded by 25 different genes, with alternative splicing giving rise to an even greater diversity of proteins (Cory *et al*., 2003b).

These proteins localize to the outer mitochondrial membrane and alter membrane permeability by interacting with the voltage-dependent anion channel porin (VDAC). Some pro-apoptotic family members such as Bak and/or Bax form a mitochondrial apoptosis induced channel once they are activated. The anti-apoptotic family members have a similar domain structure and may block apoptosis by binding to similar areas of VDAC or to the pro-apoptotic members of the family (Peña-Blanco and García-Sáez, 2018).

1.9.2. Extrinsic Pathway

The extrinsic pathway is also known as the "death receptor pathway" and is triggered by the binding of ligands to the membrane-bound death receptors of the tumour necrosis factor (TNF) gene superfamily (Kumar *et al.,* 2005). These ligands and receptors include Fas ligand/ FasR, TNF/TNF R1, Apo2L/DR4, the tumour necrosis factor receptor type 1-associated DEATH domain (TRADD) or TNF-related apoptosis-inducing ligand (TRAIL) R1 (Locksley *et al.,* 2001). This results in the formation of Death Inducing Signalling Complexes (DISC) as adaptor proteins associate with the receptor. One of these is the Fas-associated death domain (FADD) adaptor protein, which associates with the Fas receptor, while another adaptor protein TNFR-associated death domain (TRADD) binds to TNF/tumour necrosis factor receptor (TNFR) (Kumar *et al.,* 2005). Pro-caspase 8 then associates with either FADD or TRADD through its N-terminal death domain (DD), following which it is auto-catalytically activated (Lowe and Lin, 2000, Ghobrial *et al.,* 2005, Hsu *et al.,* 1995, Wajant, 2002).

Caspase 8 is able to activate the intrinsic pathway by activating BID; it also cleaves and activates Pro-caspase 3 and Pro-caspase-7 (Figure 1.8). The cleavage and myristoylation of BID by Caspase 8, allows BID to bind to BAX, forming a heterodimer. This heterodimer then forms a pore by inserting itself into the mitochondrial membrane where it stimulates the release of Cyto C. Apoptosome formation is stimulated by cytochrome release (Sax *et al.,* 2002). This pathway can be inhibited through the binding of FLICE-like inhibitory protein (cFLIP) to FADD and procaspase-8, blocking their action. The protein TOSO can also inhibit the extrinsic pathway by preventing caspase-8 biogenesis (Hitoshi *et al.,* 1998, Scaffidi *et al.,* 1999). Death

stimuli trigger the intrinsic apoptosis pathway, leading to the release of Cyto C from the mitochondria. Members of the Bcl-2 protein family control this process.

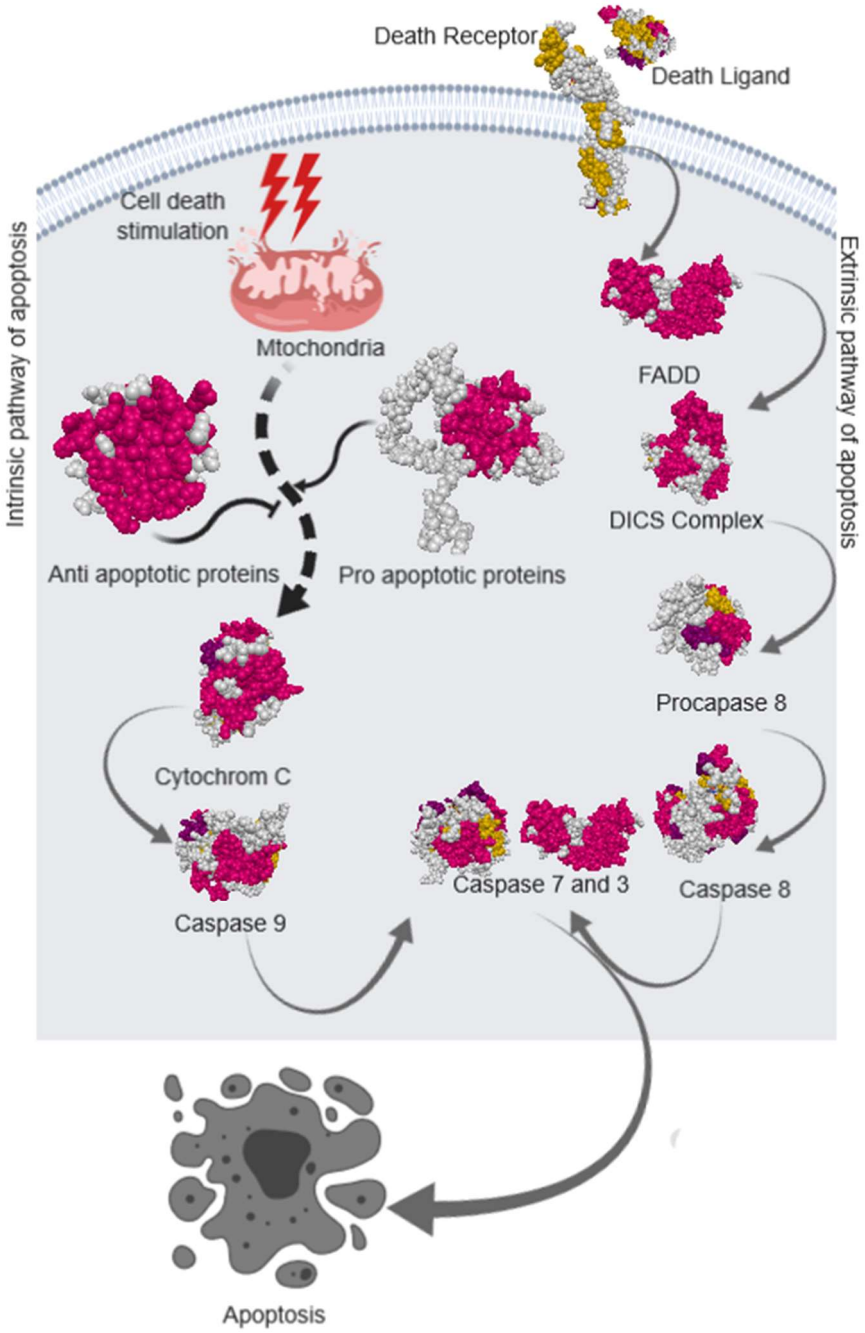


Figure 1.8 The intrinsic and extrinsic apoptotic pathways. (Adapted from Yang *et al.*, 2009)

Cyto C releases a sequence of processes, which in turn activates caspase-9, followed by activation of the executioner caspase, caspase-3. The extrinsic pathway is initiated by the binding of "death ligands" to "death receptors" which leads to the creation of multiprotein DISC complexes, which triggers caspase-8 which finally cleaves and activates caspase-3 ultimately resulting in apoptosis (Yang *et al.,* 2009).

1.9 P53

There are many known tumour suppressor genes encoding proteins that inhibit the growth of potentially cancerous cells by affecting cell cycle regulation and apoptosis (Gras *et al.,* 2001, Liggett and Sidransky, 1998, Rusan and Peifer, 2008, Scully, 2000, Tommasi *et al.,* 2008, Toyooka *et al.,* 1995). It is when tumour suppressors lose their ability to regulate the cell cycle that cancers may develop. Of these genes, P53 is one of the most studied and is often implicated in cancer progression.

p53 functions to integrate a variety of cellular stressors into collection of reactions which includes apoptosis (Abraha and Ketema, 2016). The p53 protein responds to DNA damage by activating either programmed cell death or cell cycle arrest at G1 or G2 phase of the cell cycle (Li *et al.,* 2018). This controls pro-apoptotic gene (Bax, BH3, PUMA and NOXA) transcription via binding to specific DNA sequences (Abraha and Ketema, 2016, Fridman and Lowe, 2003). Also, they inactivate some anti-apoptotic proteins (Bcl-2 and Bcl-xL) and then release of cytochrome C from mitochondria. The wild-type P53 protein can initiate apoptosis in human colon carcinoma cell lines (Shaw *et al.,* 1992). Since the function of wild type P53 protein can be blocked by mutated P53 protein, this may prevent the initiation of apoptosis (Nakayama and Oshima, 2018).

The apoptosis effector proteins such as APAF-1 and caspase 6 synthesis are also promoted by p53 (Kannan *et al.,* 2001) and also controls transcription of the important components of the extrinsic apoptosis pathway, such as the death receptor Fas and DR5 as well as the BH3 only protein Bid that links the extrinsic pathway with the intrinsic pathway (Sax *et al.,* 2002).

Besides its role in apoptosis p53 can halt cell cycle progression, initiate DNA repair, and senescence. In this way, p53 prevents cells with damaged DNA from replicating and duplicating the damaged DNA (Kannan *et al.,* 2001). Thus, the deletion of the p53 gene through the CIN pathway is an important factor in the transition of adenoma to carcinoma in CRC (Watson, 2004). It has also been demonstrated that p53 is able to affect the response of cells to chemotherapy during CRC treatment (Terranova-Barberio *et al.,* 2017, Bunz *et al.,* 1999).

Mutations of the p53 gene occur in various human tumours, including CRC (Nakayama and Oshima, 2018, Li *et al.,* 2015, Hollstein *et al.,* 1991, Wu and Levine, 1997). Some 85% of colorectal tumours have this mutation which usually occurs happens during the transition from adenoma to adenocarcinoma (Vogelstein *et al.,* 1988). The correlation between P53 protein

expression and apoptosis in colorectal neoplasms have been investigated (Merritt *et al.,* 1994). Homozygote *p53* knockout mice did not display a decreased frequency of apoptosis in mouse adenomas. Further, these mice also lacked enhanced levels of adenoma formation or adenoma progression as a result of p53 inactivation. In contrast however, the majority of studies did not show such a relationship (Paradiso *et al.,* 2000, Sinicrope *et al.,* 1995), implying that the mutant p53 protein does not play a role as an inhibitor of apoptosis in the development of CRC.

1.10 Indoline Derivatives as Anticancer Agents

Indoles are heterobicyclic compounds that have a six-membered ring fused with a five-membered pyrrole ring (Figure 1.9) and they are one of the most promising heterocyclic compounds with a wide range of biological and pharmacological properties (Kumar *et al.,* 2014).

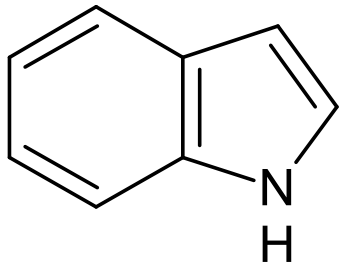


Figure 1.9 Structure of the Indole moiety (Kumar *et al.,* 2014)

Several indole derivatives were identified as effective anticancer agents (Nesi *et al.,* 2013, Zhou *et al.,* 2016, Fortes *et al.,* 2016, Prakash *et al.,* 2018). Many natural and synthetic compounds possess an indole moiety in their structure. These compounds were found to possess a wide range of therapeutic properties, including antimalarial (Xu *et al.,* 2012, Rocha e Silva *et al.,* 2012, Schuck *et al.,* 2014), antimicrobial (Gokhale *et al.,* 2017, Rajaraman *et al.,* 2017), anti-inflammatory (Sharath *et al.,* 2013, Mehndiratta *et al.,* 2014, Singh and Singh, 2018), antitumour (Shchekotikhin *et al.,* 2014, Cai *et al.,* 2016, Gehrcke *et al.,* 2018) and antitubercular (Naidu *et al.,* 2016, Khan *et al.,* 2018) activities. Structures of some of the biologically active indole derivatives and their properties are given in Figure 1.10.

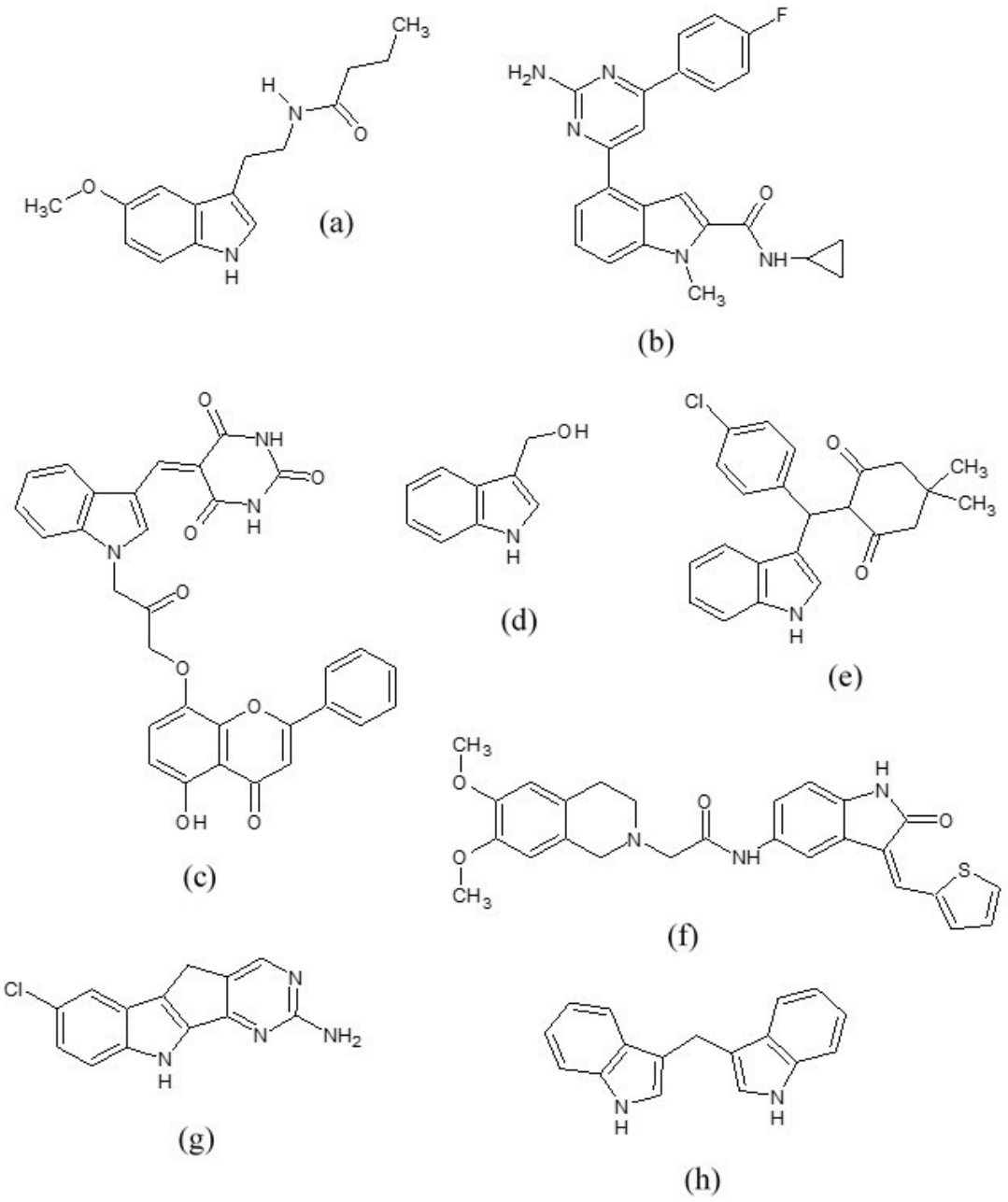


Figure 1.10 Structure of some indole derivatives that have different biological properties

(a) antimalarial, (b) antimicrobial, (c) anti-inflammatory, (d) antitumour, (e) antitubercular, and (f, g & h) anticancer. Indole derivatives were reported to have positive activity against tubulin polymerization (Brancale and Silvestri, 2007). Indoles have been reported to inhibit the formation of free radicals and affect the progression of the cell cycle. In addition, they induce apoptosis, inhibit the migration, invasion and angiogenesis of tumour cells (Lerner *et al.,* 2011, Watson, 2015).

Indoles are found in various types of plants and cruciferous vegetables, for example cauliflower, cabbage, turnip, broccoli and brussel sprouts. Indoles are classified as a phytonutrient and have been proven to be beneficial to the body in a number of ways. Consumption of cruciferous

vegetables that are rich in many phytochemicals, including indole derivatives, are reported to reduce the risk of colon, breast and prostate cancers (El Sayed *et al.,* 2015). The metabolic products of glucobrassicin derived from the cruciferous vegetables including indole-3-carbinol (I3C; Figure 1.10 d) and 3, 3'-diindolylmethane (DIM) (Figure 1.10h) were found to exhibit anti-carcinogenic properties in human cancer cells (Boone *et al.,* 1990, Hrnčiřík *et al.,* 1998, Ahmad *et al.,* 2012, Yuan *et al.,* 2011).

In a preliminary study, it has been reported that the novel indoline derivative N-(2-hydroxy-5-nitrophenyl (4'-methyl phenyl) methyl) indoline (HNPMPI) has cytotoxic activity directed against colon cancer cells (Pers Comm. J. Kandhavelu). Also, the compound was previously found to have cytotoxic activity against osteosarcoma cells (Doan *et al.,* 2016). In view of these findings, the present study sought to investigate the potential anticancer activity of HNPMPI (Figure 1.11) against colon cancer cells, representing different tumour stages. A particular focus was to query the possible induction of apoptosis by HNPMPI treatment.

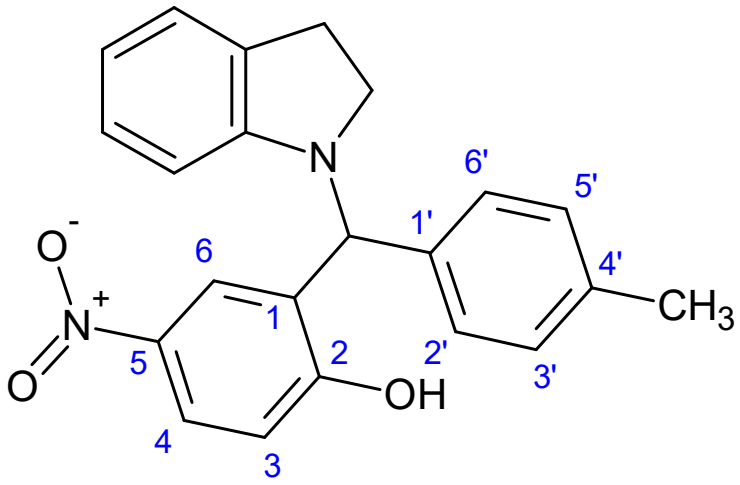


Figure 1.11 Structure of N-(2-hydroxy-5-nitrophenyl (4'-methylphenyl) methyl) indoline

1.11 Aim of this study:

The aim of the study was to compare the anticancer effects of a novel synthesized Indoline derivative on apoptosis relevant protein expression in mid (HT-29) and late stage (DLD-1) colon cancer cell lines.

