The Effect of Novel Compounds on Cell Survival And Apoptosis in Colon Cancer Cell Lines

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Declaration

I declare that the work presented in this Dissertation is, to the best of my knowledge and belief, original, except as acknowledged in the text, and that the material has not been submitted for a degree at this or any other university. It is being submitted for the degree of Master of Science in Medicine (Pharmacology) at the University of the Witwatersrand, Johannesburg.

I acknowledge that I have read and understood the University's rules, requirements, procedures and policy relating to my Dissertation. I certify that I have complied with the rules, requirements, procedures and policy of the University.

Signature:……

On this……day of……2013

Dedication

I dedicate this thesis to the following people:

- My supervisor, Dr Leonie Harmse, who has provided me with support and guidance, at every step of the way in this study. If it were not for her understanding, commitment, motivation and patience, the completion of this study would have been a distant dream. Thank you for throwing the rock that helped me break through.

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- My son, Coby Shimon of whom I love so dearly. Thank you for lightening up my spirit every second of being with you.

- Lastly, I dedicate this thesis to my husband Justin:

"The best scientist is open to experience and begins with romance - the idea that anything is possible" - Ray Bradbury

To my parents, my sister, my son and my husband, I would not be where I am if it were not for your pride in me, love and understanding.

Abstract

Colon cancer is the third most common cancer worldwide and the second most common in the western world. More than 40 % of colon cancer sufferers develop metastases and chemotherapy is often used alone or in combination with radiotherapy as adjunctive therapy for the advanced disease. A major effort has been made in the past decade to develop anti-cancer agents through both empiric screening and rational design of new compounds. These attempts are made to improve the survival rate, reduce the severe adverse effects associated with existing cancer chemotherapeutic agents as well as to reduce the development of drug resistance. In the present study, two colon cancer cell lines were exposed to novel imidazo[1,2-a]pyridines and novel nucleoside analogues, aiming to investigate the cytotoxic efficacy on the cells, the mode of cell death, and to explore the pathways by which cell death was induced.

All compounds were synthesised using standard organic chemistry techniques. The *in vitro* cytotoxic effects of the novel compounds were tested against the HT-29 and Caco-2 cell lines as well as unstimulated white blood cells. The cytotoxic effects of the two classes of compounds, was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. A colorimetric assay was used to determine caspase-3 and caspase-8 activity. Changes to the permeability of the mitochondrial membrane were detected by a JC-1 lipophilic fluorochrome and cytochrome c levels were determined by an Elisa assay. Additionally, the percentages of apoptosis were determined using the Annexin V-FITC apoptosis detection kit. The alkaline phosphatase assay was performed to determine if any of the novel compounds induced cell differentiation in the HT-29 cells.

The compounds showed cytotoxicity towards both HT-29 and Caco-2 cell lines with the cytotoxic effects of the imidazo[1,2-a]pyridines being comparable to camptothecin. The proteolytic phase of apoptosis was initiated within 2 hours after treatment with the test compounds as observed by the sequential activation of caspase-8 and caspase-3 activity. This suggests the involvement of the extrinsic death receptor pathway. These compounds caused a marked reduction in the mitochondrial membrane potential ($\Delta\psi m$) as well as a translocation of cytochrome c from the mitochondria to the cytosol after a 24 hour treatment. This together with the results from the MTT assay supports the involvement of a mitochondrion-dependent apoptotic pathway. The annexin-V binding assay demonstrated that phosphatidylserine was

translocated to the outer leaflet of the plasma membrane of the treated HT-29 and Caco-2 cells which provides further evidence that the compounds induce apoptosis.

In contrast to the imidazo[1,2-a]pyridine compounds, the mitochondrial membrane potential was unaffected by the JLP nucleoside analogues. Contrary to expectations, the nucleoside analogues caused an increase in the release of cytochrome c from the mitochondria and an increase in caspase-8 and caspase-3 activity. There are other mechanisms by which cytochrome c can translocate from the mitochondria to the cytosol such as through a protein permeable pore or channel. The JLP nucleoside analogues were more effective than the CDK and CL imidazo[1,2-a]pyridine compounds in inducing intestinal alkaline phosphatase activity with JLP 26.5 showing more alkaline phosphatase activity than acetoacetate. It is thus likely that JLP 26.5 has an effect on the HT-29 colon cancer cell differentiation and should be investigated further.

In summary, the novel compounds show cytotoxic effects comparable to camptothecin on the two colon cancer cells lines. Both groups were effective inducers of apoptosis and therefore worthy of further investigation which can include other cancer cell lines as well as a study of animal models. They can also serve as lead compounds for the development of more potent compounds.

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Research Outputs

Conference Proceedings

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Dahan NE, Panayides JL, van Otterlo WAL, de Koning CB, Harmse L and Davids H Novel nucleoside analogues in the induction of colonic cell differentiation and apoptosis. 2008. Poster presentation at the School of Therapeutic Sciences Research day, 20 August 2008, University of the Witwatersrand, Medical School.

Dahan NE, Panayides JL, van Otterlo WAL, de Koning CB, Harmse L and Davids H Novel nucleoside analogues in the induction of colonic cell differentiation and apoptosis. 2008. Poster presentation at the South African Pharmacology and Neurosciences Congress 5th – 8th October 2008 held at Rhodes, University of Grahamstown, North-West Province, South Africa

Dahan NE, Panayides JL, van Otterlo WAL, de Koning CB, Harmse L and Davids H Novel nucleoside analogues in the induction of apoptosis in colonic cancer cells. 2009. Oral presentation at the School of Therapeutic Sciences Research day, 12 August 2009, University of the Witwatersrand, Medical School.

Dahan NE, Panayides JL, van Otterlo WAL, de Koning CB, Harmse L and Davids H Novel nucleoside analogues in the induction of apoptosis in colonic cancer cells. 2009. Oral presentation at the Annual Congress of Pharmaceutical and Pharmacological Sciences (5th ICPPS), 23-23 September 2009 held at Potchefstroom Campus, North-West University.

Dahan NE, Langley C, Fernandes MA, Pelly SC, de Koning CB, Harmse L and Davids H Novel imidazo[1,2-a]pyridines in the induction of colonic cell differentiation and apoptosis 2010. Poster presentation at the 16th World Congress of Basic and Clinical Pharmacology, WorldPharma2010 17-23 July 2010 held at Copenhagen, Denmark.

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Publications

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Patents

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Abbreviations, Acronyms and Symbols

AIF	-	Apoptosis-inducing factor
Apaf-1	-	Apoptosis protease activating factor
APC	-	Adenomatous polyposis coli
ATP	-	Adenosine triphosphate
ATRA	-	All-trans retinoic acid
Bcl-2	-	B-cell lymphoma-2
Bcl-XL	-	B-cell lymphoma-extra large
BH3	-	B-cell lymphoma-2 homology domain 3
BSA	-	Bovine serum albumin
CHAPS	-	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CO_2	-	Carbon dioxide
COX	-	Cyclooxygenase
COX-2	-	Cyclooxygenase 2
CT	-	Computed Tomography
Cyt c	-	Cytochrome c
dATP	-	Deoxyadenosine triphosphate
DD	-	Death domain
DFF45	-	DNA fragmentation factor 45 kDa subunit
DISC	-	Death-inducing signalling complex
DMEM	-	Dulbecco's Modified Eagle's Medium
DMSO	-	Dimethyl sulphoxide
DNA	-	Deoxyribonucleic Acid
DTT	-	Dithiothreitol
EDTA	-	Ethylene diamine tetra-acetic acid
EGTA	-	Ethylene glycol tetra-acetic acid
EGFR	-	Epidermal growth factor receptor
FACS	-	Fluorescence activated cell sorter
FADD	-	Fas associated death domain
FBS	-	Foetal bovine Serum
FDA	-	Food and Drug Administration
FGF	-	Fibroblast growth factor