Objectives:

1. To observe the morphological changes in the cells after exposure to Indoline derivative (HNPMPI).
2. To assess programmed cell death using Annexin V assay
3. To evaluate the expression of proteins involved in apoptosis using an antibody array in both treated and untreated cells.
4. To establish the transcription levels of genes coding for selected differentially expressed proteins using real time quantitative PCR.
5. To use the protein expression data to select appropriate proteins of interest and use immunofluorescence and confocal microscopy to determine the presence and localisation of these target proteins in colon cancer cell lines, in order to map changes in protein expression.

These objectives were achieved by using *in vitro* cell culture models of colorectal cancer cell lines representing a mid-stage (stage 2) and a late-stage (stage 3) colon cancer tumour, respectively. The cell lines HT-29 (stage 2) and DLD-1 (stage 3) were purchased from the American Type Culture Collection (ATCC) and Health Science Research Resources Bank, Japan, respectively.

2.1. Cell Culture

All cell culture procedures were performed by the implementation of aseptic techniques and good cell culturing preparation to avoid contamination from bacteria, fungi and mycoplasma and also cross contamination with other cell lines. This included the consistent use of personal protective equipment, such as gloves and lab coats. As an *in vitro* cell culture study, this did not require human ethics clearance - see Appendix E for ethics waiver obtained from the Human Research Ethics Committee (Medical).

The following general cell culture procedures were executed in the cell culture room under a laminar flow cabinet. A UV germicidal lamp was used to sterilize the cabinet when it was not in use and was switched off during use. The fluorescent light and the fan were turned on, drawing air in through a HEPA filter. The flow hood was sterilized with 70% v/v ethanol before commencing with work and gloves were sterilized with 70% v/v ethanol periodically when handling different equipment. All materials (including 50mL media bottles, culture flasks, 10mL pipettes) including the surface of the flow hood were sterilized with 70%v/v ethanol.

Two cell lines were used, namely HT-29 (colorectal adenocarcinoma) (ATCC Cell Biology Collection, USA) and DLD-1 (Dukes' type C colorectal adenocarcinoma) (Health Science Research Resources Bank, Japan) (Table 2.1). Cell lines were cultured in Dulbecco's Modified Eagle Medium: Nutrient mixture F-12 (DMEM/F-12) (Invitrogen) supplemented with 10%v/v heat inactivated fetal bovine serum (FBS) (Invitrogen) prepared to a volume of 50mL, to which 100µL penicillin/streptomycin (100 IU/mL) stock solution (Lonza BioWhittaker®) was added.

Table 2.1 Cell lines used in this study

	HT-29	**DLD-1**
Organism	*Homo sapiens*	*Homo sapiens*
Morphology	Epithelial	Epithelial
Tissue	Colon	Colon
Age	44	Adult
Gender	Male	Female
Ethnicity	Caucasian	Caucasian
Culture Medium	DMEM/F12, FBS, Pen/strep	DMEM/F12, FBS, Pen/strep

2.1.1 Thawing of cells

Frozen cell lines were resuscitated by thawing after storage at -80°C and were washed with approximately 5mL of 10%v/v culture medium by centrifuging at 800rpm for 3 min to eliminate dimethyl sulfoxide (DMSO), which is a cryoprotectant that is toxic above 4°C; therefore, it is important to add culture media after thawing. This dilution helps to reduce toxicity. The supernatant was removed, and the pellet was re-suspended in 10mL of culture medium and mixed thoroughly by pipetting. The re-suspended pellet was subsequently spilt into two small (5mL/25cm^2) culture flasks. Cells were visualized using an inverted phase contrast microscope. Cells were cultured in a 37°C incubator, providing an atmosphere of 5% CO_2 and 90% humidity; and were examined daily to observe their growth and degree of confluency, as well as examining for fungal or bacterial contamination.

2.1.2 Passaging/ Sub-culturing

Once cells developed a confluent complete cell sheet (80%-90% confluency) with few intercellular spaces, they were ready to be sub-cultured. Cells were passaged every three to four days. Once the cell monolayers were observed, the cells were washed in phosphate buffered saline (PBS) (Sigma-Aldrich) after removing medium. Cells were subsequently detached from the flask surface by incubating them for 3-5 min with TrypLE (TrypLE^{TM} Express, Invitrogen), in the cell incubator. Flasks were tapped gently to aid in the detachment of any remaining cells and were visualized under an inverted microscope. Once detached, fresh 10%v/v FBS/DMEM/F-12 was added to the cell suspension to neutralize the trypsin, as trypsin is inactivated by serum. The cells were then centrifuged at 800rpm for 3 min and the supernatant was discarded. The cell pellet was re-suspended in approximately 10mL of fresh culture medium and 5mL was aliquoted into two small (25cm^2) culture flasks. Culture flasks were supplemented with 5mL of fresh culture medium and were visualized under an inverted microscope to confirm that cells were present. Finally, the flasks were placed in the 37°C incubator, in an atmosphere of 5% CO_2 and 90% humidity.

2.1.3 Cryopreservation

Once a cell line is capable of sufficient expansion, the cells can be preserved by freezing at -70°C. This allows stocks of cells to be maintained without experiencing aging as well as protecting against contamination and incubator malfunction (Freshney, 2006). Good survival

of cells is favored if there is a high density of cells at freezing (1 x 10^6 cells/mL – 1 x 10^7 cells/mL). The medium was removed, and the cell monolayer was washed in PBS. The cells were subsequently dissociated as described above. The cell pellet was re-suspended in 3mL of cryoprotective medium (Lonza) containing DMSO (Sigma-Aldrich), supplemented with 10%v/v FBS/DMEM/F-12 (50:50). The cell suspension was split into two 1.5mL cryopreservation tubes and stored at -70°C.

2.1.4 Harvesting Cells

As mentioned previously, the cells were dissociated and the supernatant was discarded, and residual supernatant was removed using a syringe or Gilson P100.

2.2 Determination of IC50 of HNPMPI and morphological changes after the exposure to drug

The working concentration of HNPMPI to use in the apoptotic activity in the CRC cell lines (HT-29 and DLD-1) is very important. Firstly, the IC_{50} of HNPMPI against each of the cell lines was determined by culturing 2x 10^5 cells/ mL of cells in 6 well plate till 60-70% confluent. Afterwards, cells were washed with PBS and serum starved overnight. Then, cells were treated with 5 different concentration of the HNPMPI and 5-FU used as control drug. After 24hrs, cell viability was calculated using Tryptan Blue assay (Details were given in Appendix B). Once established, a slightly lower concentration of the drug could be used to treat the cells without causing excessive cell mortality in the subsequent experiments. The IC50 for HNPMPI against the HT-29 and DLD-1 were pre-determined in our laboratory using a Trypan Blue assay (Data not published yet, Appendix B).

Morphological changes after the exposure of HNPMPI was evaluated under the FLoid cell imaging station with a the 20X objective.

2.3. Apoptosis Assay

The Muse^{TM} Annexin V and Dead Cell Kit was used to assess programmed cell death. Apoptosis was detected using Annexin V to detect phosphatidylserine (PS) on the external membrane of apoptotic cells. A dead cell marker (7-AAD) was also used as an indicator of cell membrane structural integrity. The HT-29 (2x 10^5 cells/ mL) and DLD-1 (2x 10^5 cells/ mL) cells were seeded into sterile 6 well plates and cultured in DMEM/ F-12 supplemented with 10% FBS and 1000U/mL pen-strep until cells were 60-70% confluent. Afterwards, cells were

washed with PBS and serum starved overnight. Then, cells were exposed to 30µM (slightly below the IC50 value) of the indoline derivative (HNPMPI) for 24 hrs, as HNPMPI was effective at 24 hr itself when compared to the standard chemotherapy drug (5-FU) which is used to treat colon cancer patients (Appendix B) Next, cells were washed with PBS and trypsinized in the incubator for 3-5 min, and culture medium containing FBS was used to inactivate trypsin. Cells were then centrifuged at 300g for 5 min and the supernatant were discarded; and again, washed with PBS and centrifuged at 300g. The pellet was resuspended in 100µl of 1% Bovine Serum Albumin (BSA). Next, 100µl of the Muse^{TM} Annexin V and Dead Cell Reagent was added to each tube for cell staining and vortexed for 3 seconds. The cell suspension was incubated in dark at room temperature for 20 mins followed by analysis of apoptosis using the Muse^{TM} Cell Analyzer.

2.4. Protein extraction

After culturing cells and treating as described above (section 2.3), the culture medium was removed, and the cell monolayer was washed in PBS. Cells were lysed using the lysis buffer l7 from the Protein profiler kit TM (R&D Bio-systems). Lysis buffer was added at a ratio of 1mL per $1x10^7$ cells. To aid in lysis, the cells were serially pipetted up and down. The lysates were agitated gently at 2-8°C for 30min. After this, the lysates were centrifuged at 14,000xg for 5 min and the supernatant were transferred into a clean test tube. Cell lysates were aliquoted and stored at - 80°C. These lysates were thawed once prior to use.

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2.4.1. Protein concentration determination

The Qubit fluorometer (Thermo Fisher) uses fluorescent dyes to determine the concentration of either nucleic acids or proteins in a sample. This assay can quantitate samples ranging from 12.5 µg⁄mL to 5 mg⁄mL and exhibits low protein to protein variation. The assay is highly selective for proteins and is designed to be accurate in the presence of reducing reagents. The concentration of the extracted protein was determined using the Qubit total protein assay (Thermo Fisher), consisting of three assay tubes for the standards (BSA) and one tube for each sample. The Qubit^{R} working solution was diluted using the Qubit^{R} Protein reagent 1:200 in Qubit^{R} Protein Buffer. Two hundred microliters of working solution was prepared for each standard and sample. Samples and standards were prepared in 0.5-mL PCR tubes according to Table 2.2. Samples and standards were vortexed for 2-3 seconds and incubated at room temperature for 15 min.

Table 2.2 Particulars of protein quantitation using the Qubit Protein assay kit

Volume	Standard	Samples
Working Solution (µL)	190µL	195µL
Standard (Kit) solution (µL)	10µL	-
Sample volume (µL)	-	5µL
Total volume in each Assay Tube (µL)	200µL	200µL

2.4.2. Qualitative SDS PAGE

The quality of the protein extractions was analysed using SDS polyacrylamide gel electrophoresis. A total of 5µg of each sample was loaded onto a 4% stacking gel with a 12% separating gel. A Colour Broad Range Protein standard molecular weight marker (New England Biolabs) was loaded onto the gel. The gel was electrophoresed for 2 hrs at 110 v. The gel was then removed from the electrophoresis apparatus and stained with 0.2% Coomassie blue G-250, overnight. Images of the stained gel were then captured on a Bio-Rad Gel Doc ^{TM} EZ Imager.

2.4.3. Protein profiling

In order to establish the expression level of proteins involved in apoptotic pathways, a Human Apoptosis kit array was used (R&D Systems). This array is very effective and economical when compared to other traditional western blot systems as it simultaneously detects 35 apoptosis-related proteins. Details of the protein profiler antibody array kit are given in appendix C.

All reagents were brought to RT before use. Samples were kept on ice. First, 2.0 mL of array Buffer 1 (blocking buffer) was added into each well of the 4-well multi-dish and it acts as a blocking buffer. Each array was removed carefully from the protective sheets using flat-tipped tweezers and placed into the well of the 4-well multi-dish provided. The array was positioned facing upwards. These arrays were incubated for 1 hour in buffer 1 on a rocking platform shaker. Samples were prepared by adding the desired quantity of lysate to 1.25mL of array buffer 1 and then adjusting to a final volume of 1.5mL with Lysis Buffer 17. The blocking buffer was removed from the arrays and 1.5ml of prepared sample was added to the array and then the arrays were incubated for overnight at 2-8^{0}C on a rocking shaker. After the overnight incubation, each array was washed with 20 mL of 1x Wash Buffer in a separate plastic container for 10 min on shaker; and the 4-well multi-dish was washed with distilled water and dried thoroughly. The washing step was repeated twice.

The detection antibody was diluted by adding 15µL of reconstituted Detection Antibody Cocktail to 1xArray Buffer 2/3 for a final volume of 1.5mL per sample, which was added to each well of the 4 well multi-dish. Each array was removed from its wash container and excess Wash Buffer was allowed to drain from the array. The arrays were returned to the 4-well multi-dish containing the diluted Detection Antibody Cocktail and incubated in the detection buffer solution for 1 hour on a rocking platform shaker. Each array was then washed 3 times for 10 min. The Streptavidin-HRP was diluted in 1x Array Buffer 2/3 using the dilution factor on the vial label and 2.0 mL was pipetted into each well of the 4-well multi-dish. Each membrane was carefully removed from the wash container. Excess Wash Buffer was allowed to drain from the membrane. The array was returned to the 4-well multi-dish containing the diluted Streptavidin-HRP and covered with a lid. The arrays were incubated in the Streptavidin-HRP solution for 30 min on a rocking platform shaker and each array was then washed. Each membrane was carefully removed from its wash container. Excess Wash Buffer was allowed to drain from the membrane by blotting the lower edge onto paper towels. Every membrane was placed on the bottom sheet of the plastic sheet protector with the identification number uppermost.

Chemi Reagent Mix was prepared by mixing Chemi Reagent Mix 1 and 2 in equal volumes, 1mL of the prepared Chemi Reagent Mix was pipetted evenly onto each membrane. The top plastic sheet protector was carefully covered. Each membrane was gently smoothened out to avoid air bubbles and to ensure Chemi Reagent Mix was evenly spread to all corners and then incubated for 1 minute. The paper towels were positioned on the top and sides of the plastic sheet protector containing the membranes and excess Chemi Reagent Mix was carefully squeezed out. The top plastic sheet protector was removed, and absorbent lab wipe was carefully layed on top of the membranes and any remaining Chemi reagent mix was blotted off. Membranes were left on the bottom plastic sheet protector. The membranes were covered with plastic wrap and any air bubbles were gently smoothened out. The excess plastic wrap was wrapped around the back of the sheet protector so that the membranes and sheet protector were completely wrapped. The membranes were placed with the identification numbers facing uppermost in an autoradiography film cassette. Membranes were then digitally imaged with a chemiluminescence camera. The template of the apoptosis protein profiler is provided in Appendix C.

2.5. Primer Design

Primers are short sequences of single stranded DNA/RNA oligonucleotides that hybridize to the complementary region on the target DNA/RNA and act as the starting point for the DNA/RNA polymerase to synthesize segments of the gene of interest present within the template DNA/RNA. Care must be taken during the primer design to exclude complementary sequences in the forward and reverse primers, which can result in the formation of homo-dimers and/or -oligomers. The design strategy is to include purine bases at the beginning and pyrimidine bases at the end of the primers to ensure low melting point and avoid miss matching.

Table 2.3 Designed forward and reverse primers for the PCR amplification

Gene	Primers	Tm (°C)
GAPDH (Control)	F: 5'-TGCMTCCTGCACCACCAACT-3' R: 3'-YGCCTGCTTCACCACCTTC-5'	53.25 52.6
BAD	F: 5'-ATCTCAGCTCCTCTAAGCCCC-3' R: 3'-CCCTGAGCTTCCCCTCGATT-5'	56.31 55.88
BAX	F: 5'-TTCATCTCAGTCCCCTGCCC-3' R: 3'-GGAGACAGGGACATCAGTCG-5'	55.88 55.88
Bcl-2	F: 5'-CCTGTGGATGACTGAGTACC-3' R: 3'-GAGACAGCCAGGAGAAATCA-5'	53.83 51.78
CASP3	F: 5'-GCTCGCTAACTCCTCACGG-3' R: 3'-TCCAATTCCTTTTCGGCCCTG-5'	55.41 54.36
P53	F: 5'- GCTCGACGCTAGGATCTGAC-3' R: 5'-CCCAGGGTTGGAAGTGTCTC-3'	60 59.90

***F – Forward primer; R – Reverse primer**

The specific genes such as BAD, BAX, BCL-2, CASP3 and P53 were chosen for evaluation following on the prior analysis of the changes in the protein expression after HNPMPI treatment. The sequences of human BAD, BAX, BCL-2, CASP3 and P53 genes were retrieved from the Gene database (Appendix D) and Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to design primers. Primer-BLAST is a general public tool that helps users to design target-specific primers. Primer-BLAST offers flexibility to accommodate different primer design needs. The primers contained 50-60% GC content and gave a PCR product of between 150 and 160bp in length. The primers were

synthesized, verified and purchased from Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa. The sequences of the forward and reverse primers for the genes of interest and their estimated Tm are given in Table 2.3. GAPDH was used as reference gene for this gene expression analysis.

2.6. RNA Purification

The HT-29 and DLD-1 were grown and treated as per section 2.3. In order to analyse gene expression, RNA was extracted from treated and untreated HT-29 and DLD-1cells using the Direct-zol™ RNA extraction kit (ZymoResearch) as per the manufacturer's protocol. Briefly, cells were lysed in Tri-reagent, which was provided in the kit. An equal volume of 95-100% ethanol was added to the lysed sample and mixed thoroughly. The mixture was then transferred into a Zymo-spin™ IIc column in a collection tube and centrifuged at 3000 rpm for 3min. The column was transferred to a new collection tube and the flow-through was discarded. The column was washed by adding 400µl Direct-zol™ RNA PreWash to the column and centrifuged at 3000 rpm for 3min. The flow through was discarded and the process was repeated once. The RNA was further washed by adding 700 µl of RNA Wash Buffer to the column and centrifuging for 2 min to ensure the complete removal of the wash buffer. The column was transferred into a RNase-free tube. The RNA was eluted by the addition of 50 µl of DNase/RNase-free water directly to the column matrix and the water was forced through the column matrix by centrifugation at 12000 rpm for 2min.

2.6.1. RNA quantification

The quality and quantity of RNA was then assessed using a Nanodrop ND-1000 spectrophotometer, which measures the absorbance of the sample RNA at 260 nm, 280nm and 230nm. The A260/A280 ratio is used to assess RNA purity. An A260/A280 ratio of 1.8 -2.1 is indicative of highly pure RNA. The spectrophotometer was blanked using nuclease free water.