FITC	-	Fluorescein isothiocyanate
5-FU	-	5-flourouracil -
h	-	Hour(s)
HEPES	-	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
IAP	-	Inhibitor-of-apoptosis
ICAD	-	Inhibitor of caspase-activated DNase
IC_{50}	-	50 % inhibitory concentration
IκB	-	Inhibitor of Kappa-B
IL-1β	-	Interleukin-1 beta
LV	-	Leucovorin
M	-	Molar
mA	-	Milliampere
mdm2	-	Mouse double minute-2
mM	-	Millimolar
µg/ml	-	Micrograms per millilitre
µl	-	Microliter
ml	-	Milliliter
µM	-	Micromolar
MPTP	-	Mitochondrial permeability transition pore
MRI	-	Magnetic resonance imaging
MTT	-	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
O_2	-	Oxygen
OD	-	Optical Density
PARP	-	Poly(ADP-ribose) polymerase
PBS	-	Phosphate Buffered Saline
PDGF	-	Platelet-derived growth factor
PPAR	-	Peroxisome proliferators-activated receptors
PUMA	-	p53 upregulated modulator of apoptosis
PI	-	propidium iodide
pNa	-	p-nitroabilide
PS	-	Phosphatidylserine
RBC	-	Red blood cell
RIP	-	Receptor interacting protein

ROS	-	Reactive oxygen species
rpm	-	Rotations per minute
SDS	-	Sodium dodecyl sulfate
SEM	-	Standard error of the mean
SMAC	-	Second mitochondria-derived activator of caspases
tBid	-	Truncated Bid
TNF-α	-	Tumour necrosis factor-alpha
TNFR1	-	Tumor necrosis factor receptor
TRADD	-	TNFR1 associated death domain
TRAIL	-	Tumor necrosis factor–related apoptosis-inducing ligand
Δψm	-	Mitochondrial membrane potential
mU/ml	-	milliunits per milliliter
v/v	-	Volume per volume
w/v	-	Weight per volume
WBC	-	White blood cell

I. Introduction

1. Cancer

Each year, more than six million people succumb to cancer. By 2030 worldwide, the incidence of cancer is estimated to increase to around 12 million cases. On a global scale, cancer causes more deaths than HIV, tuberculosis and malaria, grouped together. In 2008, cancer accounted for approximately 7.6 million deaths worldwide. Lung, stomach, liver, colon and breast cancer are the most common causes of cancer-related deaths per year (Ferlay *et al*., 2010).

Cancer is a pathological condition whereby cells proliferate uncontrollably and ultimately result in the destruction of neighbouring normal cells. There are many different types of cancers, classified according to the tissue and the cell type from which they originate. The incidence and behaviour of certain types of cancer are related to various factors such as gender, age, race, genetic predisposition and exposure to environmental carcinogens (Parkin *et al*., 2005).

Table 1 The most prominent sites of cancer and cancer deaths worldwide in 2008 (Jemal *et al*., 2011).

Type of Cancer	Estimated deaths per year
Lung	**1 378 400**
Stomach	**737 600**
Liver	**695 900**
Colorectal	**608 700**
Breast	**458 400**

1.1 Types of Cancer

Cancers are classified into four major groups namely carcinomas, sarcomas, leukaemias and lymphomas. Carcinomas are solid tumours that arise from epithelial cells and are the most common human cancers. These are often found in the skin, the cells lining the internal organs (such as in the lungs and small intestine), and in glandular tissue (such as in the breast or prostate). Sarcomas are fibrous tumours that

arise from the connective tissue of muscle and bone. Leukaemias and lymphomas arise from cells in the haematopoeitic system (Alberts *et al*., 2008. pp 1206).

1.2 Characteristics of cancer cells

Cancer cells display a characteristic set of features, such as a disorganised cytoskeleton and a relatively large nucleus when compared to normal cells (Hanahan and Weinberg., 2000). Cancer cells differ from that of normal cells in that they display uncontrolled proliferation, defective differentiation, invasiveness and metastasis, which are not observed in normal cells.

Throughout the process of differentiation and the growth of tissues and organs, normal cells develop specific spatial relationships with one another. In contrast, cancer cells do not remain confined within a particular area; the cells can invade surrounding tissues. The defective differentiation of cancer cells is related to the uncontrolled proliferation and the failure of cancer cells to undergo apoptosis. Metastasis is a process that occurs when a tumour cell leaves the primary tumour, travels to a distant site via the circulatory system and establishes a secondary tumour. Cancer cells also show other characteristics like immortality, decreased dependence on growth factors to support proliferation, loss of anchorage-dependence for growth, loss of cell cycle control, failure to enter apoptosis, and increased genetic instability (Hanahan and Weinberg., 2000).

1.3 Causes of Cancer

Cancers arise as a result of accumulated gene mutations in the somatic cells. In a normal cell cycle, the action of growth-promoting oncogenes is balanced by the action of growth-inhibiting tumour suppressor genes. The growth of tumour cells are promoted by functional mutations of proto-oncogenes which result in oncogene formation, as well as by genetic mutations which cause tumour suppressor proteins to be dysfunctional. Both types of mutations are necessary for a cell to become malignant (Goyns and Hancock., 1991; Sherr., 2004).

The telomerase enzyme is also involved in the development of cancer (Sykorova and Fajkus., 2009). Telomerase is a ribonucleoprotein polymerase that attaches DNA sequence repeats (telomeric sequences - TTAGGG) to the 3′ ends of DNA strands. This occurs in the telomere region, found at the end of the chromosomes. The telomere region is a characteristic DNA sequence that prevents shortening of the chromosomal DNA with each round of replication. In senescent non-dividing cells, telomerase activity is inhibited. However, cancer cells express functional telomerase (Alberts *et al*., 2008 pp 294).

1.3.1 Oncogenes

Proto-oncogenes encode proteins that control cell proliferation. The mutated version of a proto-oncogene is called an oncogene. A proto-oncogene can be converted into an oncogene by a deletion or point mutation in the coding sequence, gene amplification or chromosomal rearrangement.

Proto-oncogenes are essential for normal human development as well as for the maintenance of organs and tissues. Oncogenes display an increased production of cell cycle regulatory proteins, leading to an increase in cell division, decreased cell differentiation and a reduction of cell death, all which are the common features of cancer cells. Examples of oncogenes include i) *sis* – the gene that codes for platelet-derived growth factor (PDGF), ii) *EGFR* - the gene that codes for the epidermal growth factor receptor **(**EGFR) family , iii) *int-2* - the gene that encodes the fibroblast growth factor (FGF) growth factor family (Wilkinson 1988, Croce and Carlo., 2008).

1.3.2 p53 Tumor suppressor gene

Tumor suppressor genes are essential in the development and maintenance of organs as they prevent uncontrolled cell division. More often than not a mutation in a tumor suppressor gene is a deletion which results in a defective protein (Park and Vogelstein., 2003). The most prominent tumour suppressor is the product of the p53 tumour suppressor gene (Levine and Oren., 2009). The product of the p53 gene is a protein of 53 kilodaltons and plays an essential role in control of the cell cycle and in apoptosis. Under normal conditions, a small amount of p53 is found in the majority of all cells. When DNA damage occurs in the specific cell or if the cell has been

impaired in other ways, p53 halts the cell cycle. If there is minimal damage, p53 halts cell division until the damage is repaired, whereas if there is major damage that cannot be repaired, p53 triggers the cell to self-destruct by apoptosis. The p53 gene is not functional or functions incorrectly in many human cancers (Brosh and Rotter., 2009).

There are several ways whereby p53 function can be altered in human cancers. p53 can be inactivated indirectly through binding to viral proteins, due to alterations in the mouse double minute-2, (mdm2) protein (a negative regulator of the p53 tumor suppressor) or by localization of the p53 protein to the cytoplasm. Several *in vitro* experiments have shown that apoptosis in colon cancer cell lines is induced by the expression of wild type (normal) p53 (Geske *et al*., 2000).

1.4 Terminal differentiation and cancer

Cells that have permanently lost the ability to divide during the differentiation process, are described as being terminally differentiated. In general, differentiation of a cell greatly reduces its replication potential. Since cancer cells are generally less differentiated than normal cells, increasing or restoring differentiation in cancer cells may be one method of reducing or removing their replicative or invasive potential (Gagna *et al*., 2001). Thus, the search for compounds that cause a permanent loss of proliferative activity or induce differentiation may lead to the design of new therapeutic strategies aimed at targeting the differentiation of cancer cells. One such compound all-*trans* retinoic acid (ATRA), a derivative of vitamin A, has been shown to promote differentiation and decreases the proliferation of leukemic promyelocytes, thus allowing for growth of normal haematopoietic cells (Huang *et al*., 1988; Degos and Wang., 2001).

Cellular alkaline phosphatase is increasingly recognized as an important marker of differentiation in human malignancies (Dabare *et al*., 1999). Alkaline phosphatase (ALP) is mainly derived from the liver, bones and in smaller amounts from the intestines, placenta, kidneys and leukocytes (Maldonado *et al*., 1998; Saif *et al*., 2005). Alkaline phosphatase is used as a biochemical marker for gastrointestinal differentiation (Pinto *et al*., 1982).

1.5 Cancer therapeutics

In general, solid tumours are treated by surgical removal, radiation therapy and chemotherapy. Non-solid tumours are not amenable to surgery and are usually treated with chemotherapy and radiation. Clinicians tend to measure five year survival rates when assessing the outcomes of many cancers. The chance of cancer survival improves with early diagnosis. Prostate, thyroid and testicular cancer have the highest 5 year survival rate whereas lung, pancreatic and liver cancer have a low 5 year survival rate (Parkin *et al*., 2002).

1.6 Prevalence and morbidity of colorectal cancer

Worldwide, over 1.2 million new colorectal cancer cases are diagnosed annually (Ferlay *et al*., 2010). Colorectal cancer is the second most common cancer in industrialised countries and was responsible for approximately 608 700 deaths in 2008.

After breast cancer, colon cancer is the second most prevalent cancer, with around 1 233 700 million people affected worldwide (Jemal *et al*., 2011). There are a number of factors that influence the risk of developing colon cancer, such as age, diet and a genetic predisposition (Kovvali *et al*., 2003). The rates of incidence are highest in North America, Australia / New Zealand. The countries with a high incidence of colon cancer correlate with high red meat consumption. The incidence rates are much lower in Africa and Asia and intermediate in southern parts of South America (Figure 1.1).

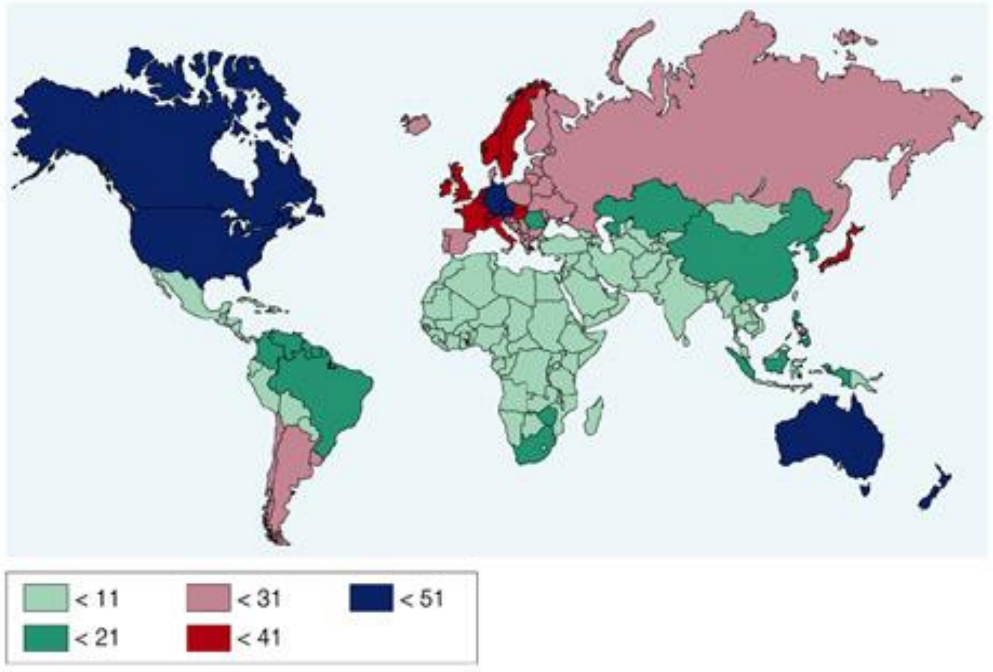


Figure 1.1 Incidence of colon cancer around the world
Countries with a high incidence of colon cancer (cases per 100,000 people) are indicated in blue (North America, Australia, and New Zealand); countries with moderate levels are indicated in pink or red; and countries with low incidence in green (Asia, Africa). (Bingham & Riboli., 2004).

2. Colon cancer pathology

The majority of colon cancers develop from adenomatous polyps. Adenomatous polyps arise from dysplastic aberrant crypt foci, also known as adenomatous crypts or microadenoma. The polyps are usually benign and approximately 5% become malignant over a period of 10 years. Mutations of the adenomatous polyposis coli (APC) tumour suppressor gene, the *K-ras* proto-oncogene and the p53 gene have been observed to lead to the formation of polyps and the subsequent development of cancer in the large intestine (Bendardaf., 2006).

2.1 Risk factors

There are various risk factors for the development of colon cancer. They include age, diet, or a family history of colon or rectum cancer, history of ulcers lining the large intestine and several hereditary diseases like Lynch Syndrome and familial adenomatous polyposis. Although over 90 % of all colon cancer patients are above the age of fifty, it has been observed in younger populations. The risk increases in people

who have had a history of chronic bowel inflammation or a previous adenomatous polyp (Boyle and Leon., 2002).

Many studies have shown that a diet high in fat, refined carbohydrates, red meat, and an inactive lifestyle increases the chances of developing colorectal cancer whereas dietary fats such as fish oils and dietary fibre protects against colon tumour formation (Norat and Riboli., 2003, Chao *et al*., 2005, Willett., 2001). Heavy consumption of alcohol as well as a low intake of folate and methionine, has been shown to increase the risk of developing colon cancer as well as other cancers (Pietinen., 2004).

Several epidemiological and experimental studies have shown that an increased intake of calcium has a protective function and may reduce the risk of developing colon cancer. Supplementation with calcium causes decreased proliferation of colonic epithelial cells and influences cellular processes such as differentiation and apoptosis by acting directly through the calcium sensing receptor (Chakrabarty *et al*., 2003). Calcium may decrease the risk of colonic tumors by binding bile and fatty acids in the bowel and hence, reduces the exposure of the colonic epithelium to compounds which may possibly be carcinogenic (Peters *et al*., 2004). Cyclo-oxygenase inhibitors have been shown to prevent the formation of colon cancer (Cao and Prescott., 2002).

2.1.1 Bacterial Carcinogenesis

The specific geographical location and spread of colon cancer suggests that environmental factors may play a role in the resistance of certain populations to colon cancer. There is a connection between the incidence of colon cancer and a specific strain of *Escherichia coli* (Pitari *et al*., 2003). An infection of *Helicobacter pylori* may also induce cancer through an inflammatory mechanism (Zumkeller *et al*., 2007).

2.1.2 APC gene mutation

The proapoptotic adenomatous polyposis coli (APC) gene is referred to as a gatekeeper gene (gene products that prevent growth of potential cancer cells) and when mutated it shifts the balance in favour of excess cell generation as opposed to cell death. The commencing stage for the induction of colorectal polyps is often due to an inherited mutation in the APC gene. The protein that is produced by the APC

gene plays an important role in several cellular processes that determines whether a cell will develop into a tumor. Irregularities in APC-dependent signaling are found in approximately 90 % of sporadic colorectal adenomas (Iwamoto *et al*., 2000). A mutation in the APC gene has also been shown to up-regulate the expression of several genes such as the *c-myc* oncogenes and its antagonist *Mad*-1 (He *et al*., 1998).

The *C-myc* gene product has an important role in regulating the transcription of ornithine decarboxylase, the first enzyme in polyamine synthesis. The wild type APC has been observed to decrease the expression of *c-myc.* As a result, there is a decrease in the expression of ornithine decarboxylase which corresponds to reduced colon tumorigenesis. Mutant APC has been observed to increase the activity of ornithine decarboxylase which resulted in more polyamines and an increase in colon tumorigenesis (Martinez *et al*., 2003).

2.2 Symptoms of colon cancer

The most common symptoms of colorectal cancer are changes in bowel habits and bleeding. The signs and symptoms vary, depending on the location of the lesion. If the tumor is right sided, the most common symptoms are anaemia, abdominal pain, weight loss or changes in bowel habits, whereas left sided tumors present most commonly with a change in bowel habits, rectal blood loss and abdominal pain. Changes in bowel habits may include a change in the frequency or consistency of the stool, diarrhoea, constipation, a feeling of fullness after having a bowel movement and narrowing of the stools. If the tumour is located on the rectum, there may be fresh red blood on the stool surface whereas haemorrhagic proximal lesions result in dark stools (Hamilton and Sharp., 2004).