2.6.2. Synthesis of cDNA from RNA

Prior to quantification of gene expression, synthesis of ss-cDNA from the total RNA was carried out using the Applied Biosystems™ High-Capacity RNA-to-cDNA kit (catalogue number 4387406), following the manufacturer's instructions. The components of the kit are listed in Table 2.4. The kit components were thawed on ice and the volume of the required components was calculated based on the number of reactions. The final reaction volume for each sample was 20µl, using the kit components as described in Table 2.5. The tubes containing

20µl of RT reaction mixture each were sealed and briefly centrifuged to eliminate any air bubbles. They were then placed on ice until ready to load tubes into the Applied Biosystems™ 7500 Real-Time PCR instrument. The optimized conditions for the thermal cycling for use with the High-Capacity RNA-to-cDNA™ as described in the kit's user guide were set and the cycling conditions are given in Table 2.6.

Table 2.4 Components of the Applied Biosystems™ High-Capacity RNA-to- cDNA kit

Components	50 reactions	500 reactions
2X RT Buffer Mix (50 x 20µL reactions	500µL	5 x 1000µL
20X RT Enzyme Mix (50 x 20 µL reactions)	50µL	500 µL

Table 2.5 Preparation of the RT reaction mixtures

		2x RT Buffer Mix	20x RT Enzyme Mix	RNA sample	Nuclease – free H_20	Total Volume
HT - 29	**Untreated Control +ve**	1x (10.0µl)	1 x (1.0µl)	2µg (1.43µl)	7.57µl	20µl
	Untreated Control -ve	1x (10.0µl)	-	-	10µl	20µl
	Treated (Indoline Derivative) +ve	1x (10.0µl)	1 x (1.0µl)	2µg (7.22µl)	3.95µl	20µl
	Treated (Indoline Derivative)-ve	1x (10.0µl)		-	10µl	20µl
DLD-1	**Untreated Control +ve**	1x (10.0µl)	1 x (1.0µl)	2µg (1.63 µl)	7.37µl	20µl
	Untreated Control -ve	1x (10.0µl)		-	10µl	20µl
	Treated (Indoline Derivatie) +ve	1x (10.0µl)	1 x (1.0µl)	2µg (2.27µl)	6.73µl	20µl

	Treated (Indoline Derivatie) -ve	1x (10.0µl)		-	10µl	20µl

Table 2.6 The thermal cycling conditions used for reverse transcription

Setting	Step1	Step2	Step3
Temperature	37ºC	95ºC	4ºC
Time	60 min	5 min	∞

2.7. Real-time – PCR

Primer annealing temperatures for RT-PCR were optimized, and the Real Time PCR was performed on the Applied Biosystems Informatics (ABI) 7500 Real-Time PCR machine, using the Quantitect SYBR one-step qRT-PCR kit (Qiagen) following the manufacturer's instructions. SYBR Green I dye was used to detected PCR product. Amplification and melting curves were performed for each sample. Briefly, 2x QuantiNova SYBR Green PCR Master Mix, QuantiNova Yellow Template Dilution Buffer, template cDNA, primers, QN ROX Reference Dye, and RNase-Free water were thawed on the ice for 15-20 min. The reaction mixture solution was prepared according to Table 2.7.

Table 2.7 Real Time reaction set up

Component	Volume		Final Concentration
	Reaction mix for 10 reactions	**per Reaction in a 96 well plate**	
2x Quanti Nova SYBR	50µl	5µl	1x
QN ROX Reference Dye*	1µl	0.05µl	1x
Primer F	10µl	1µl	0.7µM
Primer R	10µl	1µl	0.7µM
H_2O	20µl	1.95µl	-
cDNA	-	1µl	≤100 ng/reaction
Total Reaction volume	-	10µl	-

*Corresponds to a 1:200 dilution for a low ROX dye cycler such as the Applied Biosystems 7500 Real-Time PCR System

Due to the hot-start method, it was not necessary to keep samples on ice during reaction setup. The reaction mixture was mixed thoroughly and 8µl were dispensed into 18 wells of a 96 well reaction plate. Template cDNA was added (≤100ng/reaction) to the individual wells containing the reaction mixture. The real-time cycler was programmed as outlined in Table 2.8. Data acquisition was performed during the combined annealing/extension step.

Table 2.8 Real-Time cycler conditions

Step	Time	Temperature
Initial activation step	2min	95°C
Denaturation	5s	95°C
annealing/extension	10s	60°C
Number of cycles	40	-

2.8. Microscopy

2.8.1. Seeding cells onto coverslips

Subcellular protein expression in control and drug treated cells was assessed using indirect immunofluorescence together with confocal microscopy. Foremost**,** each coverslips were cleaned by soaking in 70% v/v ethanol for 30 min. Cells were cultured, harvested and pelleted as before. A volume of 6mL DMEM/F12/FBS (10%v/v) was added to re-suspend the pellet. The re-suspended pellet (1mL) was diluted with approximately 8mL of DMEM/F12/FBS (10% v/v). With the aid of a forceps, each coverslip was sterilized by heat in a flame. The sterilized coverslip was placed in the center of a sterile petri dish. Assiduously, 500 µL of re-suspended cells (1 x 10^3 cells/mL) in culture medium were added onto the coverslip, the petri dishes were closed and incubated at 37°C for 5 hrs to enable the adherence of the cells to the coverslips. 2mL of DMEM/F12, FBS10%v/v) was then added to the petri dishes to immerse each coverslip. Fresh cell solution was prepared with the excess cells, which were transferred into two new culture flasks and incubated at 37°C in an atmosphere of 5% CO_2 and 90% humidity. Upon reaching 70% confluence, cells were treated as described previously.

2.8.2. Fixing of cells to the coverslips

A fixative solution was prepared by diluting, 30% v/v of formaldehyde with PBS to obtain a Formaldehyde/PBS solution (Fixative) (3% v/v). The cells were then washed three times with PBS after removing the culture medium. After washing, 1mL-1.5mL of 3% v/v fixative solution was added to the coverslip and incubated for 15 min at room temperature. Then, the coverslips were washed three to four times with PBS. The dishes were sealed with Parafilm after adding PBS to the petri dishes containing the fixed cells. These were stored in a refrigerator at 4°C until ready for immunofluorescence staining.

2.8.3. Indirect immunofluorescence

2.8.3.1. Incubation of fixed cells with primary antibody

BSA) solution (0.5% w/v) was prepared in 100mL of PBS. To 125µL of Triton X-100 (Sigma-Aldrich), 50mL of BSA/PBS (0.5% v/v) was added to make up a 0.25% v/v solution of Triton X-100. PBS was discarded, after which Triton X-100/BSA solution (0.25% v/v) was added to the fixed cells for 10 min. Triton X-100 being a detergent permeabilises the cell membrane, to allow the primary antibody to enter the cell The Triton X-100 solution was then carefully discarded, and coverslips were washed with 0.5% v/v BSA/PBS. Following this the coverslips were subsequently transferred onto a dry labelled slide, with the cells facing uppermost. A 1:200 dilution of primary antibody was prepared with 0.5% v/v BSA/PBS. 100µL of Bcl-2 (C-2) mouse monoclonal IgG (Santa Cruz Biotechnology) and P53 rabbit polyclonal IgG (R&D Systems) antibodies were each was added to the coverslips. Slides containing coverslips were then incubated overnight at 4°C in a moist container.

2.8.3.2. Incubation of cells with secondary antibody

After overnight incubation, the coverslip was removed with forceps from the slides and carefully blotted edge on, onto tissue paper. Residual antibody was removed by sequentially dipping (10 times) the coverslips into beakers containing 0.5% v/v BSA/PBS. The coverslips were then transferred back onto the slides, with the cell side facing upwards. Each of Alexa Fluor® 488 conjugated donkey anti-mouse (Molecular Probes) and Alexa Fluor®488 conjugated donkey anti-goat (Molecular Probes) secondary antibodies were prepared in a 1:200 dilution with 0.5% v/v BSA/PBS, after which 100µL of secondary antibody was added to each coverslip. Slides containing coverslips were stored in a container lined with wet filter paper for 1 hour at room temperature (25°C) in a dark cupboard. Each coverslip was then removed from each slide and drained onto tissue paper to remove excess liquid and rinsed as described previously and replaced with the cells uppermost onto a slide for further staining.

2.8.3.3. Fluorescent staining of the nucleus

To stain the nuclei, 100µL of 300nM DAPI (Boehringer Mannheim) was added to each coverslip. A 1:10 000 dilution of DAPI was prepared with PBS. DAPI or 4', 6-diamidino-2-phenylindole is a blue-fluorescent nuclear stain, which preferentially stains double-stranded DNA and nuclei with little or no labelling of the cytoplasm. Slides containing coverslips were stored in a container lined with wet filter paper for 10 min at room temperature (25°C) in a dark cupboard, as DAPI is light sensitive. Each coverslip was then removed from each slide and drained onto tissue paper to blot away the excess DAPI solution. The coverslips were then dipped approximately ten times into each beaker containing 0.5%v/v BSA/PBS to remove any residual DAPI. Coverslips were then mounted onto the new microscope slides with cells facing downwards-using Gel Mount^{TM} aqueous mountant (Sigma-Aldrich). Coverslips were left to set and stored at 4°C until they were ready to be viewed under the Zeiss LSM 780 laser scanning microscope, using a 63X objective (see software information in Appendix A).

Fluorescence staining was assessed qualitatively using the following scale:
+ + - minimal/low fluorescence
++ ++ - medium intensity fluorescence
+++ +++ - high intensity fluorescence

Statistical analysis

All the experiments were done either in duplicate or in triplicate. The Mean and Standard deviation were calculated in the annexin V assay and for RT– PCR.

Proteome array Data analysis: A template to analyse pixel density for each spot of the array was created and the signal values were exported to an Excel sheet. The average signal (pixel density) of the pair of duplicate spots representing each apoptosis-related protein was determined and then an averaged background signal from each spot was subtracted. The corresponding signals on duplicate arrays were compared to determine the relative change in apoptosis-related protein levels between samples. The fold change was calculated between the control and treated samples.

3.1. Effect of HNPMPI on Morphological Changes

The IC_{50} of the HNPMPI was determined as 39.3118 µM ± 7.036 for DLD-1 and 31.8748 µM ± 1.252 for HT-29 cells, respectively (Appendix B). In order to investigate whether loss of cell viability in colon cancer cells after treatment with HNPMPI was due to apoptosis, the cells were subsequently observed under a light microscope. This visual observation indicated that sequential morphological changes occurred in both HT-29 and DLD-1 cells at 24 h after treatment. These changes included the loss of cell adhesion, membrane shrinkage and membrane blebbing as shown in Fig 3.1, where the control groups showed clear intact cell margins. As this data indicated that, the synthetic HNPMPI might induce apoptosis in these colon cancer cells, an annexin V apoptosis assay was carried out to assess this.

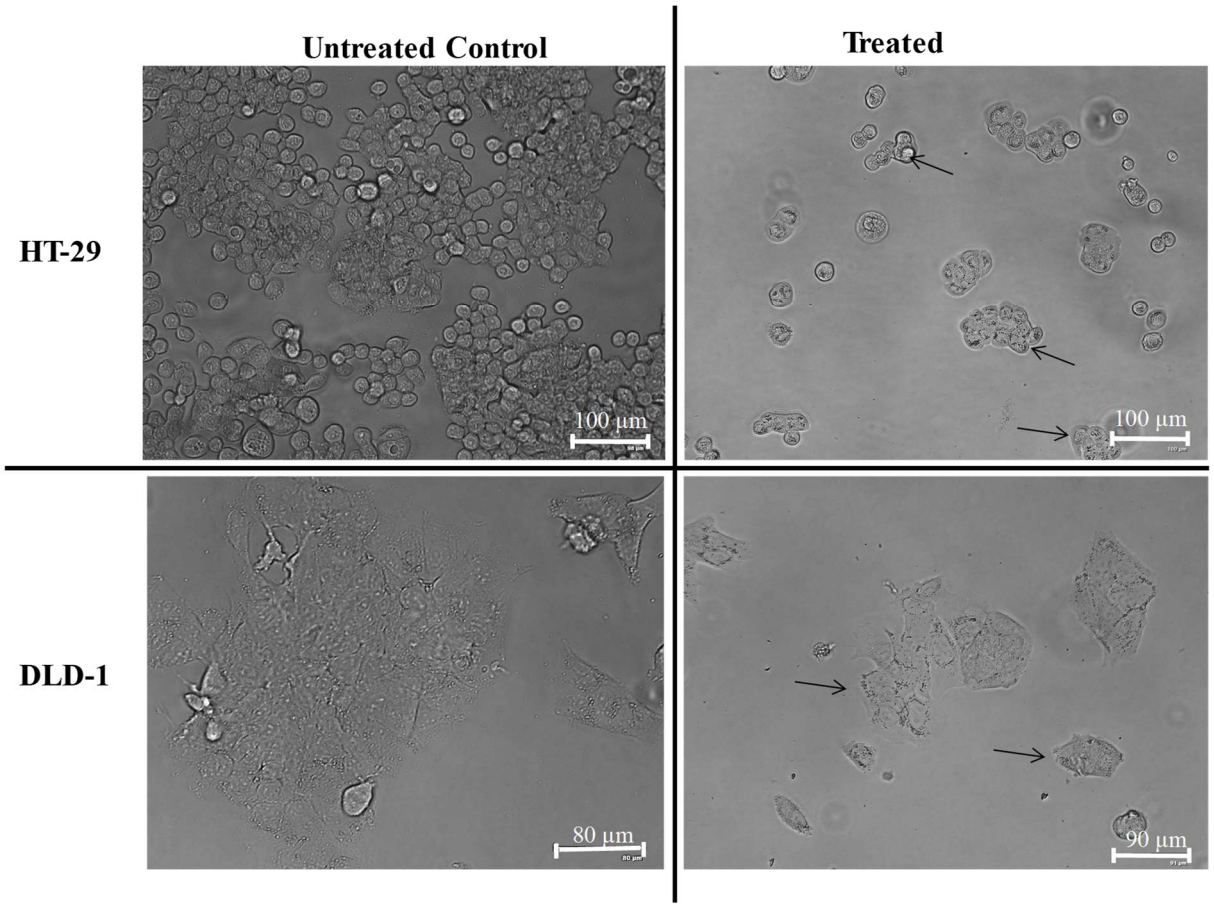


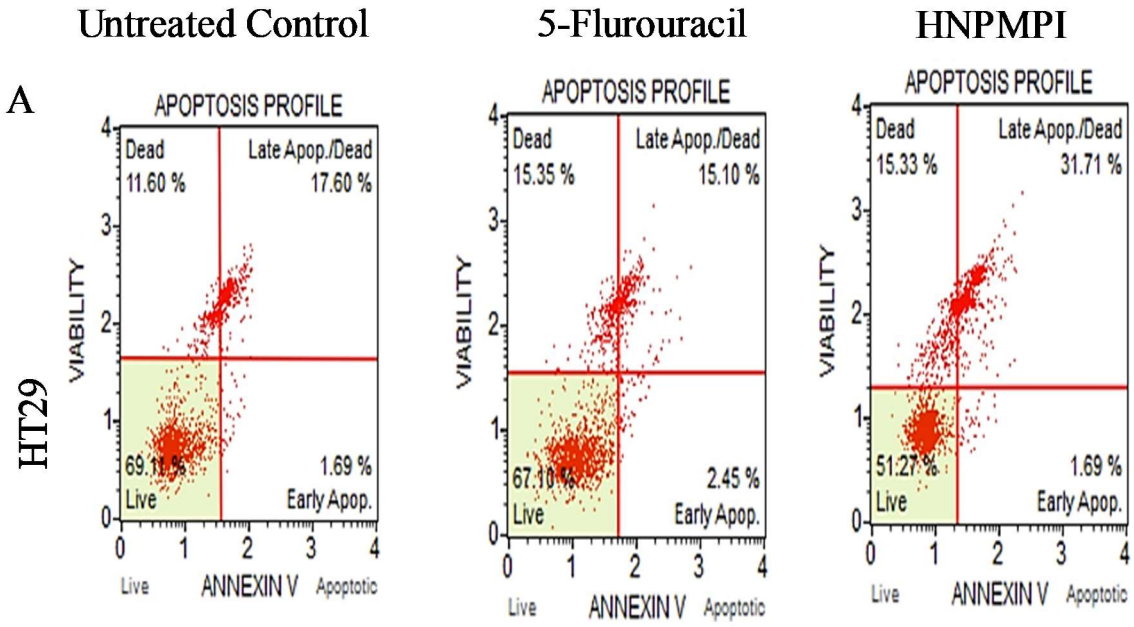
Figure 3.1 HNPMPI induces morphological changes in Mid (HT- 29) and Late Stage (DLD-1) colon cancer cells. The cells were seeded and cultured for 24 h and then treated with 30 µM of the Indoline Derivative for 24 h. As observed, HNPMPI induces cell death with morphological changes resembling those associated with apoptosis after treatment. The arrows indicate membrane shrinkage and membrane blebbing. All images were taken using a FLoid cell imaging system (Objective 20X)

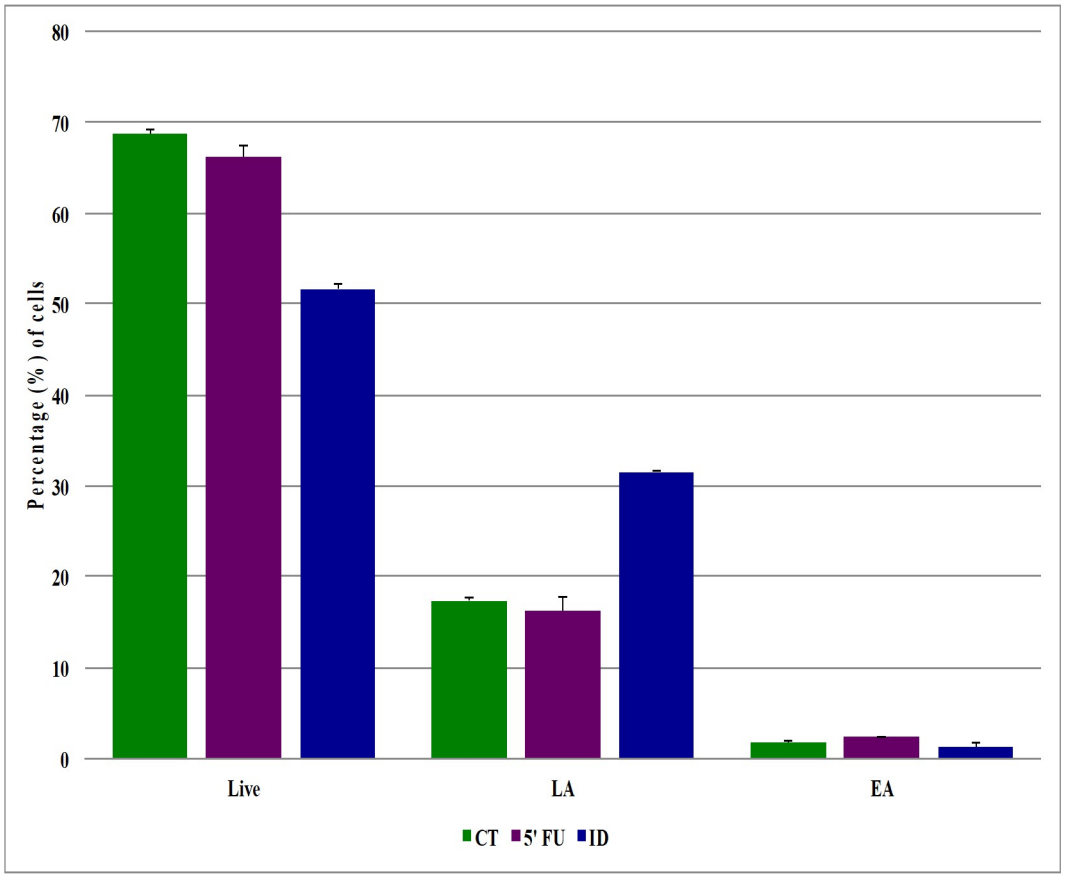
3.2. Detection of apoptosis using Annexin-V

Both cell lines (HT-29 and DLD-1) were treated with 30 µM of HNPMPI and 100µM of 5-Fluorouracil for 24 h and their effect on apoptosis was analysed using the Muse - Annexin V assay. In this study 5- Fluorouracil (5-FU), an FDA approved chemotherapeutic drug was used as a positive control for apoptosis.