2.3 Diagnosis

In colorectal cancer, screening tests are done to search for pre-malignant, benign polyps. The screening procedure involves a physical examination, laboratory tests and visualizing the lining of the colon (Wils., 2007).

The physical investigation begins with a rectum examination, rectoscopy and checking for blood in the faeces. A radiological investigation is performed often with the use of an air contrast barium enema to detect cancerous or pre-cancerous polyps.

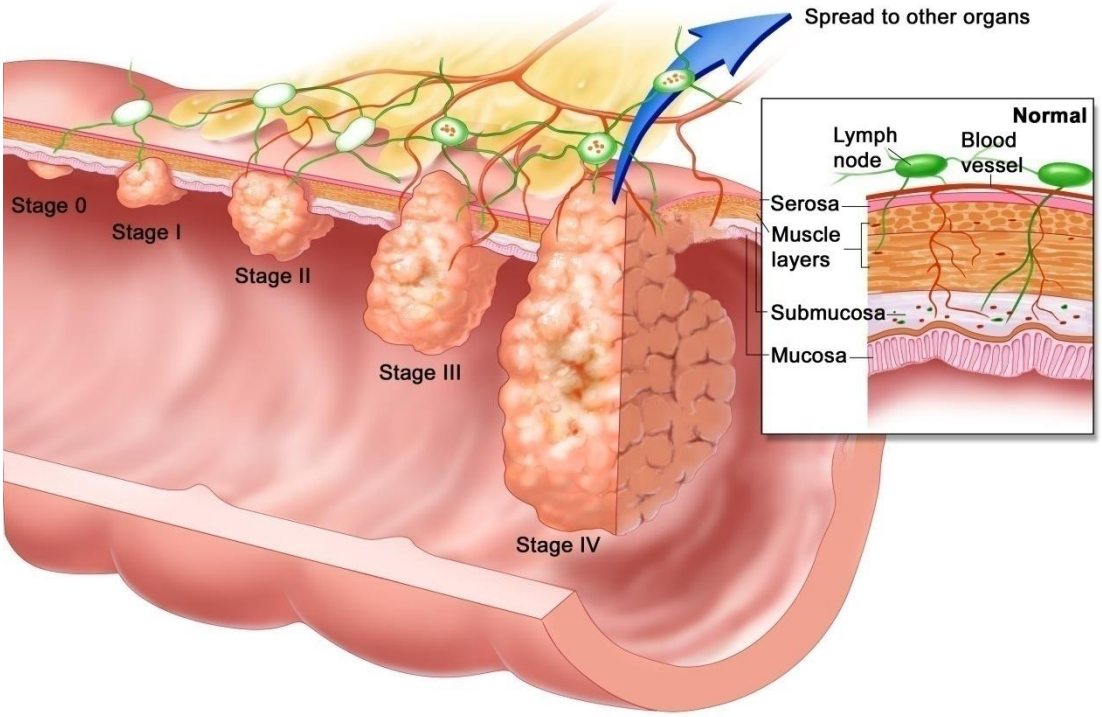
Endoscopic investigations of the rectum and colon are also performed. These include both a sigmoidoscopy and a colonoscopy. Biopsies of any abnormal lesions in the colon are also carried out in order to make a definite diagnosis. The extent of local tumour growth is determined by Ultrasound, CT and MRI methods. These methods are also performed to determine if there has been metastatic spread to the lymph nodes or other organs (Wils., 2007).

2.4 Management of colorectal cancer

The therapeutic management of colon cancer has improved in the past few years. When the diagnosis of cancer is confirmed, its clinical stage can be assigned using the characteristics of the primary tumor, its depth of bowel penetration, the degree of lymph node involvement and the presence or absence of distant metastasis. The staging of the tumor plays an important role in determining the best treatment strategy and it is also the most critical factor in predicting survival of the patient. Colorectal cancer is initially treated by surgery followed by chemotherapy depending on the stage of the tumour. Radiation therapy is also used in metastatic disease (Leslie and Steele., 2002; Cho *et al*., 2007; Wils., 2007).

The following criteria is used to assign stages I through IV of colon cancer (Figure 1.2).

- Stage 1: the tumor is confined to the intestinal wall
- Stage II: the tumor has penetrated through to the outer wall of the colon or has gone through it, with a possible invasion of other local tissues.
- Stage III: the tumor is associated with some lymph node involvement.
- Stage IV: the tumor is now also associated with distant metastasis and has spread to nearby lymph nodes and several parts of the body.



<u>Figure 1.2</u> The different stages of colon cancer illustrating the relative size and penetration of the tumour (Winslow., 2005).

2.5 Chemotherapeutic drugs used in colon cancer

Chemotherapy is useful in reducing the development of metastasis, reducing the tumor burden or slowing tumor growth (O'Neil and Goldberg., 2008).

The standard chemotherapeutic treatment of colon cancer is intravenous 5-flourouracil (5-FU) or capecitabine combined with leucovorin (LV) for a period of 6 – 12 months. Although 5-FU is recognized as the agent with the most clinical efficacy, about 80% of patients do not benefit from this treatment either because surgery was sufficient or because their tumor was not responsive to 5-FU based chemotherapy (Chabner *et al*., 2011).

2.5.1 5-Fluorouracil and leucovorin

5-FU is a pyrimidine antimetabolite that requires metabolic activation to yield ribosyl and deoxyribosyl nucleotide metabolites. One of these metabolites, 5-fluoro-2-deoxyuridine-5-monophosphate (FdUMP), inhibits the enzyme thymidylate synthase.

Thymidylate synthase converts deoxyuridine monophospate (dUMP) to thymidine monophosphate (dTMP), which is later phosphorylated to thymidine triphosphate for use in DNA synthesis and repair. Thymidylate synthase is inhibited when 5-FU forms a covalently bound ternary complex with the enzyme and $N^{5,10}$-methylenetetrahydrofolate (Benson and Goldberg., 2003).

5-FU is converted to 5-fluorouridine-5-triphosphate (FUTP), which is integrated into RNA, where it interferes with the processing and translation of RNA. 5-FU can also be converted to 5-fluorodeoxyuridine-5-triphosphate (FdUTP), which, when integrated into cellular DNA, inhibits DNA synthesis and function. The cytotoxic effects of 5-FU is therefore experienced in both DNA- and RNA-mediated events. (Chabner *et al*., 2011). 5-FU is used in combination with irinotecan for the treatment of metastatic colorectal cancer (Benson and Goldberg., 2003).

Leucovorin, a 5-formyl derivative of tetrahydrofolic acid, is often given in combination with 5-FU. 5-FU can only bind to thymidylate synthase when leucovorin has locked the enzyme in an active conformation. It stabilises the binding of 5-FU to thymidylate synthase and causes a fatal deficiency in dTMP. Leucovorin, has no intrinsic cytostatic activity (Meyerhardt and Mayer., 2005; Chabner *et al*., 2011).

2.5.2 Capecitabine

Capecitabine is a fluoropyrimidine carbamate prodrug that is enzymatically converted into 5-FU in the tumor where it stops DNA synthesis and reduces the growth of the tumor **(**Chabner *et al*., 2011).

2.5.3 Irinotecan

Irinotecan is a topoisomerase inhibitor. Topiosomerases are enzymes that uncoil DNA for replication and transcription. They cut DNA and also catalyse the religation of the cut DNA ends. Irinotecan interacts with DNA topoisomerase 1 and prevents the cut DNA from being repaired efficiently, hence causing fragmented DNA (Jeffrey and Mayer., 2005).

2.5.4 Oxaliplatin

Oxaliplatin is a platinum-based chemotherapeutic agent and belongs to the same family as cisplatin and carboplatin. The exact mechanism of action of oxaliplatin is still unclear, however, the cytotoxicity of platinum compounds is considered to be as a result of DNA synthesis inhibition (Chabner *et al*., 2011). Oxaliplatin is used in combination with 5-flourouracil (Jeffrey and Mayer., 2005).

2.5.5 Cetuximab

Cetuximab is a monoclonal antibody to the epidermal growth factor receptor. The receptor is a transmembrane glycoprotein that is required in many signalling pathways that affect and stimulate cell growth, proliferation, differentiation and apoptosis. The receptor is located on the surface of normal epithelium and has been observed to be overexpressed in some tumours including those of colorectal cancer. Cetuximab targets the extracellular binding domain of the receptor and some findings have shown its effectiveness in overcoming cellular resistance to irinotecan (Jeffrey and Mayer., 2005; Chabner *et al*., 2011).

2.5.6 Bevacizumab

Bevacizumab is a monoclonal antibody directed against the vascular endothelial growth factor, a soluble protein that stimulates angiogenesis. Several studies have shown that the supplementation of bevacizumab to current therapeutic strategies has a positive effect on overall survival (Jeffrey and Mayer., 2005).

3. Apoptosis and cancer

3.1 Mechanisms of cell death

Physiologically, the balance between cell death and cell generation is essential in all living organisms to maintain tissue homeostasis (Tapon *et al*., 2002). Cell death may occur by one of two mechanisms, necrosis (injury) or apoptosis (programmed cell death). If a cell's death is due to injury, a passive and uncontrolled process occurs that

involves cell swelling and lysis. This results in the leakage of the cell's contents, accompanied by inflammation of the surrounding tissues.

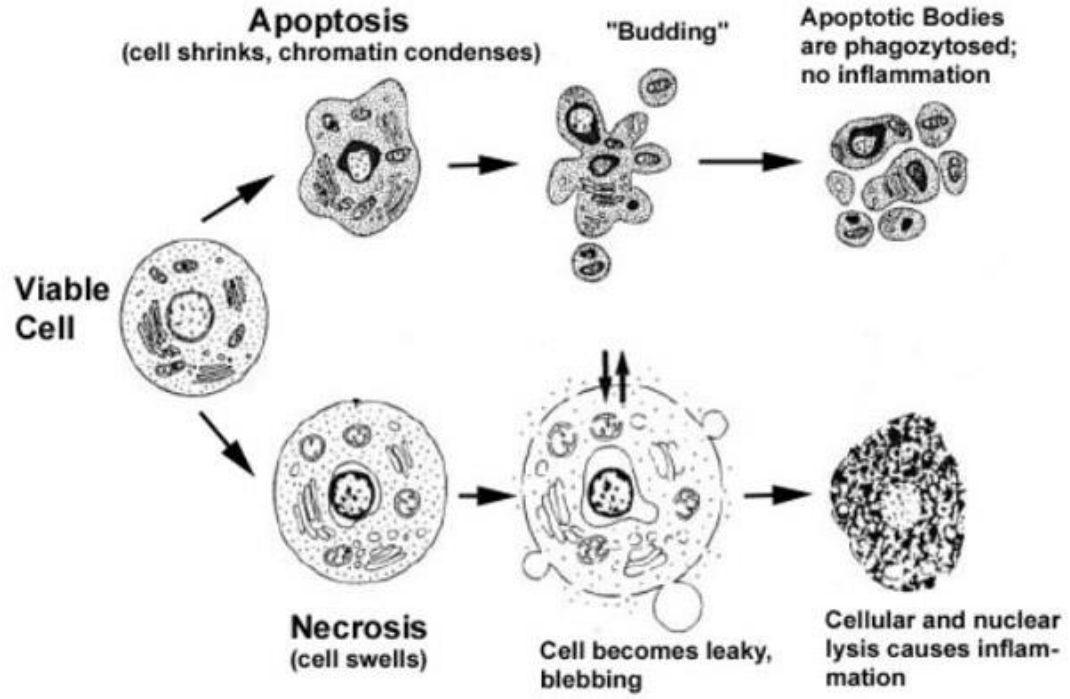
Apoptosis was first described in 1972 by Kerr *et al*. and is a highly controlled process, where a sequence of molecular events is initiated that results in cell death. The apoptotic process is a dynamic interplay of several molecules with many different upregulatory and downregulatory properties that is largely dependent on the type of cell. Apoptosis is initiated from the cytoplasm of a cell and can occur within 2 hours (Elmore., 2007).

Apoptosis has an essential role in sculpting the shape and organisation of organs during embryonic development by eliminating various groups of cells at specific stages (Abud., 2004). This process is tightly controlled and the inability to regulate apoptosis may lead to a number of diseases such as ischaemic injury, stroke, multiple organ failure syndrome and neurodegenerative disease. Inefficient apoptosis or apoptotic failure may result in cancer and several autoimmune disorders (Bjelakovic *et al*., 2005).

Cells undergoing apoptosis have the following morphological features: cell shrinkage, development of bubble-like plasma membrane blebbing on the surface, encapsulation of the cell membrane and chromatin degradation (Wyllie., 1997). Apoptosis has been characterized by certain biochemical changes, which includes the exposure of phosphatidylserine (PS) on the outer leaflet of the plasma membrane (Denecker *et al*., 2001), changes in mitochondrial membrane permeability (Kroemer and Reed, 2000), the release of mitochondrial proteins such as cytochrome c from the intermembrane space (Van Loo *et al*., 2002) and DNA cleavage (Figure 1.3). Numerous molecules on the cell surface also change to ensure that the apoptotic cells will be recognised and engulfed by phagocytes or neighbouring cells, thus resulting in little or no inflammation (Lopaczynski and Zeisel., 2001).

Apoptosis can take place in response to a number of external and internal stimuli. Examples of external stimuli includes ultraviolet radiation, hormones and antibiotics, while the internal stimuli include regulators of transcription and DNA damage (Kaufman and Earnshow., 2000).

The failure of cancer cells to enter the apoptotic state despite genetic instability is an innate part of the carcinogenic process and is also implicated in the resistance to chemotherapeutic drugs (Johnstone *et al*., 2002). During the course of tumor development, mutations in apoptotic mechanisms (e.g. the loss of p53) can contribute to both intrinsic and acquired drug resistance. Modern cancer chemotherapeutic approaches aim to stimulate apoptotic cell death as opposed in contrast to necrosis since apoptotic cells are cleared by phagocytosis without inducing the inflammatory response (Shacter *et al*., 2000) which is detrimental to the patient.



<u>Figure 1.3</u> Differences between necrosis and apoptosis

The different characteristic changes of a cell's structure due to necrosis and apoptosis. Necrosis results in cell and organelle swelling, an early loss of plasma membrane integrity and a leakage of the cytoplasmic material into the extracellular space. Apoptosis, includes characteristics such as nuclear condensation and membrane shrinkage, development of apoptotic bodies and subsequently engulfment of the apoptotic bodies (Majno and Doris., 1995; Van Cruchten and Van den Broeck., 2002).

The currently used chemotherapeutic agents are not very effective, hence there is a need to evaluate novel compounds for their cytostatic effects as well as their ability to induce apoptosis. Novel chemical agents that target tumor-specific alterations in apoptotic pathways, either alone or in combination with conventional chemotherapeutic agents, may improve the clinical outcome of the disease (Wolpin *et al.*, 2008; Chibaudel *et al.*, 2012).

3.2 Apoptotic Pathways

Cells are stimulated to enter apoptosis through two overlapping, caspase-dependent pathways namely:

I) the extrinsic or death receptor pathway or
II) the intrinsic or mitochondrial pathway.

The death receptor pathway differs from cell type to cell type while the mitochondria has been observed to be the principal target of the intrinsic pathway (Evan and Vousden, 2001). Caspases are proteolytic enzymes that cause cleavage of a variety of proteins that elicit cell death (Kumar., 2007). The caspases are divided into two groups namely the initiator (example caspase-8, 9 and 10) and the effector caspases (example caspase-3, 6 and 7), based on their roles in cell death (Kumar., 2007; Lamkanfi *et al*., 2007; Sakamaki and Satou., 2007).

The process of apoptosis can also take place by means of intracellular inducers which are not caspase-mediated. Examples of these include apoptosis inducing factors (AIF), endonuclease G, calpains and cathepsins. These mediators are able to cause DNA fragmentation (Broker *et al*., 2005). When apoptosis inducing factors are released into the cytosol from the intermembrane space of the mitochondria, they interact with cyclophilin A. In neurons, cyclophilin A participates in the nuclear translocation of apoptosis-inducing factor which become a DNAse (Zhu *et al*., 2007). As soon as translocation to the nucleus occurs, DNA fragmentation commences (Urbano *et al*., 2005).