As this assay combines Annexin V and 7-AAD it thus allows for the classification of the percentage of apoptotic cells into four groups, as follows: live cells [Annexin V (−) and 7-AAD (−)]; dead cells [Annexin V (−) and 7-AAD (+)]; early apoptotic cells [Annexin V (+) and 7-AAD (−)]; late apoptotic or dead cells [Annexin V (+). These categories are represented in the scatter plots in Fig 3.2 below.

In the HT-29 cells, treatment with 5-FU and HNPMPI resulted in a significantly increased percentage of cells in the late stages of apoptosis with 16.2 and 31.58 % of cells being in the late stages of apoptosis, respectively (Fig 3.2 A). In comparison in DLD-1 cells the numbers of both early (7.88%) and late (4.70%) stage apoptotic cells increased, when compared with the control (1.05% and 1.85%, respectively) and 5-FU treated cells (7 and 4.02%) (Fig 3.2 B). Overall, HNPMPI showed an ability to induce apoptosis to a larger degree in HT-29 cells than in DLD-1 cells. All the experiments were performed in triplicate and the mean value of the percentage of the live and dead cells were calculated with the standard deviation and represented graphically (Fig 3.2)


A
HT29
Untreated Control
5-Flurouracil
HNPMPI
APOPTOSIS PROFILE
Dead
11.60 %
Late Apop./Dead
17.60 %
69.11 %
Live
1.69 %
Early Apop.
VIABILITY
ANNEXIN V
Live
Apoptotic
APOPTOSIS PROFILE
Dead
15.35 %
Late Apop./Dead
15.10 %
67.10 %
Live
2.45 %
Early Apop.
VIABILITY
ANNEXIN V
Live
Apoptotic
APOPTOSIS PROFILE
Dead
15.33 %
Late Apop./Dead
31.71 %
51.27 %
Live
1.69 %
Early Apop.
VIABILITY
ANNEXIN V
Live
Apoptotic


Percentage (%) of cells
80
70
60
50
40
30
20
10
0
Live
LA
EA
CT
5' FU
ID

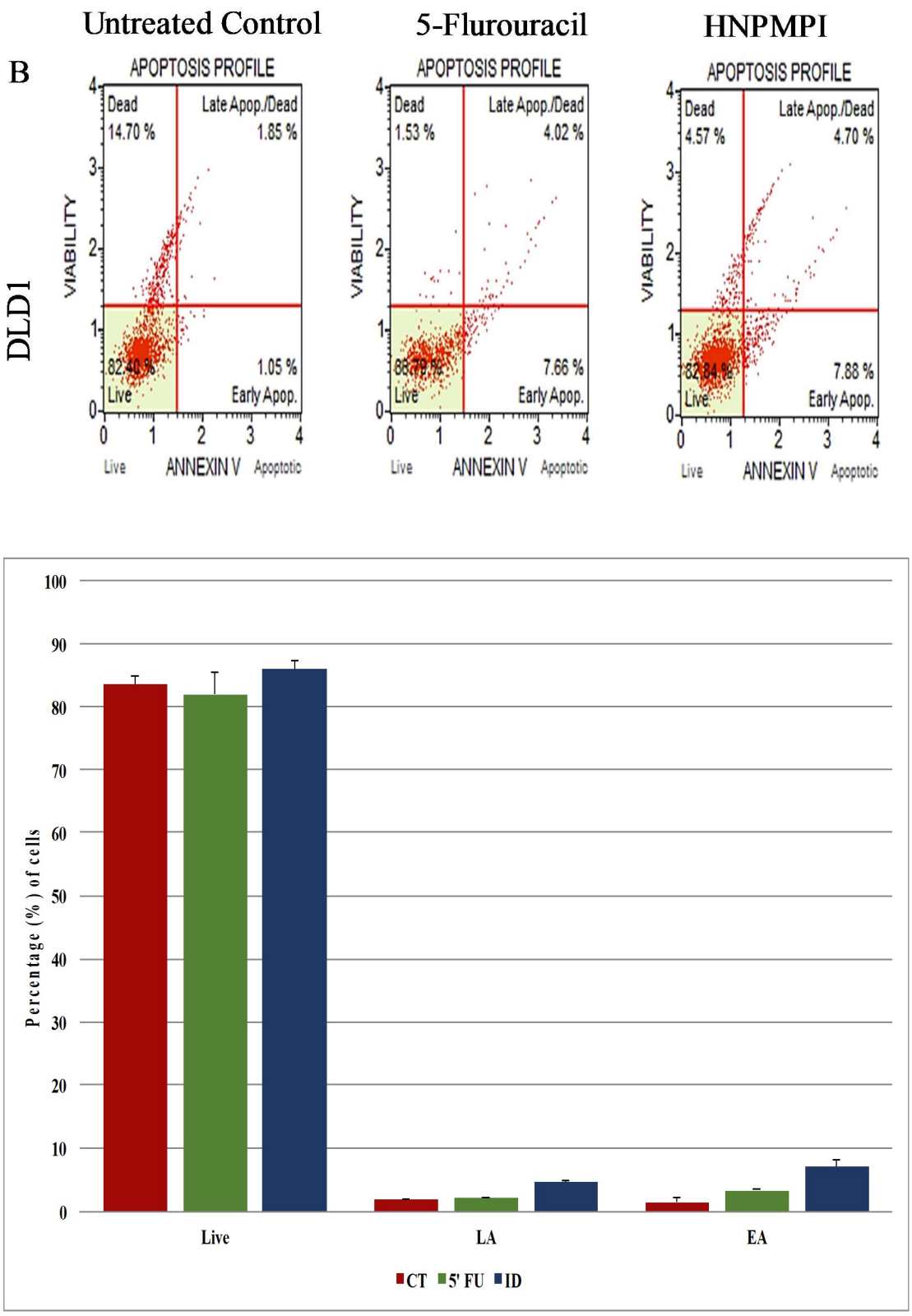


Figure 3.2 HNPMPI induces apoptosis in HT-29 and DLD-1 colon cancer cell lines. The percentage of apoptotic cells was determined using the Muse – Annexin V assay. Cells in the mid stage showed Annexin V (+) and 7-AAD (−) staining and late apoptotic or dead cells showed Annexin V (+) and 7-AAD staining. Scatter plot and Graphs showing the effect of HNPMPI in HT-29 (A) and DLD-1 (B). Graph shows the mean value of the samples and Triplicate samples were used to obtain the mean and standard deviation. EA=Early Apoptosis; LA= Late Apoptosis; CT- Untreated Control

3.3. Protein Extraction and Quantification

Both HT-29 and DLD-1 cell lines were cultured as described previously and they were treated with 30 μM HNPMPI and the proteins were extracted following 24h of treatment. The concentration of the extracted proteins was determined using the Qubit protein assay relative to a standard curve (Example of the standard curve shown in Appendix C). All the samples contained a similarly high concentration of proteins (26.5μg/mL) and with some being higher than this value (Table 3.1). The high protein concentration indicated a successful extraction procedure.

Table 3.1 Protein Quantification as determined using the Qubit® Assay

Samples	Protein Concentration
HT-29 - Control	26 μg/mL
HT-29 - Treated	26 μg/mL
DLD-1 -Control	26.5μg/mL
DLD-1 - Treated	> 26.6 μg/mL

3.4. Determination of the quality of the extracted protein using SDS-PAGE

In order to determine if the extracted protein was of good quality with little signs of proteolysis, equivalent amounts of the extracted samples were electrophoresed on a 12% SDS polyacrylamide gel with a 6% stacking gel. For each sample, 5 μg of protein was loaded onto the gel. The banding pattern (Figure 3.3) showed that the protein samples were of good quality with little or no degradation as indicated by the even distribution of protein bands in each sample, thus allowing for further downstream analyses of the protein extracts. These extracts were then analysed for apoptotic protein expression using a protein profiler antibody array.

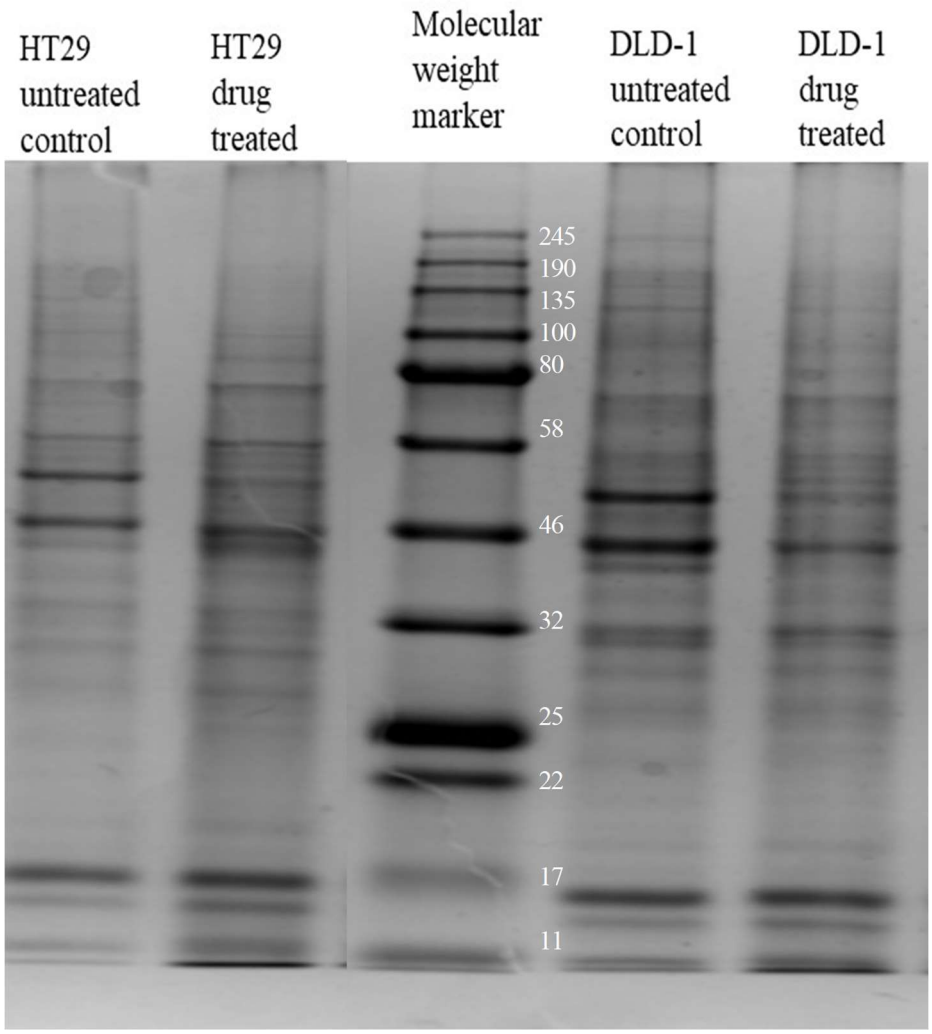


Figure 3.3 SDS PAGE electrophoresis of protein extracts from HT-29 and DLD-1 colorectal adenocarcinoma cells. The protein concentration was determined and 5µg of protein from each sample was analysed using 12% SDS PAGE. The gel showed that the protein extracts were of a consistently good quality, suitable for downstream analyses.

3.5. Changes in the expression of apoptotic proteins following the treatment of HT-29 and DLD-1 cell lines with HNPMPI

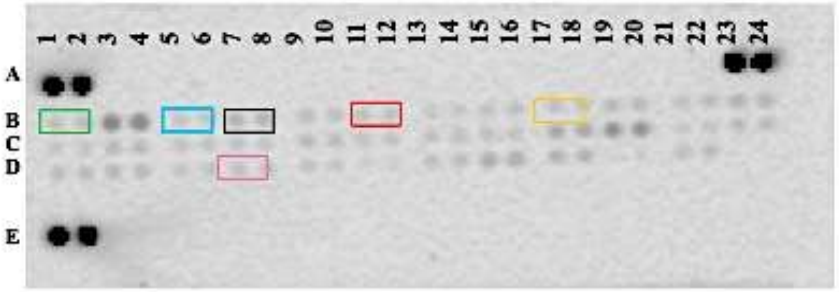
As previously shown in this study, HNPMPI was found to act upon cancer cells to induce apoptosis. It was thus queried here which specific apoptosis-related proteins were directly linked to the HNPMPI induced apoptosis in the HT-29 and DLD-1 cell lines. This was determined using a human apoptosis protein profiler array. This technique utilizes capture and control antibodies spotted onto a nitrocellulose membrane. The membranes were visualized using a chemi-luminescent camera and the intensity of the antibody signal was recorded as an optical density reading.

Following normalization of the signals, the graphed relative expression levels clearly showed that HNPMPI influences the expression of pro and anti-apoptotic proteins in both HT-29 and DLD-1 cell lines (Figure 3.4 and 3.5). A list of proteins whose expression changes upon the treatment of cells with HNPMPI and their role in apoptosis (pro or anti apoptotic) are provided below in Table 3.2.

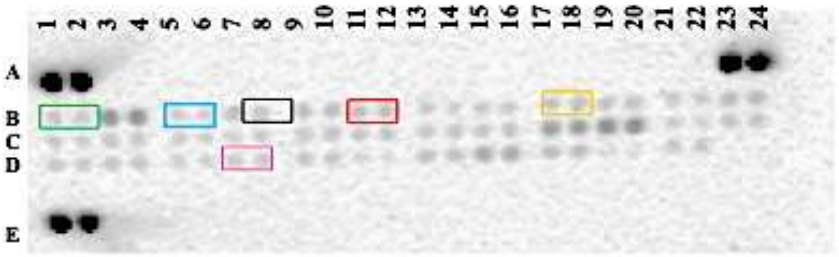
In HT-29 cells, proteins related to apoptosis, for example, caspase-3, TNF-related apoptosis-inducing ligand (TRAIL) receptor 1/DR4, and tumour necrosis factor receptor 1 (TNF R1), Fas/TNFRSF6/CD95 were upregulated (Fig 3.4 C). Cleaved Caspase 3 was increased 1.6-fold after the treatment; while TNF R1, Fas/TNFRSF6/CD95 and TRAIL had a 0.8, 0.2and 0.2-fold increase, respectively, when compared to the control. This result showed that extrinsic pathway of apoptosis is triggered in HT 29 cells after the treatment.

As earlier reflected in annexin V assay, DLD-1 cells displayed a different protein profile as compared to HT-29 cells. Most of the apoptotic proteins didn't show any significant difference, but all forms of Caspase proteins (1.6 fold) and TNF-related apoptosis-inducing ligand (TRAIL) were upregulated. Also, all the forms of p53 were downregulated (0.02 fold) after the treatment. The opposite pattern occurred in the HT-29 treated cells. Similarly, Cytochrome C is downregulated in DLD-1 and Upregulated in HT-29. Moreover, as all of the Bcl-2 family proteins were downregulated, this confirms that HNPMPI does not induce the mitochondrial mediated apoptosis pathway, but rather Caspase mediated apoptosis in DLD-1 cells.