3.2.1 Death receptor pathway (extrinsic)

The death receptor pathway is initiated by extracellular stimuli and is mediated either by cell surface receptors including the Fas receptor and the tumor necrosis factor (TNF) receptor system, or by perforin and granzyme B which are released from activated, cytotoxic lymphocytes. The extrinsic pathway is able to activate a caspase cascade within seconds of binding to a ligand, hence apoptosis is induced very rapidly by this mechanism. The most common death receptors are CD95 (also referred to APO-1 and Fas), tumour necrosis factor receptor 1(TNF-R1), TNF-related apoptosis-

inducing ligand receptor 1 (TRAIL-R1, also known as APO-2), and TNF-related apoptosis-inducing ligand receptor 2 (TRAIL-R2). TNF-R1 and CD95 (Fas/Apo-1) have been identified to trigger apoptosis by their natural ligands or specific agonist antibodies. The receptors possess a conserved cytoplasmic domain, known as the death domain (DD), which binds other DD-containing proteins (Figure 1.4). The most commonly known DD-containing molecules are TNF-R1-associated DD (TRADD), Fas–associated death domain (FADD) and receptor interacting protein (RIP) (Jin and El-Deiry., 2005).

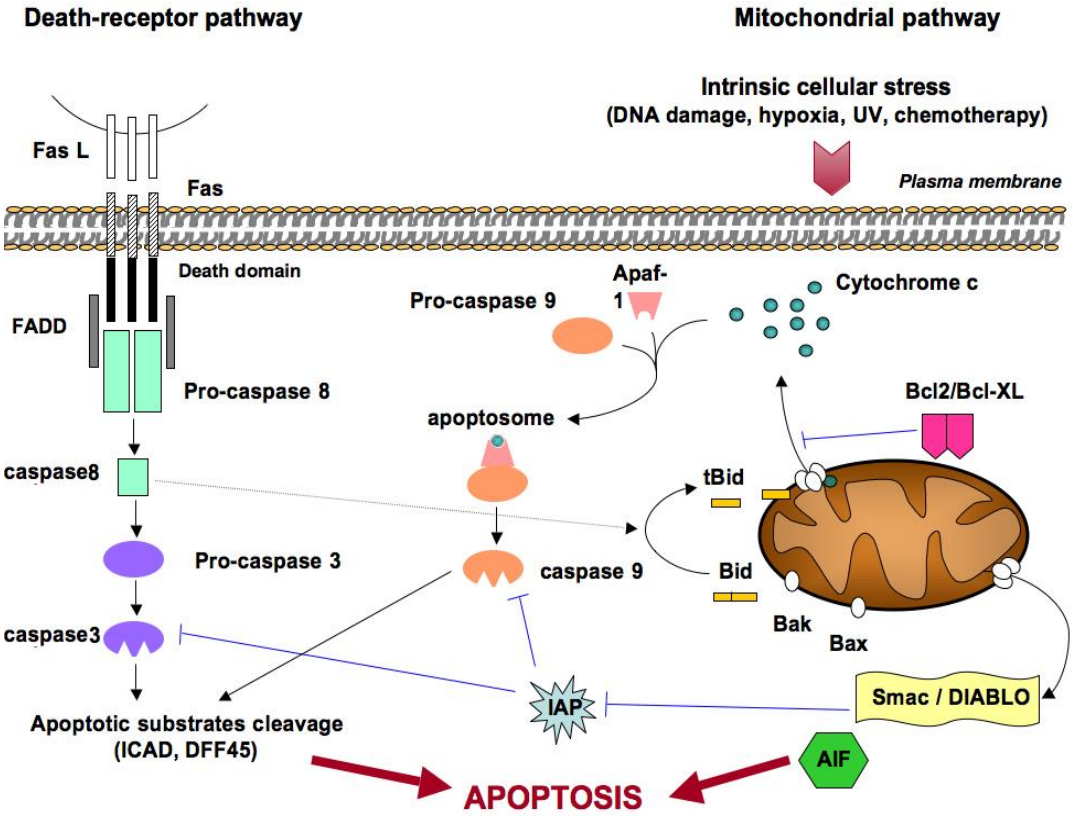


Figure 1.4 Schematic representation of different pathways involved in apoptosis.

Death receptor pathway: Death ligands such as FasL trigger apoptosis by binding to death receptors. The death receptors recruit the Procaspase-8 by means of an adaptor protein such as Fas associated death domain protein (FADD). After cleavage the active caspase-8 then directly activates the effector procaspase-3 to form active caspase 3 or cleaves the BH3-only protein Bid. Truncated Bid (tBid) interacts with Bax and Bak leading to a Bax/Bak oligomerisation which forms a pore in the outer mitochondrial membrane through which cytochrome c (Cyt c) is released. Other apoptosis-inducing molecules released by the mitochondria include second mitochondria-derived activator of caspase/direct inhibitor-of-apoptosis (IAP) protein binding protein with low pI (Smac/DIABLO)] and apoptosis-inducing factor (AIF). The release of cytochrome c and AIF is inhibited by the B-cell lymphoma 2 (Bcl-2) family of proteins which includes B-cell lymphoma-extra large (Bcl-XL). Mitochondrial pathway: The mitochondria release Cyt c in response to stress. Cyt c binds to apoptotic protease activating factor-1 (Apaf-1) which forms the apoptosome. This results in the proteolytic activation of the procaspase 9. Active caspase-9 can then proteolytically activate the executioner caspase, caspase-3 which cleaves the inhibitor of caspase-activated DNase (ICAD), also known as DNA fragmentation factor 45 kDa subunit (DFF45) which cleaves DNA (Hengartner., 2000; Nadege *et al*., 2009).

3.2.1.1 Fas-Fas ligand-mediated apoptosis

Fas (APO1/CD95) was the first death receptor to be identified. The Fas-mediated apoptosis cascade requires clustering of the receptors by a trimeric ligand and a homotypic binding of receptors with cytoplasmic adaptor proteins. A membrane-bound or soluble ligand, called Fas ligand (FasL/CD95L) produced by cells of the immune system binds to Fas on T cells and Natural Killer cells (Griffith *et al*., 1995). This will lead to interactions with the cytoplasmic adaptor protein that displays corresponding Fas-associated proteins with a death domain (FADD) (Figure1.4). This 'adapter protein' in turn binds to homologous domains (death effector domain (DED) in initiator caspases (such as caspase-8), resulting in an autocatalytic activation to trigger procaspases-3 and -7 (Figure 3.3). Caspase-3, in turn, cleaves and thereby activates procaspase-6 (not shown on diagram), which eventually results in the cleavage of cellular components such as lamin, poly(ADP-ribose) polymerase (PARP), and DNA fragmentation (Ethell and Buhler, 2003). The adaptor, receptor, and caspases all exist as a closed, single complex before ligand binding. This complex of proteins is called the death-inducing signaling complex (DISC) (Zangemeister-Wittke and Simon., 2001).

3.3 Mitochondrial pathway (intrinsic)

The mitochondrion is vital for cell homeostasis, energy production and survival. The mitochondria play a role in the regulation of cell death by releasing certain apoptogenic factors during apoptosis such as cytochrome *c*, apoptosis-inducing factor, Smac/Diablo, and endonuclease G (Voux., 2011). The mitochondrial apoptotic pathway is initiated from within the cell and is characterised by mitochondrial dysfunction or endoplasmic reticulum stress (Saelans *et al*., 2004). Damage of the inner mitochondrial membrane results in the opening of the mitochondrial membrane transition pore (MPTP) (Kroemer and Reed., 2000).

Apoptosis is related to mitochondrial membrane potential ($\Delta\psi m$), especially in cancer cells (Hu and Kavanagh, 2003; Elmore., 2007). A decreased mitochondrial membrane potential ($\Delta\psi m$) has been shown to be a vital factor in controlling the induction of apoptosis (Kroemer and Reed., 2000). It has also been described, for different cell types and different apoptosis-inducing triggers, that this loss of mitochondrial

membrane potential (Δψm) occurs very early in the apoptotic process and results from the asymmetrical distribution of protons on both sides of the inner mitochondrial membrane, giving rise to a chemical (pH) and electrical gradient, which is essential for mitochondrial function (Castedo *et al*., 2002). Petit and coworkers (1995) have also reported that a disruption in the Δψm and subsequent nuclear apoptosis are strictly co-regulated, i.e. whenever a cell loses its Δψm it dies by apoptosis, and whenever a cell is protected against apoptosis, it maintains its Δψm.

A decrease in the mitochondrial membrane potential results in activation of mitochondrial pro-apoptotic factors (Hu and Kavanagh., 2003). Various conditions cause a change in mitochondrial membrane permeability such as an overload of Ca^{2+} or oxidants. The collapse of the Δψm during apoptosis has been noticed in several studies and it has thus been suggested that the depolarisation of the mitochondria is one of the first events which occurs in apoptosis and could be a prerequisite for the release of cytochrome c and the activation of apoptosis (Ly *et al*., 2003).

The B-cell lymphoma-2 (Bcl)-2 protein (an integral mitochondrial membrane protein) is a member of a large family of structurally, closely related proteins, some of which will cause cell survival while others cause cell death. The MPTP is regulated by the relative proportions of anti-apoptotic (Bcl-2, Bcl-xl) and pro-apoptotic (Bax, Bad) Bcl-2 family members within the cell (Mikhailov *et al*., 2003).

The Bax/Bak protein forms a high conductance channel with a voltage-dependent anion channel, resulting in a loss of membrane potential, thus allowing the passage of cytochrome c to the cytoplasm. Subsequently, cytochrome c and AIF are released from the mitochondria. In addition to the release of the mentioned mitochondrial factors, the dissipation of the MPTP also causes a loss of the biochemical homeostasis of the cell and results in the uncoupling of oxidative phosphorylation.

Adenosine triphosphate (ATP) formation from ADP is halted and redox molecules such as NADH, NADPH, and glutathione are oxidized, and reactive oxygen species (ROS) generated (Kroemer and Reed., 2000). The high levels of ROS causes the oxidation of lipids, proteins, and nucleic acids, thereby enhancing the disruption of the inner mitochondrial transmembrane potential. Reactive oxygen species are thus

released into the cytosol, mediating mitochondrial Ca^{2+} accumulation (Kroemer and Reed., 2000).

In the cytoplasm, cytochrome c then binds Apaf-1, caspase-9 and ATP or deoxyadenosine triphosphate dATP to form the apoptosome. This in turn activates the effector caspases, such as 3, 6 and 7. The activation of these execution caspases leads to the degradation phase of apoptosis (Fadeel *et al*., 2003). The MPTP also leads to the release of endonuclease G, a pro-apoptotic protein resulting in the cleavage of chromosomal DNA without activating any caspases.

The second mitochondria-derived activator of caspases (SMAC), also known as DIABLO is a mitochondrial protein that is released from the mitochondria and prevents the inhibitor of apoptosis protein (IAP) from interacting with caspase-9, thus resulting in apoptosis. Heat shock proteins influence both pathways of apoptosis. They may have proapoptotic and antiapoptotic properties (Zhivotovsky and Orenius., 2003).

The death receptor pathway is connected to the mitochondrial pathway via the following: caspase-8 cleaves the proapoptotic cytosolic protein BID (Bcl-2 Interacting Domain) to a truncated tBID, which thereafter translocates to the mitochondrial membrane and binds to the BAD (Bcl-2-associated death promoter), a proapoptotic protein, causing the release of cytochrome c (Pan *et al*., 2001; Elmore., 2007).

3.4 The execution phase

The mitochondrial and death receptor pathway meet at the activation of the execution caspases -3, -6, and -7, which leads to the activation or cleavage of various substrates (Figure 1.5). Caspase-3 activation converts caspase-activated DNase (CAD) into an active form by cleaving an inhibitor of the enzyme (ICAD) (Coleman and Olsen., 2002).

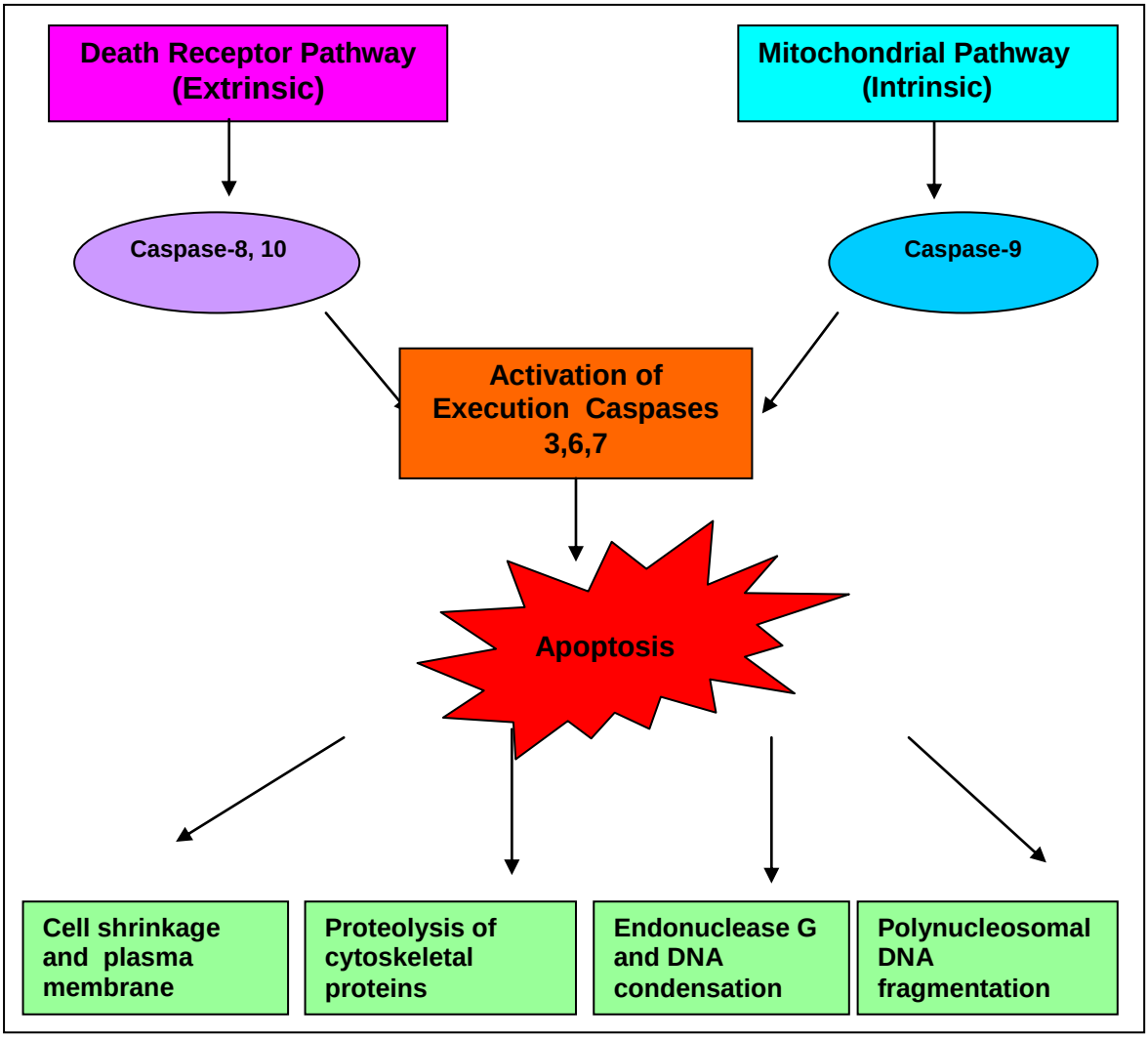


Figure 1.5 The execution phase of apoptosis The initiator caspases (8,9,10) result in activation of the execution caspases (3,6,7), which cleave vital cellular substrates, and ultimately results in death of the cell.

3.5 Caspases

The most important executioners of apoptosis are the caspases, a group of proteolytic enzymes. Mammalian caspases are homologous to one another and contain many evolutionary conserved regions. If the caspase activity is decreased, the apoptotic process is reduced or inhibited completely (Lamkanfi *et al*., 2007). They implement cell death by cleaving a variety of intracellular proteins that cause the death of a cell in a controlled manner (Evan and Vousden., 2001, Lamkanfi *et al*., 2007). The caspases are cysteinyl aspartate-specific proteases that cleave peptide bonds on the carboxyl side of aspartate. They bring about the various morphological changes which

are observed in cells undergoing apoptosis (Kumar., 2007). Caspases are synthesised as inactive zymogens and upstream signals convert these into the active proteases (Hengartner., 2000). Active caspases are $\alpha_2\beta_2$ tetramers composed of two 17-35 kDa and two 10-12 kDa subunits (Lamkanfi *et al*., 2007).