A) Untreated HT-29 cells



B) HT-29 cells treated with 30µM HNPMPI



C) Comparison of expression of apoptosis related proteins in treated and untreated HT-29 cells

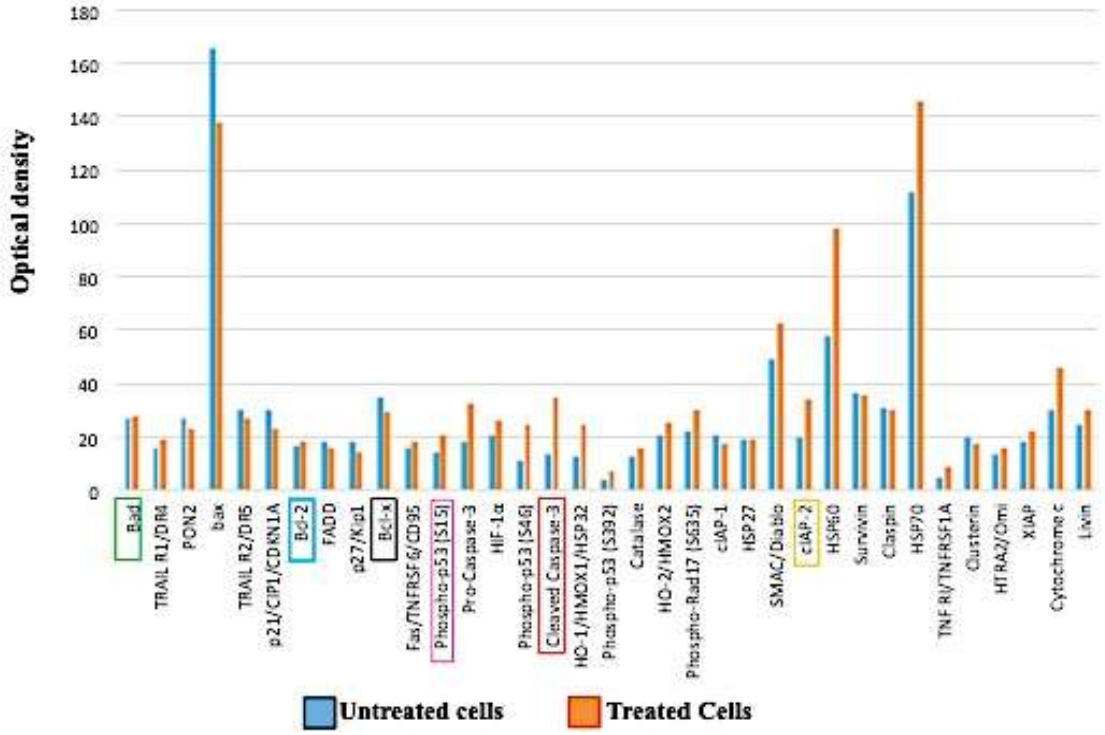


Figure 3.4 Changes in the expression of the apoptosis-related proteins in HT-29 cells following treatment with HNPMPI. Array membranes showing the protein expression (A) before treatment, (B) after treatment in HT-29 cells and (C) the expression of the apoptosis-related proteins in untreated versus treated cells were compared.

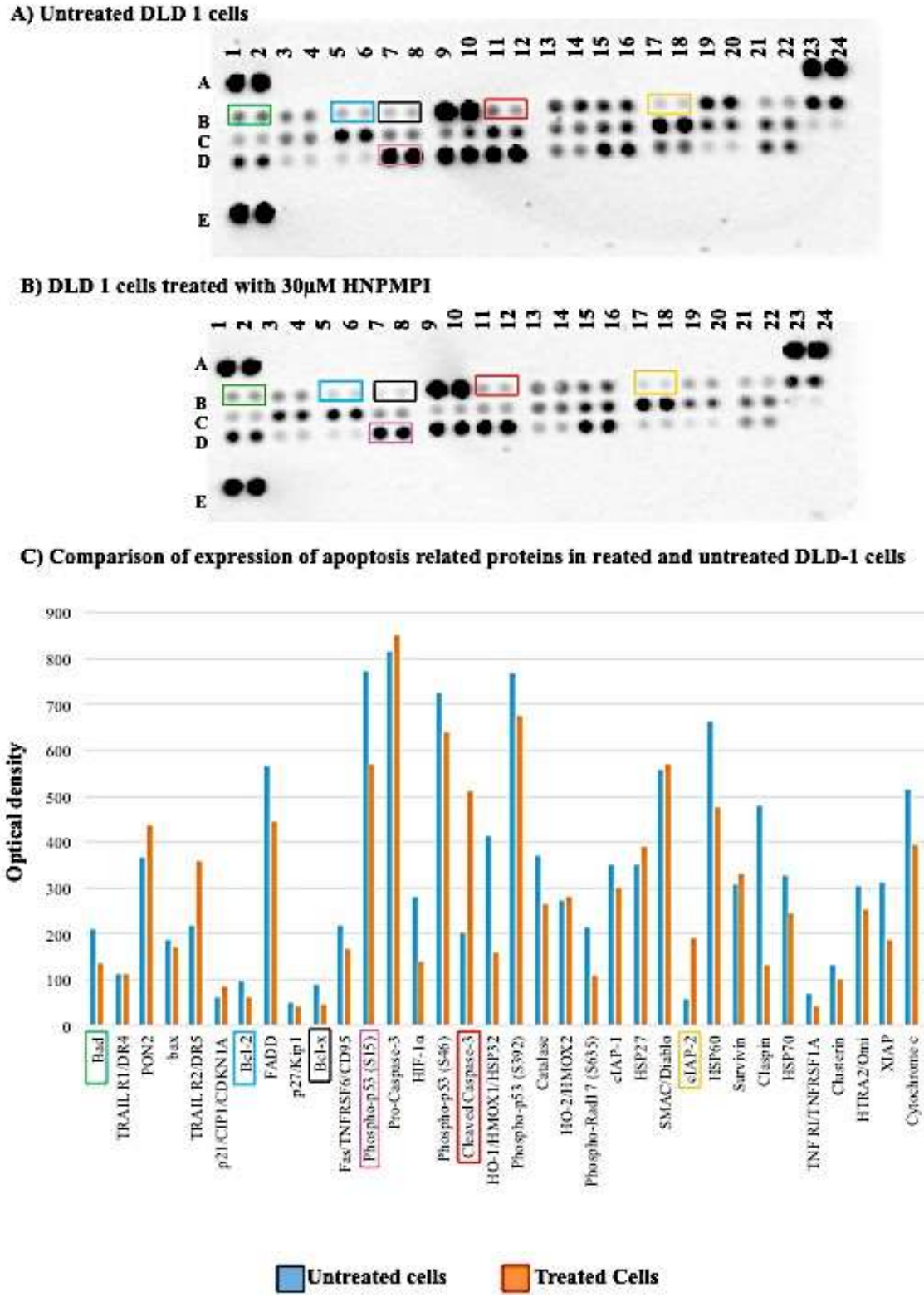


Figure 3.5 Changes in the expression of the apoptosis-related proteins in DLD-1 cells following treatment with the HNPMPI. (A) and (B) Apoptosis array membranes showing the protein expression before (A) and after treatment (B) in DLD-1 and (C) the expression of the apoptosis related proteins in untreated versus treated cells was compared.

Table 3.2 Summary of protein expression results obtained using the protein profiler array

Gene name	Role in apoptosis	Expression following HNPMPI treatment	
		DLD-1	HT - 29
BAD	Pro-apoptotic	Decrease	No change
BAX	Apoptotic activator	Decrease	Decrease
Bcl-2	Anti-apoptotic	Decrease	Increase
Bcl-x	Bcl-xL- anti-apoptotic Bcl-xS- pro-apoptotic	Decrease	Decrease
Pro-Caspase-3	Inactive precursor of pro-apoptotic protein	Increase	Increase
Cleaved Caspase-3	Pro-apoptotic	Increase	Increase
Catalase	Protects cells from apoptosis	Decrease	Increase
cIAP-1	Apoptosis inhibitor	Decrease	Decrease
cIAP-2	Apoptosis inhibitor	Increase	Increase
Claspin	Cell cycle arrest	Decrease	Decrease
Clusterin	Protects the cell from apoptosis	decrease	Decrease
Cytochrome c	Induces apoptosis	Decrease	Increase
TRAIL R1/DR4	Induces cell apoptosis	No change	Increase
TRAIL R2/DR5	Induces cell apoptosis	increase	Decrease
FADD	Induces cell apoptosis	No change	Decrease
Fas/TNFRSF6/CD95	Induces cell apoptosis	Decrease	Increase
HIF-1α	Induces apoptosis with p53 or	Decrease	Increase
HO-1/HMOX1/HSP32	Protects cells from apoptosis	Decrease	Increase
HO-2/HMOX2	Protects cells from apoptosis	Increase	Increase
HSP-27	Inhibition of apoptosis	Increase	No change
HSP60	Prevents apoptosis	Decrease	Increase
HSP70	Inhibits apoptosis	Decrease	Increase
HTRA2/Omi	Pro-apoptotic	Increase	Increase
Livin	Inhibits apoptosis	Decrease	Increase
PON2	Inhibits apoptosis	Increase	Decrease
p21/CIP1/CDKN1A	Cell cycle arrest	Increase	Decrease

p27/Kip1	Cell cycle arrest	decrease	Decrease
Phospho-P53 (S15)	Initiate apoptosis in response to DNA damage	Decrease	Increase
Phospho-P53 (S46)		Decrease	Increase
Phospho-P53 (S392)		Decrease	Increase
Phospho-Rad17 (S635)	Cell cycle arrest in response to DNA damage	Decrease	Increase
SMAC/Diablo	Pro-apoptotic	Increase	Increase
Survivin	Inhibits apoptosis	Increase	Decrease
TNF RI/TNFRSF1A	Induces cell apoptosis	Increase	Increase
XIAP	Inhibits apoptosis	Decrease	Increase

The proteins highlighted in the blots and the plots with coloured boxes were those selected for transcript level analysis using RT-PCR.

3.6. RNA Extraction

In order to establish if the changes in protein expression are reflected as changes in the transcriptome, Real Time PCR was performed using primers designed to amplify genes coding for proteins whose expression may change following the drug treatment. An increase or a decrease in protein expression may be due to a change in the level of transcription of the genes encoding these proteins. However, this is not always the case and discrepancies between RNA and protein levels may reflect different forms of control of gene expression. mRNA was extracted from HT-29 and DLD-1 cells treated with HNPMPI and from untreated cells. The concentration and purity ratios of these extractions is given in Table 3.3. The concentration of all the samples was relatively high. The purity ratios show that the samples were pure and relatively free of protein contamination.

Table 3.3 Concentration and purity ratios of RNA extracted from treated and untreated DLD-1 and HT-29 cells

Sample	**Concentration ng/µl**	**Purity (A260/A280)**	**Purity (A260/A230)**
HT- 29 Untreated	1395.2	2.00	2.29
HT-29 Treated	395.5	1.99	2.34
DLD-1 Untreated	1224	2.01	2.32
DLD-1 Treated	878.8	1.8	2.28

3.7. Assessment of Apoptosis gene expression in HT-29 and DLD-1 cell lines

RT-PCR was performed to determine whether the changes in the protein expression are accompanied by the changes in the gene transcription. To determine this cDNA was synthesized from the mRNA extracted from the HNPMPI treated and untreated control samples of both HT-29 and DLD-1 cell lines individually; and RT-PCR was performed using primers designed to amplify BAD, BAX, Bcl-2, CASP3 and p53 genes as described in methods.

The expression levels of the transcripts upon HNPMPI treatment are shown in Figure 3.6. The results indicate that the transcription of the pro-apoptotic genes BAD, CASP3 and the anti-apoptotic gene Bcl-2 was found to be increased, while the pro-apoptotic gene BAX and p53 expression levels were both decreased with HNPMPI treatment in HT-29 cells and DLD1 (1 and 1.5-fold, respectively).

3.7.1. CASP3 gene expression

In DLD-1 cells, only transcription of the CASP3 gene was increased, whereas, in the other genes transcript levels were decreased upon treatment with HNPMPI. The transcription of CASP3 was increased both in the HT-29 and DLD-1 cells, that is CASP3 was highly expressed in both the mid (0.3 times) and late stage (0.4 times) CRC cell models. The CASP3 protein expression was also found to be increased in both HT-29 and DLD-1 cells.

3.7.2. BAD gene expression

In assessing transcription of the BAD gene after treatment, it was more highly expressed (2.5 times) in HT-29 cells, when compared to all the other genes. The change in the BAD gene expression was however not reflected in the BAD protein expression (Figure 3.4C). In DLD-1 cells transcription of BAD was slightly decreased (0.1 times) which was also reflected in the corresponding protein expression (Figure 3.5C).

3.7.3. Bax and Bcl2 gene expression

When treated with HNPMPI, the transcription of BAX genes decreased by 0.75 times and 0.1 times in both HT-29 and DLD-1 cells, respectively. This was also found to be reflected in BAX protein expression in the respective cell lines (Figure 3.4C and 3.5C). Bcl2 transcription decreased by 0.5 times in DLD-1 cells but remained unchanged in HT-29 cells. This matches the protein expression pattern in DLD-1 cells. However, Bcl-2 protein expression in drug treated HT-29 cells was high as compared to the low level of Bcl-2 gene transcribed as detected by RT-PCR.

3.7.4. p53 gene expression

The transcription of p53 decreases in both cell lines following the treatment with HNPMPI. In HT-29 cells, the decrease is larger, that is 1.5-fold, while in DLD-1 it is only 0.3-fold.

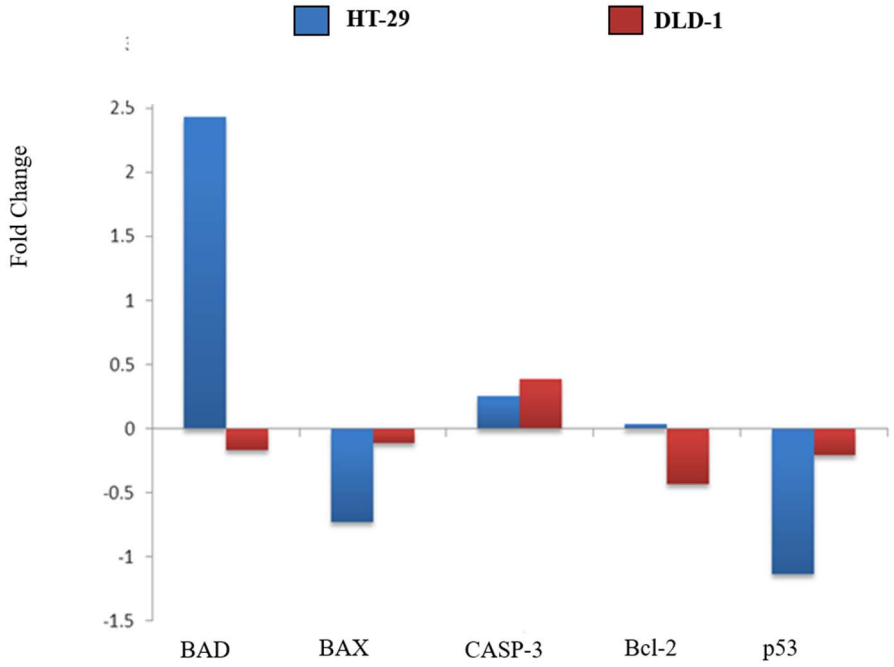


Figure 3.6 RT-PCR analysis of transcript levels. The above graph illustrates the fold change (Log Phase 2) in the target gene transcription level following the treatment of HT-29 and DLD-1 cell lines with HNPMPI.

The decrease in p53 transcription in DLD-1 cells is accompanied by a decrease in expression of p53 protein, but there is an increase in the expression of p53 protein in the treated HT-29 cells.

3.8. Confocal microscopy analysis of Bcl-2 and p53 Protein expression in colon cancer cell lines after HNPMPI treatment

Two proteins, Bcl-2 and p53 were selected for microscopic analysis in HNPMPI treated cells. The microscopic visualization of the cells shows distinct morphological differences between untreated and treated cells, suggesting that the drugs particularly affected Bcl- 2 gene expression. Also, cells when treated with HNPMPI change from an irregular to a more oval shape with a seeming lower cytoplasmic to nuclear ratio (Figure 3.7).

3.8.1. Bcl-2 cellular expression

In the untreated HT-29 cells, Bcl-2 was little observed in the cytoplasm and around the nucleus (+++). Following treatment with HNPMPI it was detected within the cytoplasm and predominantly around the nuclear periphery (indicated by arrows in Figure 3.7). This microscopy-based observation of increased Bcl-2 levels supports the relative gene expression and protein profile data showing an up-regulation in Bcl-2 expression following HNPMPI treatment (+++++).

In both control and treated HT-29 cells, Bcl-2 expression is perinuclear (+ in controls; ++ in drug treated), with minimal localisation in the cytoplasm (+, in controls and ++ in treated cells). In DLD-1 cells, Bcl-2 localisation was more dispersed around the nuclei in both control and treated cells. In treated DLD-1 cells the staining intensity was (++) compared to control cells (++++) (Figure 3.7). Bcl-2 was not localized to nuclei in either cell line. In treated cells nuclei appeared enlarged relative to untreated cells (see arrows).

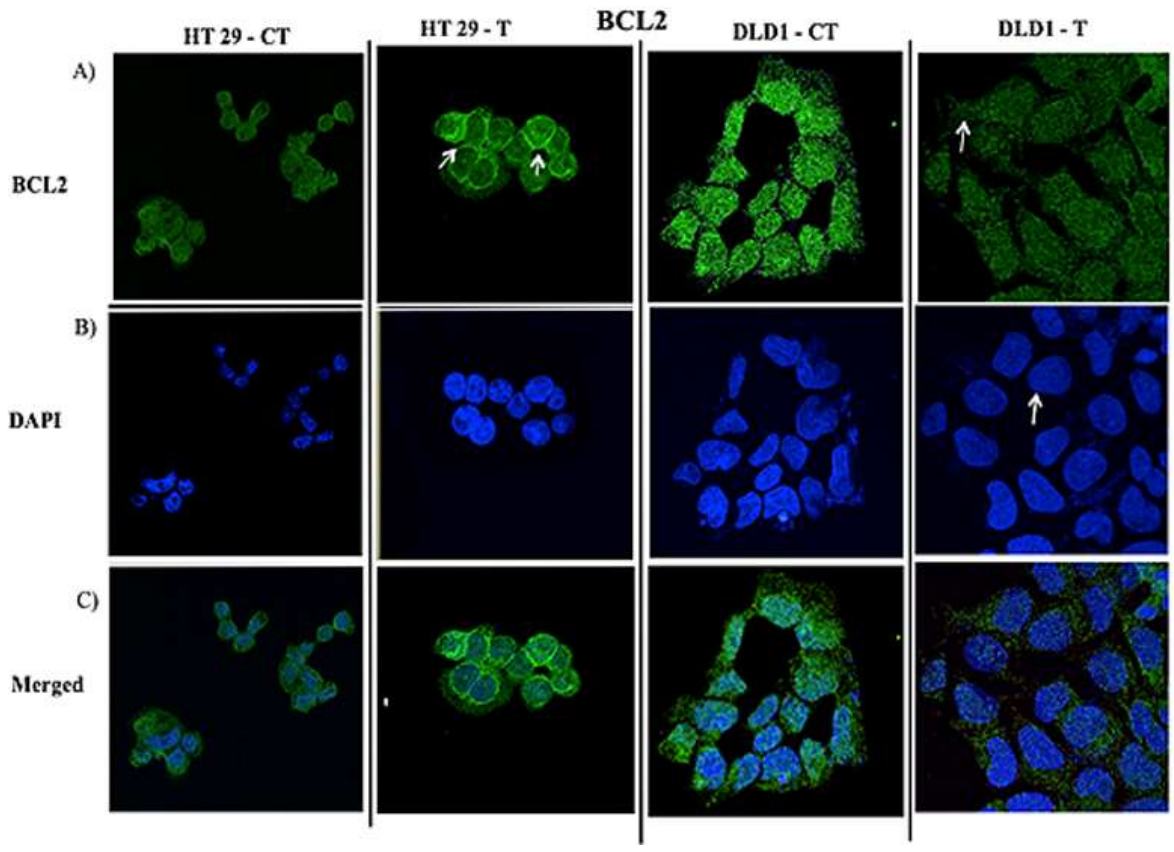


Figure 3.7 Effect of the HNPMPI on Colon cancer cell line. A confocal microscopy images (63X) shows the expression of Bcl-2 protein expression in HT-29 and DLD-1 cells and its localization. Bcl2 mouse monoclonal IgG was detected with Alexa Fluor® 488 conjugated donkey anti-mouse IgG (A) and Nuclei were

stained with DAPI (B). The merged image includes the green and blue fluorescence overlays in a single image (C). Blue – DAPI; Green – Bcl-2; CT= control; T= treated.

3.8.2. Effect of HNPMPI on P53 cellular expression in colon cancer cell lines

Similar to Bcl-2 expression, p53 protein expression was upregulated in the HT-29 cells after treatment with HNPMPI. In control cells some of the cells only expressed nuclear p53 (see arrows in Fig 3.8 A). After drug treatment, there was a marked expression of nuclear p53 (+++++), when compared to untreated cells (+++).

In DLD-1 treated cells, there was a change in the cell size relative to the untreated control (as indicated by arrows); the cytoplasm was reduced in size and in volume and the cells have an irregular shape. The nuclei appear condensed, an indication of apoptosis. p53 was expressed more in nucleus in untreated cells (+++++) when compared to treated cells (+++). The expression of Nuclear P53 expression was downregulated in DLD-1 cells after treatment.

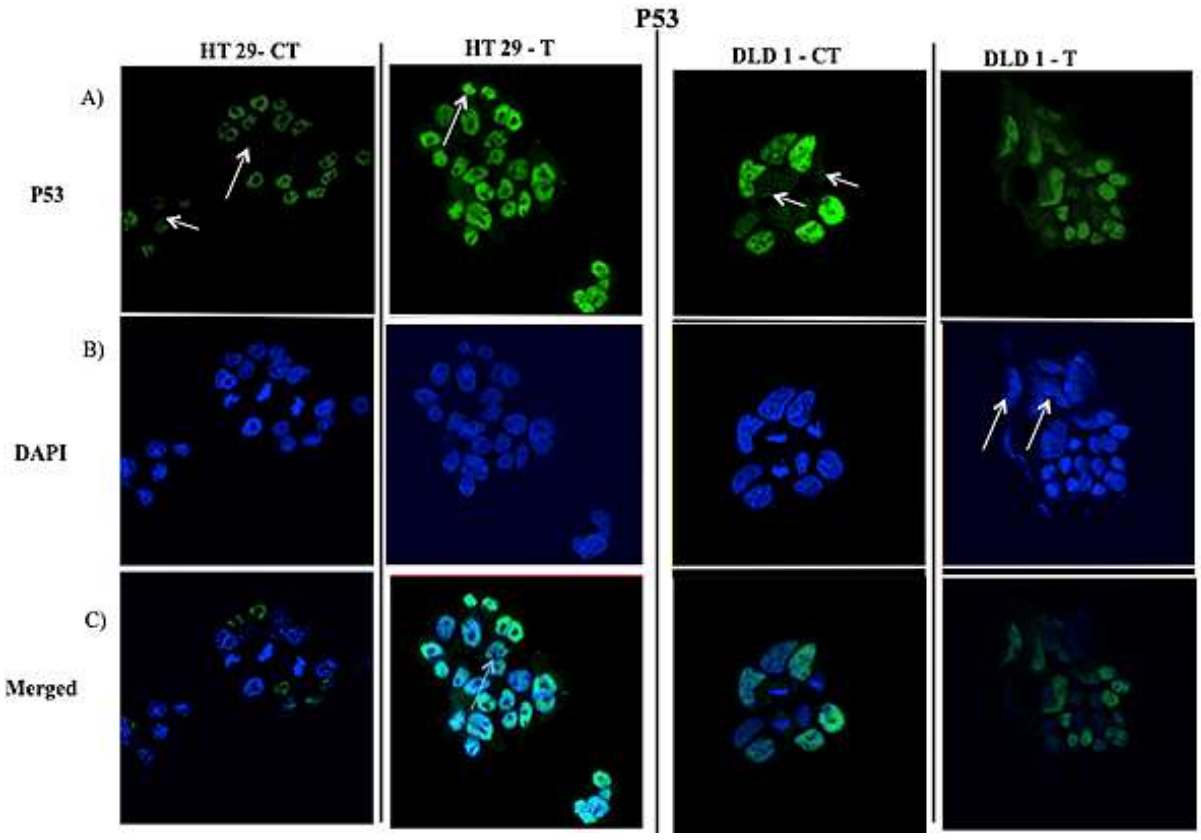


Figure 3.8 Effect of the HNPMPI on HT-29 and DLD_1 colon cancer cells. Confocal microscopy images (63X) show the expression of P53 protein in HT-29 and DLD-1 cells. p53 was stained with goat polyclonal IgG antibody and detected with Alexa Fluor®488 conjugated donkey anti-goat IgG. Blue -DAPI (B); Green-P53 (A). The merged image includes both the green and blue fluorescence overlays in a single image (C).

CHAPTER 4: DISCUSSION

4.1. Introduction

In many emerging nations, such as South Africa, the prevalence of CRC is reported to be increasing and may be related to the increased adoption of western diets and lifestyles. However, unlike wealthier western countries, these nations are unable to deal with the extra burden this disease places on the public health sector due to financial constraints. Current drugs used to treat this disease are expensive, relatively toxic and the cancer cells may develop resistance against these drugs. Thus, it is important to identify new anticancer agents that may act as lead compounds for the development of new drugs. These compounds generally target one or more of the so-called hallmarks of cancer that differentiate cancer cells from normal healthy cells of the body. Such compounds belong to several structural classes such as enediynes, anthracyclines, isoprenoids, indolocarbazoles, polyketide macrolides, non-ribosomal peptides including glycopeptides, some with anti-cancer activity (Rawls and McGinty, 1998, Xue *et al.,* 1999). As a fundamental trait of cancer cells is to evade apoptosis providing tumour cells with a pathway to avoid drug-induced apoptosis (Wong and Goeddel, 1994), compounds that selectively induce apoptosis in cancer cells are thus a particular focus in the development of new anticancer drugs (Martin *et al.,* 2002, Reed, 2000, Goldstein, 2001). One of the most promising synthetic drugs in medicinal chemistry is the Indoles, that contain an electron-rich pyrrole moiety (Megna *et al.,* 2016). In this study, the anti-cancer activities of the novel HNPMPI indole derivative was assessed on both mid stage (HT-29) and late stage (DLD-1) colon cancer cell lines.

4.2. Background to HNPMPI

The present study followed on from a pilot study wherein HNPMPI was shown to exhibit strong cytotoxicity in a concentration dependent manner against the DLD-1 and HT-29 cancer cell lines. In a prior study, the IC_{50} of HNPMPI was determined to be 39.31µM ± 7 and 31.87µM ± 1.2 for HT-29 and DLD-1 cells, respectively. Therefore, the mid stage colon cancer cells required a lower concentration of the drug to induce cytotoxic effects, compared to the late stage colon cancer cell line (pers comm, J Kandhavelu). With regards to the prior findings described above, for the purposes of the present study, an HNPMPI concentration of 30µM being slightly below the IC50 for both DLD -1 and HT-29 cells, was chosen. This was to allow

for the compound to have measurable effects on the gene expression levels in these cells, without killing the majority of them.

4.3. HNPMPI and the induction of apoptosis

As has been well described, apoptosis may be defined as cell death, where there is cytoplasmic and nuclear condensation (pyknosis), nuclear fragmentation (karyorrhexis), and an intact plasma membrane with signs of blebbing. These changes are followed by the cell breaking down into apoptotic bodies (Galluzzi *et al.,* 2007). This plays an important role in the elimination of unwanted cells, including malignant/cancerous cells. However, many cancer cells have the ability to escape from apoptosis (Hanahan and Weinberg, 2011), this being pertinent since most of the current conventional anticancer agents induce apoptosis. As described above, following on from a prior pilot cytotoxicity study, the present study evaluated the potential anticancer activity of HNPMPI and its possible role in inducing apoptosis in two tumour stage specific colon cancer cell lines. In the present study, light microscopy analysis of HNPMPI treated cells showed an induction of apoptosis in both the HT-29 and DLD1 cells. Treated cells had features of apoptosis, including decreased cell adhesion, signs of membrane blebbing and the breakdown of cells into apoptotic bodies. In support of this, there are a few reports on natural indole phytoalexins from plants or synthetic derivatives that induce apoptosis or cell cycle arrest in cancer cells (Brard *et al*., 2008, Lee *et al*., 2004). As an example, Esmaeelian *et al.,* 2013 reported that an indole derivative from *Dicathais orbita* induced apoptosis and cell cycle arrest in both the HT-29 and Caco2 colon cancer cell lines; and a synthetic indole derivative induced apoptosis in the HCT116 colon cancer cell line (Tischlerova *et al.,* 2017). Similarly, in the present study, the synthetic indole derivative, HNPMPI induced apoptosis. This is discussed further below.

To confirm the microscopy analysis of apoptosis in response to drug treatment, an Annexin V assay was used, this being a calcium-dependent phospholipid-binding protein that has a high binding affinity with cell membrane associated phosphatidylserine that is translocated to the outer membrane surface in early stage apoptotic cells. (Tait *et al.,* 1989, Andree *et al.,* 1990, van Heerde *et al.,* 1995).

Here, HNPMPI treatment effectively increased the percentage of colon cancer cells (HT-29 and DLD-1) undergoing apoptosis, with the highest percentage increase of cells in late stage apoptosis being observed in the HT-29 (mid-stage) cells as compared to DLD-1 cells. In

comparison, the positive control drug, 5-FU had far less effect than HNMPI in inducing apoptosis in both cell lines; and induced a lower percentage of cells into early rather than late apoptosis.

The induction of apoptosis by 5-FU is via the process of thymine less death, wherein the substitution of thymine with uracil interferes with DNA replication, causing DNA damage in rapidly dividing cells. The cellular responses to the indole derivative indicates that it has a different mode of action to 5-FU, either by inducing DNA damage in an alternative way or through a different mechanism, such as oxidative stress or membrane damage.

The differential apoptotic effect of HNPMPI on the HT-29 cells may be linked to their representing the mid-stage (stage 2) of a colorectal cancer tumour. This may imply that changes taking place in more advanced tumour stages, such as the ability of late stage cells to undergo epithelial to mesenchymal changes and metastasize could alter the effectiveness of HNPMPI reported on here in the mid stage colon cancer cells.

4.4. HNPMPI alters the expression of apoptosis related proteins

The ability of cancer cells to evade the induction of apoptosis is a key hallmark of cancer. Thus compounds that are able to induce apoptosis are of especial interest in the quest for new anticancer drugs (Trump *et al.,* 1997). Therefore, in the present study changes in the expression patterns of proteins involved in apoptosis, are indicators of particular apoptotic pathways activated by HNPMP1; and that will eventually result in the apoptotic death of the treated cells.

Apoptosis has three major pathways: these being the extrinsic pathway (receptor pathway of apoptosis), the intrinsic pathway (mitochondria-mediated apoptosis); and the signalling pathways that indicate death-associate proteolytic activities (Kaufmann and Earnshaw, 2000, Lee *et al.,* 2001). All of these pathways commonly stimulate the effector caspase 3 that leads to the degradation of cellular proteins, resulting in apoptosis. Figure 4.1 summarises the interactions between the different proteins assayed and their involvement in apoptosis; while Figure 4.2 compares the changes in protein expression in HT-29 and DLD-1 cells following treatment with HNPMP1. The extrinsic and intrinsic pathways in association with the cell lines are discussed in further detail below.

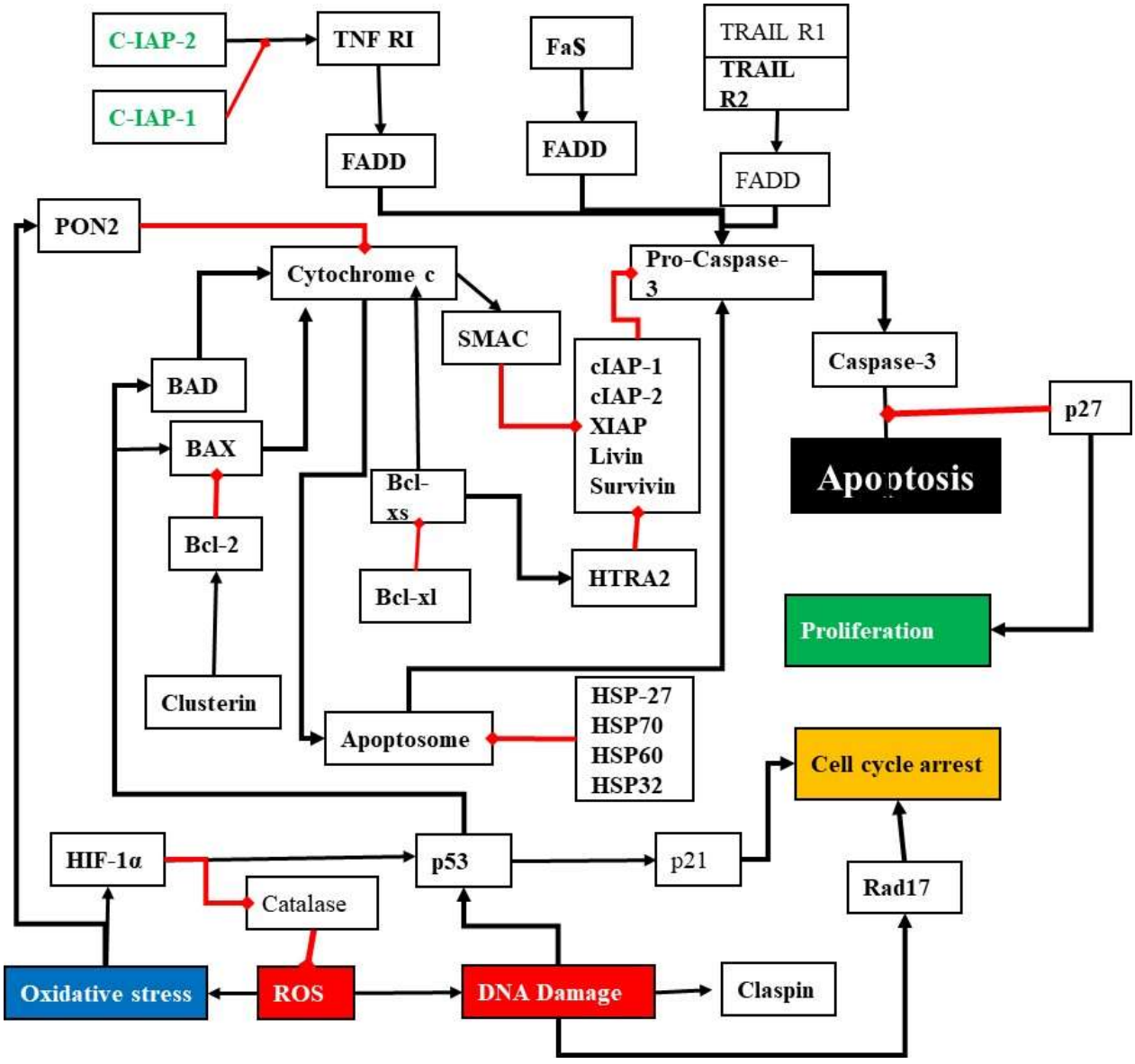


Figure 4.1. Diagrammatic representation of the interactions between the different proteins assayed and their involvement in apoptosis.

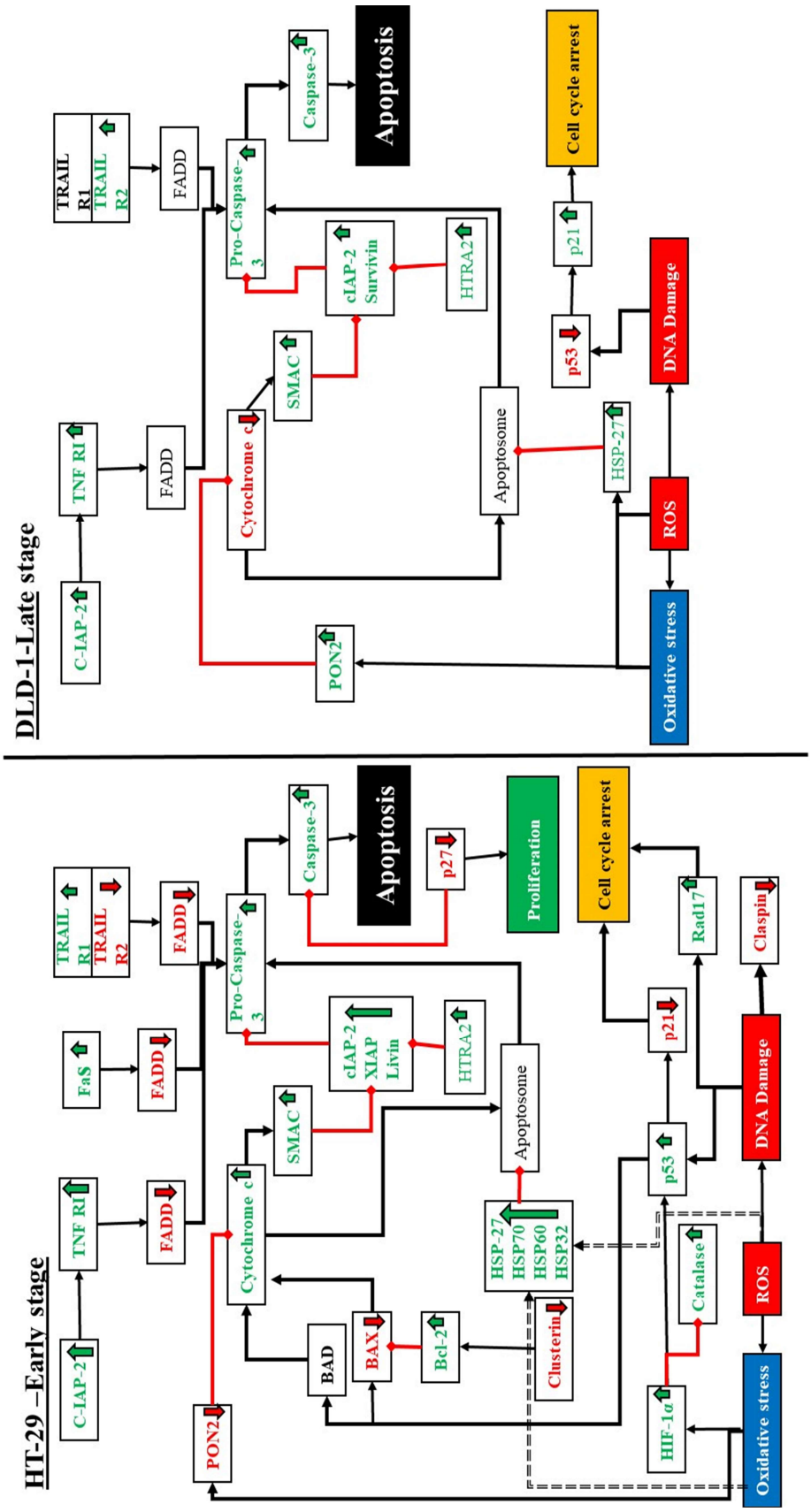


Figure 4.2. Comparative changes in protein expression in HT-29 and DLD-1 cells following treatment with HNPMP1

4.4.1. The extrinsic apoptotic pathway

The induction of apoptosis via the extrinsic pathway originates with the Fas, TNF and Trail signal receptors leading to activation of Caspase 8, which in turn activates Caspase 3 *(Sattler et al*., 1997, Cory *et al*., 2003a).

As shown in the present study, the expression of the TNFR1 death receptor was upregulated in both colon cancer cell lines, seeming to play an important role in promoting apoptosis (Ebach *et al.,* 2005). However, in the DLD-1 cells, FAS levels decreased, while TRAIL R2 increased, but with no change in TRAIL R1. Despite this, the apoptosis due to HNPMP1 treatment seemed to be largely dependent on the extrinsic pathway. This is likely due to HNPMPI increasing the levels of pro-caspase 3 and caspase 3, but without an increase in cytochrome c or BH3 domain proteins, that would characteristically occur in the activation of the intrinsic pathway. This is despite the observation that the levels of Fas-associated protein with death domain (FADD), the death domain adaptor protein, did not increase. This may be due to the binding of ligands to the cell surface receptors that leads to the recruitment of FADD to the signalling complex, but not necessarily to an increase in the transcription and expression of FADD.

In HT-29 cells representing mid stage colon cancer, FADD levels decreased following HNPMPI treatment, suggesting that the extrinsic pathway does not play a major role in the apoptotic process these cells undergo. When FADD activity is not required, it can be blocked by competitive inhibitors, but a rapid decrease in the levels of FADD occurs via its proteolytic degradation by the proteasome after being labelled with ubiquitin molecules by the E3 ligase Makorin Ring Finger Protein 1 (MKRN1) (Lee *et al*., 2012).

4.4.2. The intrinsic apoptotic pathway

The intrinsic pathway involves the pro and anti-apoptotic Bcl-2 family of proteins, which inhibit or activate the release of cytochrome C from the mitochondria by forming permeability transitioning (PT) pores in the mitochondria (Sattler *et al*., 1997, Cory *et al*., 2003a). The role of the pro and anti-apoptotic Bcl-2 protein family in the present study are discussed further below.

The intrinsic pathway does not seem to play a role in the apoptotic pathways induced following treatment of DLD-1 cells with HNPMPI. This finding is supported by the decreased level of cytochrome C and the decrease in the expression of Bcl-2 family members.

In comparison however, in the HT-29 cells, the intrinsic pathway seems to play a dominant role in apoptotic signalling after HNPMPI treatment. This process is due to the increased levels of cytochrome C and the decreased levels of FADD.

The BCL-2 family of proteins includes the anti-apoptotic proteins such as Bcl-2, Bclx, Bcl-w, Mcl-1 and A1; and the pro-apoptotic proteins, such as Bak, Bax, PUMA, Bok, Bad, Bik, Blk, Hrk, BNIP3 and Bim proteins. These proteins have been shown to regulate apoptosis in response to chemotherapy, both *in vivo* and *in vitro* (Fridman and Lowe, 2003, Adams and Cory, 1998). The mitochondria-mediated apoptosis is indicated by measuring the ratio of Bax and Bcl-2 protein expression where if the ratio is more than 1 this indicates the compound had pro-apoptotic effects (Sun *et al.,* 2014).

In this study, as raised levels of the pro-apoptotic protein, Bax were not observed, it was not possible to determine the Bax/Bcl2 ratio. The anti-apoptotic Bcl-2 was upregulated, and may be responsible for blocking BAX, despite the increased expression of p53, which should lead to the increased expression of BAX (Miyashita and Reed, 1995). BAX activity is also inhibited by the Golgi chaperone CLUSTERIN, which is down regulated in HT-29 cells following HNPMPI treatment. While BAD expression remained the same, increased levels are not required to effect a change in the apoptotic pathway. BAD is normally phosphorylated causing it to homodimerize. However, once dephosphorylated, it forms a heterodimer with the anti-apoptotic proteins Bcl-2 and Bcl XL, leading to BAK/BAX induced apoptosis (Downward, 2004). Therefore, the pro-apoptotic activation of BAD can be accomplished rapidly by conformational changes and does not require an increase in protein expression.

The expression of five members of the inhibitors of apoptosis family of proteins was also evaluated here. These include cIAP-1, cIAP-2, XIAP, Livin and Survivin. Generally, these proteins act by binding to Caspases and ubiquitinylating them, leading to their degradation. In this study, treatment of either cell line with HNPM1 led to the up-regulation of some family members and the downregulation of others. In HT-29 cells, cIAP-2, XIAP and Livin were all upregulated. The CIAP-2 protein only binds weakly to caspases.

The downregulation of XIAP is most likely due to the increased expression of SMAC/DIABLE observed in DLD-1 cells following treatment with HNPMPI. SMAC/DIABLO does not directly target XIAP for degradation, but it does promote its auto-ubiquitination and degradation (Yang and Du, 2004). The pro-apoptotic protein SMAC/DIABLO was also upregulated here,

following exposure of cells to HNPMPI. This protein is activated by cytochrome C and acts by inhibiting the inhibitor of apoptosis (IAP) family of proteins. Although there was upregulation of apoptosis inhibitors such as cIAP-2, HSP70, Livin and HSP60, there was an overexpression of SMAC/Diablo, which most likely inhibited the inhibitors of apoptosis in the HT-29 cells.

4.4.3. The role of p53 in apoptosis

Usually, following exposure of a cell to a variety of stressors, intracellular p53 levels will increase. The usual short half-life of P53 becomes extended by the inhibition of proteins that are normally responsible for its degradation. At the same time conformational change in the p53 protein's N terminal domain activates its transcription factor function (Hafner *et al.,* 2019). Various protein kinases are responsible for this activation, with different kinases activating p53 in response to different stressors. During physiological stress, for example, oxidative stress, osmotic shock, heat shock and membrane damage, the MAPK family of kinases, including JNK1-3 and ERK1-2 are expressed. Other important protein kinases are the genome integrity checkpoint kinases that activate p53 in response to DNA damage. These include ATR, ATM, CHK1, CHK2, DNA-PK, CAK and TP-53RK (Yogosawa and Yoshida, 2018).

4.4.4. RAD17 and Claspin expression is altered by HNPMPI in HT-29 cells

The RAD17 protein is expressed in response to DNA damage and is responsible for cell cycle arrest and DNA damage repair (Bartek and Lukas, 2007). At the same time Claspin is a large scaffolding protein normally found at replication forks, that functions to phosphorylate and activate Chk1 through the signaling by the upstream kinase, ATR, in response to DNA damage.

In the present study, expression levels of RAD17 and Claspin were altered in HT-29 cells following treatment with HNPMPI. Since the levels of RAD17 increased here and together with the increased expression of p53, this implies that the treatment of these cells induced some form of DNA damage.

Claspin is an unstable protein that is degraded during the G2 and M phase of the cell cycle (Smits *et al.,* 2019). During apoptosis, CLASPIN is targeted for degradation by caspase 3 (Azenha *et al.,* 2017). Thus, the observed decrease in Claspin expression after HNPMPI treatment in HT-29 cells suggests that the resultant DNA damage lead to the removal of CLASPIN, preventing the cell from initiating a cell cycle checkpoint and DNA repair, to ensure that the cell undergoes apoptosis.

4.4.5. HNPMPI decreases Cyclin-Dependent Kinase inhibitor, p21 expression

Another protein involved in the regulation of the cell cycle, is the Cyclin-Dependent Kinase inhibitor, p21. This protein normally assists in cell cycle arrest and its transcription is activated by p53. By inducing cell cycle arrest, p21, is a pro-survival gene and acts to modulate or inhibit apoptosis (Karimian *et al.,* 2016).

The decrease observed in p21 expression following drug treatment of HT-29 cells, confirms that the drug induced DNA damage, committing the cells to apoptosis rather than DNA repair. In contrast however, p21 was increased in DLD-1 cells, implying that cell cycle arrest and associated DNA repair were underway.

4.4.6. HNPMPI stimulates the production of ROS in HT-29 and DLD1 cells

HNPMPI treatment of the colon cancer cell lines resulted in the altered expression of proteins linked to increased expression of ROS. This included Hypoxia inducible factor 1α, (HIF-1α), the antioxidant enzyme Paraoxonase 2 (PON2), and heat shock proteins. These changes in protein expression are discussed below.

The transcription factor HIF-1α was up regulated in HT-29 cells after HNPMPI treatment. This protein is important in activating gene transcription in response to hypoxic conditions. Following hypoxic stress HIF-1α induces the expression of p53, p21and Bcl-2. However, HIF-1α transcription and expression is also activated by ROS (Bonello *et al.,* 2007), as is the antioxidant enzyme Paraoxonase 2 (PON2).

PON2 is commonly up regulated in cancer cells where it helps the tumour to resist the effects of chemotherapy drugs designed to kill the cell through enhanced EOS generation (Kruger *et al.,* 2015). This enzyme was also up regulated in DLD-1 cells following treatment with HNPMPI.

Another important category of proteins upregulated by ROS are heat shock proteins (HSP) which help to inhibit the release of cytochrome C, protecting the cell from apoptosis. This is especially true for HSP70 and HSP27 (Slimen *et al.,* 2014).

In this study, after drug treatment, the expression of HSP27 increased in DLD1 cells; while the expression of HSP32, HSP60 and HSP7 increased only in HT-29 cells. This suggests that while the drug treatment generates reactive oxygen species in both cell lines, the effect thereof, or the response in HT-29 cells was more acute than in the DLD 1 cell line.

4.5. Evaluation of gene transcription following HNPMPI treatment

4.5.1. Transcript levels determined by RT-PCR do not always agree with protein expression

Real-time PCR was performed on mRNA extracted from HT-29 and DLD-1 cells before and after treatment with HNPMPI, for key genetic indicators of apoptosis that had demonstrated significant changes in their protein expression levels. The genes selected for analysis here included BAD, BAX, Bcl-2, CASP-3 and p53; and these are discussed below.

4.5.2. BAX and Bcl-2 expression

The changes observed in the expression of the pro-apoptotic BAX protein was matched by a decrease in the transcription levels of bax in both cell lines. In comparison in DLD-1 cells both BAX and Bcl-2 protein levels and associated transcript levels decreased after HNPMPI treatment.

With regards to BAD protein levels, although unchanged in HT-29 cells, transcription of *bad* increased. As noted above BAD activity is controlled through its phosphorylation state, with dephosphorylated BAD being an inducer of apoptosis. BAD regulation is therefore a combination of BAD expression and phosphorylation making its phosphorylation a chemotherapy target (Bui *et al.,* 2018).

It has been reported that transcription level changes leading to changes in protein level would be a poor way of regulating the role of Bcl-2 proteins in apoptosis. Rather the function of these proteins is controlled through changes in protein conformation and the cellular localisation of the proteins depending on their interaction with other Bcl-2 proteins (Edison *et al.,* 2017). Hence, it would seem that HNPMPI possibly increased the expression of Bcl- 2 and decreased that of BAX post-transcriptionally. This regulation could be achieved through the control of protein stability and degradation (Edison *et al.,* 2017), or through the control of translation by non-coding RNA (Liao *et al.,* 2018).

4.5.3. Caspase 3 expression

The expression level of CASP3 and transcription of caspase3 increased in both cell lines following treatment with HNPMPI. The transcription of caspase3 is controlled through the E2F family of transcription factors, which itself may be initiated through growth factors and reactive oxygen species (Ramana *et al.,* 2010).

4.5.4. p53 expression

The protein and transcript levels of p53 in DLD-1 cells both decreased following treatment with HNPMPI. However, in HT-29 cells the protein expression level of P53 increased after HNPMPI treatment, while the transcript level was decreased. One of the mechanisms employed by cells to control the apoptotic function of p53, is the change in its sub-cellular localization, where decreased nuclear localization of p53 is associated with a decreased incidence of apoptosis. The human homologue of MDM2 (Mouse double minute 2 homolog), HDM2 prevents p53 activation by binding to it and exporting it from the nucleus. It then labels the p53 with ubiquitin, leading to its degradation by the proteasome (Hafner *et al.,* 2019, Li *et al.,* 2003). This implies that changes in the protein activity and level of p53 would depend on the extension of its half-life and inhibition of its degradation.

4.6. Changes in cellular localization and expression levels of p53 and Bcl-2

Since one of the most important means of regulating the apoptotic role played by some proteins is due to changes in their subcellular localization, immunofluorescence was used to assess possible changes in the localization of the Bcl-2 and p53 proteins following drug treatment. Also, the fluorescence intensity reflecting decreased or increased cellular protein expression was compared to protein expression levels obtained in the antibody array.

4.6.1. Subcellular localization and expression levels of Bcl-2

In both cell lines, Bcl-2 was localized cytoplasmically around the periphery of the nucleus but with an absence of nuclear staining. Following treatment of HT-29 cells with HNPMPI, the subcellular expression of Bcl-2 increased, reflecting the expression result obtained with the antibody array. Following drug treatment, the localization pattern did not change, with the protein remaining in the cytoplasm with some peri-nuclear staining. This was as expected, as Bcl-2 is normally localized to the membranes of the endoplasmic reticulum, nuclear envelope and the mitochondrial outer membrane. There is no evidence suggesting that the localization of this protein changes following the induction of apoptosis (Schinzel *et al.,* 2004). A similar cytoplasmic localization pattern was observed for Bcl-2 in DLD-1 cells before and after treatment, with little to no nuclear staining. However, after drug treatment, fluorescent staining intensity decreased, confirming the reduced expression of Bcl-2 obtained using the antibody array.

4.6.2. Subcellular localization and expression levels of p53

In both cell lines, p53 was localized in nucleus and in mid stage (HT-29) colon cells the expression was upregulated; while in late stage cells, the expression was downregulated after treatment with HNPMPI. The expression of p53 as detected by immunofluorescence matched the results acquired from the antibody arrays, where in the treated HT-29 cells, p53 expression increased; and in DLD-1 cells there was decreased p53 expression.

In untreated HT-29 cells, some of the cells lacked p53 positivity. In unstressed cells p53 is degraded to prevent its activation. As long as p53 is bound to the HDM2 molecule, it is inactive and sequestered in the cytoplasm (Ostermeyer *et al.,* 1996). However, with HNPMPI treatment, p53 was mostly located in the cytoplasm of HT-29 cells. In association with this, cells showed morphological changes, including reduced cytoplasmic volume and cell shrinkage, suggesting the onset of apoptosis.

4.7. Conclusion

In conclusion, this study provides evidence that the indole derivative, HNPMPI, induces apoptosis in both mid and late stage colon cancer cells, represented by the HT-29 and DLD-1 cell lines, respectively. Interestingly, the intrinsic pathway was activated in HT-29 cells and the extrinsic pathway in the DLD1 cells. In both cell lines, it would appear that cellular damage occurs because of the generation of reactive oxygen species and oxidative stress. In this regard, characterization of oxidative stress markers such as superoxide dismutase (SOD), catalase (CAT) and GSHPx should be carried out to confirm this. The stage specific differing modes of activation of apoptosis could be further investigated via the caspase signalling pathway and by determining the extent of DNA fragmentation in response to drug treatment. An evaluation of the effects of HNPMPI on additional “hallmarks of cancer”, such as proliferation, cell adhesion, cell migration and invasion will optimize the role of this drug as an anticancer treatment.

An improved understanding of the molecular mechanisms of drug induced apoptosis in cancer cells could lead to the development of novel therapeutics in colon cancer. In this regard, HNPMPI has promising pro-apoptotic properties inducing both intrinsic and extrinsic pathways of cell death in colon cancer cell lines (see summary Fig 4.3 below). Its effects on apoptotic regulatory factors and the modulation of apoptosis predispose it as a potential therapeutic for colon cancer.

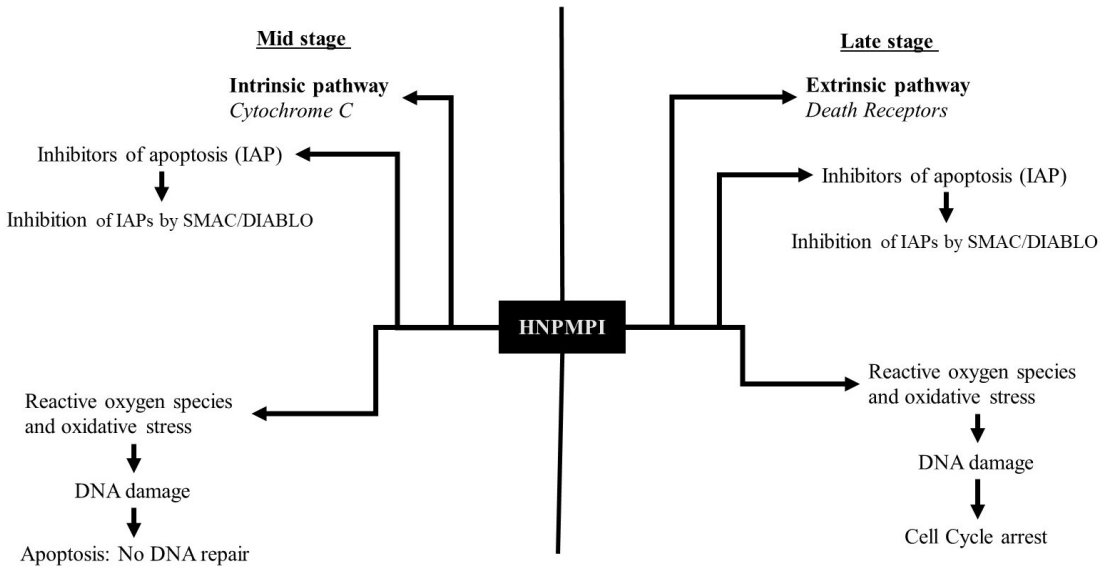


Figure 4.3. Model summarising the pro-apoptotic effects of HNPMPI leading to the induction of the intrinsic apoptotic pathway in mid-stage colon cancer cells; and to the induction of the extrinsic pathway in late stage colon cancer cells.

APPENDIX A: Solutions and Recipes

Solution	Component	Concentration/ volume/amount	Manufactures
Dulbecco's modified eagle medium, nutrient mixture F-12 (10%v/v FBS/DMEM/ F12)	DMEM/F-12		Invitrogen
	foetal bovine serum (FBS)	10%v/v	Invitrogen
	Streptomycin	1X	Lonza BioWhittaker ®
10%v/v FBS/DMEM/ F12	DMEM/F12	50mL	Lonza BioWhittaker ®)
	fetal bovine serum	5mL	GIBCO Invitrogen
	10 000U penicillin/mL 10 000ug streptomycin/mL	100µL	Lonza BioWhittaker ®
50:50 Cryoprotective medium	DMSO	50%v/v	
	FBS/DMEM/F-12	50%v/v	
Indoline derivative	Indoline derivative	30 µM	
	DMSO	1%	
10X TBE Prepared to 1L pH 8.3	80g Tris	8%	Saarchem
	55g Boric acid	5.5%	Sigma-Aldrichig
	9.3g EDTA	0.93%	Merck
1X TBE	10X TBE	100mL	
	dH_2O	900mL	

1.5%w/v Agarose gel	Agarose powder	1.5g	Bio-Rad
	1X TBE	100mL	
	Ethidium bromide	6.5µL	Promega
Phosphate buffered saline	1X PBS tablet		Sigma-Aldrich
	dH2O	200mL	
	Formaldehyde	3% v/v	Univar
0.5% v/v BSA/PBS	bovine serum albumin	0.5g	Sigma-Aldrich
	PBS	100mL	Sigma-Aldrich
0.25%v/v BSA/PBS/Triton X	0.5%v/v BSA/PBS	50mL	Sigma-Aldrich
	Triton X	125µL	Lonza)
Primary antibody 1:200 Rabbit polyclonal IgG 200µg/mL	primary antibody	1µL	
	0.5%v/v BSA/PBS	199µL	Santa Cruz Cat. no. sc-20691
Secondary antibody 1:200	secondary antibody	1µL	Santa-Cruz
	0.5%v/v BSA/PBS	199µL	
Alexa Fluor® 594 conjugated donkey anti rabbit		0.5mL	Life Technologies
Alexa Fluor® 488 conjugated donkey anti mouse		0.5mL	Life Technologies
DAPI 1:10 000	DAPI	1µL	Boehringer Mannheim
	PBS	9 999µL/~10mL	

Microscope and software information

Software: analySIS FIVE, Olympus Soft Imaging Systems, Germany

Zeiss Laser Scanning Microscope, LSM 780, ZEN Software (2011)

Appendix B: Indoline derivative

Chemical information

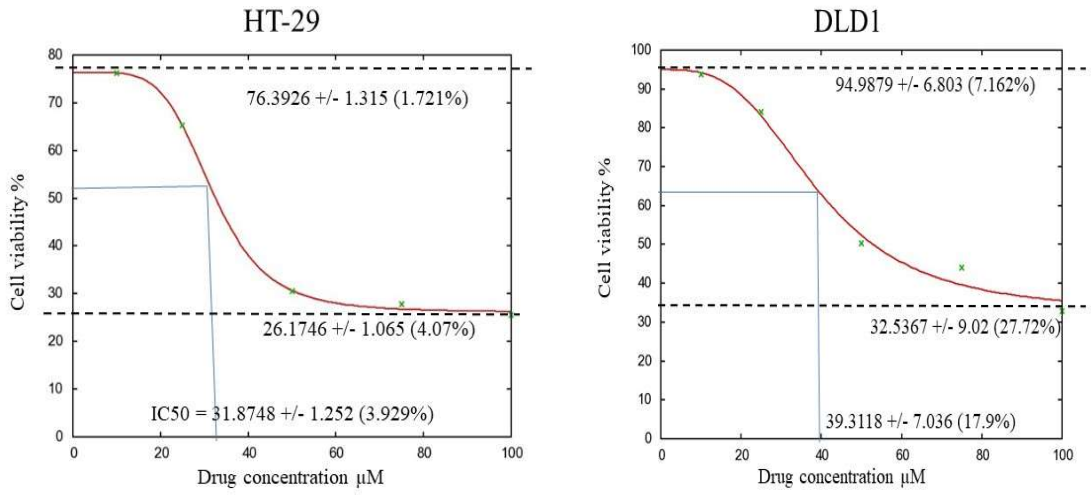
Empirical formula: C22H20N2O3

Molecular weight: 360,413

Solubility: DMSO – 16mg/mL

Determination of the IC_{50} of HNPMPI

Trypan blue assays were performed on HT- 29 and DLD-1 cells lines. The cells were treated with five different concentrations of the drug (10, 25, 50, 75 and 100 µM). The percentage of viable cells were plotted against drug concentration. The IC_{50} of the HNPMPI was determined as 39.3118 µM ± 7.036 for DLD-1 and 31.8748 µM ± 1.252 for HT-29 cells, respectively. This shows that the late stage of the colon cancer cell lines needs a higher concentration of the drug when we compared with mid stages of the colon cancer cell line to induce apoptosis. From this, 30 µM of HNPMPI was chosen for further experimental purpose for both cell lines in this study.

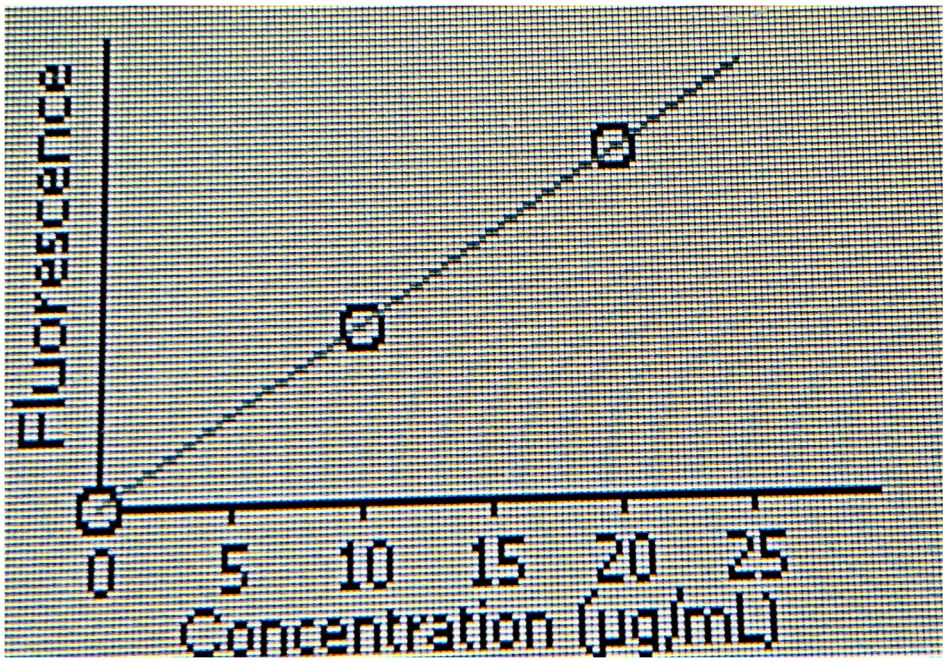


Determination of IC_{50}: A Trypan Blue assay was performed with a range of concentrations of HNPMPI. From the plots of cell viability against drug concentration depicted above, it can be

determined that the IC_{50} of the HNPMPI is 39.3118 ± 7.036 µM (17.9%) and 31.8748 ± 1.252 µM (3.929%) at 24 hrs in both the DLD-1 and HT-29 cell lines, respectively

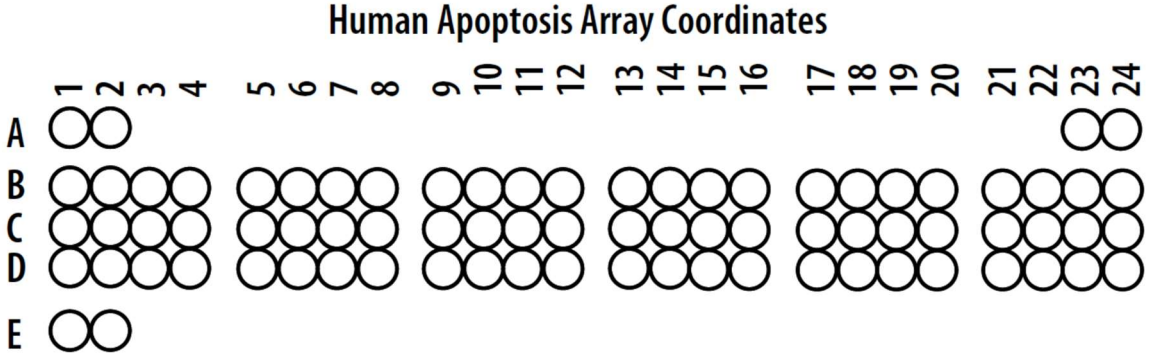
Appendix C: Human apoptosis protein profiler Array

Protein concentration standard curve



Standard curve for Qubit 2.0 to determine protein concentration

Protein profiler array co-ordinates and list of target proteins



Template showing the positioning of the spots in the apoptosis proteome array to coordinate the genes.

The table below for the Human Apoptosis Array coordinates

Coordinate	Target/Control	Coodinate	Target/Control
A1, A2	Reference Spots	C13, C14	HO-2/HMOX2
A23, A24	Reference Spots	C15, C16	HSP27
B1, B2	Bad	C17, C18	HSP60
B3, B4	Bax	C19, C20	HSP70
B5, B6	Bcl-2	C21, C22	HTRA2/Omi
B7, B8	Bcl-x	C23, C24	Livin
B9, B10	Pro-Caspase-3	D1, D2	PON2
B11, B12	Cleaved Caspase-3	D3, D4	p21/CIP1/CDKN1A
B13, B14	Catalase	D5, D6	p27/Kip1
B15, B16	cIAP-1	D7, D8	Phospho-p53 (S15)
B17, B18	cIAP-2	D9, D10	Phospho-p53 (S46)
B19, B20	Claspin	D11, D12	Phospho-p53 (S392)
B21, B22	Clusterin	D13, D14	Phospho-Rad17 (S635)
B23, B24	Cytochrome c	D15, D16	SMAC/Diablo
C1, C2	TRAIL R1/DR4	D17, D18	Survivin
C3, C4	TRAIL R2/DR5	D19, D20	TNF RI/TNFRSF1A
C5, C6	FADD	D21, D22	XIAP
C7, C8	Fas/TNFRSF6/CD95	D23, D24	PBS (Negative Control)
C9, C10	HIF-1α	E1, E2	Reference Spots
C11, C12	HO-1/HMOX1/HSP32		

Appendix D: Gene and primer sequences.

GAPDH

Homosapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH), transcript variant 1, mRNA

NCBI Reference Sequence: NM_002046.7

NM_002046.7 *Homo sapiens* glyceraldehyde-3-phosphate dehydrogenase (GAPDH), transcript variant 1, mRNA

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TCCTCACAGTTGCCATGTAGACCCCTTGAAGAGGGGAGGGGCCTAGGGAGCCGCACCTTGTCATGT
ACCATCAATAAAGTACCCTGTGCTCAACCA

GAPDH (Control)	F: 5’-TGCMTCCTGCACCACCAACT-3’ R: 3’-YGCCTGCTTCACCACCTTC-5’	TM 53.25 52.6

BAD

Homo sapiens BCL2 associated agonist of cell death (BAD), transcript variant 1, mRNA

NCBI Reference Sequence: NM_004322.3

>NM_004322.3 Homo sapiens BCL2 associated agonist of cell death (BAD), transcript variant 1, mRNA

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CCGGGACCCGAGCCCCCGCGATGCCCTGGGGGCCGTGCTCACTACCAAATGTTAA
TAAAGCCCGCGTCTGTGCCGCCGAAAAAAAAAAAAAAAAA

BAD	F: 5'-ATCTCAGCTCCTCTAAGCCCC-3' R: 3'-CCCTGAGCTTCCCCTCGATT-5'	TM 56.31 55.88

Bax

Homo sapiens chromosome 19, GRCh38.p12 Primary Assembly

NCBI Reference Sequence: NC_000019.10

>NC_000019.10:48954825-48961798 Homo sapiens chromosome 19, GRCh38.p12 Primary Assembly

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	R: 3’-GGAGACAGGGACATCAGTCG-5’	55.88

Bcl-2

Homo sapiens BCL2, apoptosis regulator (BCL2), transcript variant alpha, mRNA

NCBI Reference Sequence: NM_000633.2

>NM_000633.2 Homo sapiens BCL2, apoptosis regulator (BCL2), transcript variant alpha, mRNA

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BCL2	F: 5'-CCTGTGGATGACTGAGTACC-3' R: 3'-GAGACAGCCAGGAGAAATCA-5'	TM /53.83 51.78

Caspase 3

Homo sapiens caspase 3 (CASP3), transcript variant 1, mRNA

NCBI Reference Sequence: NM_004346.4

>NM_004346.4 Homo sapiens caspase 3 (CASP3), transcript variant 1, mRNA

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CASP3	F: 5’-GCTCGCTAACTCCTCACGG-3’ R: 3’-TCCAATTCCTTTTCGGCCCTG-5’	TM 55.41 54.36

P53

Homo sapiens chromosome 17, GRCh38.p12 Primary Assembly

NCBI Reference Sequence: NC_000017.11

>NC_000017.11:c7687550-7668402 Homo sapiens chromosome 17, GRCh38.p12 Primary Assembly

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AGTTAAGGGTTAGTTTACAATCAGCCACATTCTAGGTAGGGGCCCACTTCACCGT
ACTAACCAGGGAAGCTGTCCCTCACTGTTGAATTTTCTCTAACTTCAAGGCCCATA
TCTGTGAAATGCTGGCATTTGCACCTACCTCACAGAGTGCATTGTGAGGGTTAAT
GAAATAATGTACATCTGGCCTTGAAACCACCTTTTATTACATGGGGTCTAGAACT
TGACCCCCTTGAGGGTGCTTGTTCCCTCTCCCTGTTGGTCGGTGGGTTGGTAGTTT
CTACAGTTGGGCAGCTGGTTAGGTAGAGGGAGTTGTCAAGTCTCTGCTGGCCCAG
CCAAACCCTGTCTGACAACCTCTTGGTGAACCTTAGTACCTAAAAGGAAATCTCA
CCCCATCCCACACCCTGGAGGATTTCATCTCTTGTATATGATGATCTGGATCCACC
AAGACTTGTTTTATGCTCAGGGTCAATTTCTTTTTTCTTTTTTTTTTTTTTTTCTT

TTTCTTTGAGACTGGGTCTCGCTTTGTTGCCCAGGCTGGAGTGGAGTGGCGTGATC
TTGGCTTACTGCAGCCTTTGCCTCCCCGGCTCGAGCAGTCCTGCCTCAGCCTCCGG
AGTAGCTGGGACCACAGGTTCATGCCACCATGGCCAGCCAACTTTTGCATGTTTT
GTAGAGATGGGGTCTCACAGTGTTGCCCAGGCTGGTCTCAAACTCCTGGGCTCAG
GCGATCCACCTGTCTCAGCCTCCCAGAGTGCTGGGATTACAATTGTGAGCCACCA
CGTCCAGCTGGAAGGGTCAACATCTTTTACATTCTGCAAGCACATCTGCATTTTCA
CCCCACCCTTCCCCTCCTTCTCCCTTTTTATATCCCATTTTTATATCGATCTCTTATT
TTACAATAAAACTTTGCTGCCACCTGTGTGTCTGAGGGGTG

P53	F: 5’- GCTCGACGCTAGGATCTGAC -3’ R: 3’- CCCAGGGTTGGAAGTGTCTC -5’	TM 60 59.90

APPENDIX E: Ethical Clearance

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

22/06/2018

Ref: W-CBP-180622-01

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: Ms V Naidoo
Student No. (if appropriate): 1353730
Staff No. (if appropriate):

Supervisor: Professor CB Penny

School: Clinical Medicine
Department: Medicine
Internal Medicine Division
Oncology Division
Medical School

Project title: *Effect of indoline derivative on different stages of colon cancer cell lines*

Reason: In vitro study.
No human participants will be involved in the study.

Dr N Naran
Chairperson: Human Research Ethics Committee (Medical)

Copy – HREC (Medical) Secretariat: Zanele Ndlovu and Rhulani Mkansi.

Research Office Secretariat:
Physical address: Phillip Tobias Building, 3rd Floor, Office 302, Corner York Road and Princess of Wales Terrace, Parktown, Johannesburg 2193.
Postal address: Private Bag 3, Wits 2050
Tel Nos. +27 (0)11-717-1234/2656/2700/1252
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