The caspases are divided into two classes namely initiator and effector caspases, based on their roles in cell death. Caspases are synthesized as inactive proenzymes comprising an N-terminal peptide (prodomain) together with one large and one small subunit. Caspases-8 and 9 are the main initiator caspases (Figure 2.2) and once activated, they cleave and activate the effector caspases (Bao and Shi., 2007). The effector or downstream caspases, are involved in most of the proteolytic events that disassemble the cell and are characterized by a short N-terminal prodomain. Caspase-3, 6 and 7 are the common effector caspases and once activated, by either the extrinsic (death receptor) or the intrinsic (mitochondrial) pathway, they cleave polypeptides that ultimately undergo proteolysis in the apoptotic cells (Figure 2.2). Caspase-3 is the most important effector caspase since it cleaves most of the cellular substrates in apoptotic cells. It is activated by proteolytic cleavage by caspase-8 or -9 and by cytochrome c (Porter and Janicke., 1999; Liu *et al*., 1996).

The sequence of events leading to caspase activation will vary depending on the apoptotic stimulus. The caspase cascade can be activated by several mechanisms. One of the best characterised systems is that of the cytotoxic T lymphocyte component granzyme B which directly activate caspases-3, 7, 8 and 10, as well as causing the ligation of the Fas (APO-1/CD95) receptor which ultimately results in the activation of Caspase-8 (Talanian *et al*., 1997). Nevertheless, for many other stimuli, targeting the mitochondria to release pro-apoptotic factors has been assumed to be the crucial step in caspase activation (Barnhard *et al*., 2003).

In cancer, defects in the activation of the caspase-3 proteolytic system after treatment with chemotherapeutic compounds is associated with resistance to apoptosis and is thus linked to chemotherapy failure (Soung *et al*., 2004).

4. Apoptosis and colon cancer

4.1 Colon cancer and the death receptor pathway:

4.1.1 The Fas receptor and its ligand

Despite the fact that colon cancer tumors and colon cancer cell lines express Fas receptors (Fas R), research has shown that some colon cancer cell lines are resistant to Fas-mediated apoptosis, implying a defect in the Fas-mediated apoptosis signaling pathway. In contrast, the normal colonic epithelium continues to be responsive to Fas-mediated apoptosis. As a result, efficacy of the Fas ligand (FasL) or Fas ligand agonists like tumour necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) in chemotherapy is diminished since the cancer cells would be resistant to apoptosis. In contrast, normal colonic cells would easily apoptose thus favouring the survival of the cancer cells (O'Connell *et al*., 1997).

The resistance to Fas mediated apoptosis plays an essential role in providing colon cancer cells an opportunity to evade the immune system. The immune system gets rid of unwanted cells by bringing about apoptosis on cells with Fas receptors (Guillen-Ahlers *et al*., 2010).

4.1.2 The death receptor TRAIL

The tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), an Apo2 ligand, is a member of the TNF family. When TRAIL binds to DR4 or DR5 receptors which are expressed in colonic mucosa and colon carcinomas, it activates the TRAIL death receptor pathway of apoptosis (Koornstra *et al*., 2003). Colon cancer cells have been shown to be sensitive to TRAIL-mediated apoptosis whereas the normal colonic epithelium shows resistance to TRAIL-mediated apoptosis in colon cancer cell lines and colon cancer tumours. The expression of the TRAIL receptor DR4 has been suggested to have a protective role against metastatic diseases and therefore it may be important in removing tumour cells by the immune system (Strater *et al*., 2002).

4.1.3 The caspase enzymes and colon cancer

The initiation of all apoptotic events is linked to the activation of the caspases. One potential mechanism to evade apoptosis is the inactivation of caspase-8 by genetic or epigenetic mechanisms (Hong *et al*., 2008). The impaired expression or function of caspase-8 has been shown to stimulate the formation of tumors (Fulda S., 2010).

Caspase-8 (FLICE), is one of the molecules that is crucial for cell death induction, especially via the CD95 (APO-1/Fas) death receptor pathway. Caspase-8 plays a crucial role in apoptosis triggered by the interaction of ligand with death receptors, such as Fas, tumor necrosis factor receptor (TNFR), and TNF-related apoptosis inducing ligand receptor (TRAILR) (Fulda., 2010).

4.2 Colon cancer and the mitochondrial apoptotic pathway

4.2.1 Bcl-2, Bax and cytochrome c

Bcl-2 is an inhibitor of apoptosis and is overexpressed in colorectal adenomas and carcinomas (Poincloux., 2009). Several studies have shown that the expression of Bcl-2 increases in the initial stages of development of colon cancer and decreases as the tumour develops into the late stages of the adenoma-to-carcinoma sequence. This increase causes a decline in p53 and supports tumour development (Saleh *et al*., 2000).

Over 50 % of colon cancer adenocarcinomas also have Bax mutations (Zhang *et al*, 2000a). Several *in vitro* studies have shown that cells lacking active Bax are more resistant to TRAIL-mediated apoptosis which aids in tumour development (Ravi and Bedi., 2002).

The release of cytochrome c is primarily determined by the ratios of the mitochondrial pro-apoptotic and anti-apoptotic mediators. The induction of apoptosis in colon cancer cells has been associated with a decrease in the Bcl-2:Bax ratio. Past research on the occurrence of Bcl-2 and Bax proteins in colorectal adenocarcinomas has indicated an increased levels of Bcl-2 and decreased levels of Bax (Zhang *et al*., 2000a).

4.2.2 The p53 tumor suppressor gene and p53 upregulated modulator of apoptosis (PUMA)

The major tumor suppressor gene product p53, has a crucial role in regulating the cell cycle and, is involved in preventing cancer. The p53 gene is often absent or mutated in many human cancers (Vousden 2002). Tumor progression is promoted by mutations of the p53 gene (Forslund *et al*., 2002; Shaw *et al*., 1992). When p53 is mutated, it prevents apoptosis of faulty cells and allow damaged cells to proliferate.

Apoptosis is indirectly stimulated by p53 since it represses the levels of the survivin protein (Hoffman *et al*., 2002). Survivin belongs to the inhibitors of apoptosis (IAP) family and has been shown to be over expressed in 64% of colon cancer patients. The expression of survivin is decreased by both p53 and Nuclear Factor kappa B **(**NFκB) (Hoffman *et al*., 2002).

The p53 up-regulated modulator of apoptosis (PUMA) is one of the Bcl-2 Homology domain 3 (BH3) only group of proteins. The BH3-only members of the Bcl-2 protein family are important initiators of apoptosis. The PUMA protein binds to Bcl-2, concentrates in the mitochondria, and induces the release of cytochrome c (Yu *et al*., 2003).

4.2.3 Nuclear factor kappa B (NFκB) and *c-FLIP*

Nuclear Factor Kappa B (NFκB) proteins are a family of transcription factors involved in the control of numerous cellular processes such as immune and inflammatory responses, cellular growth and developmental processes (Sen and Baltimore 1986; Li and Verma 2002; Sethi *et al*., 2008). In the cytoplasm, NFκB activity is inhibited by Inhibitor of Kappa B (IκB) proteins. When NFκB is bound to IκB, the complex is primarily in the cytoplasm. The phosphorylation of IκB leads to its enzymatic degradation. The uncoupling of IκB and NFκB results in the activation of NFκB by permitting its movement into the nucleus. Once in the nucleus, it induces the transcription of IAPs, hence leading to the inhibition of the execution caspases. NFκB has an important role in regulating the transcription of Bcl-2 and the inhibition of NFκB results in cells being more susceptible to TRAIL-mediated apoptosis (Cusack., 2003).

Cellular caspase-8 (FLICE)-like inhibitory protein (***c-FLIP***) is a very strong inhibitor of caspase-8 activity. It binds to FADD and inactivates caspase-8. Several *in vitro* studies on colon cancer cells have shown that c-FLIP activates NFκB and therefore induces apoptosis inhibitors (Katoaka *et al*., 2000). In colon cancer, several studies have shown that low levels of c-FLIP induces apoptosis, whereas higher levels inhibit (Chang *et al*., 2001; Longley *et al* 2006).

4.3 Other factors involved in colon cancer apoptosis

4.3.1 Ceramide

Ceramide is composed of sphingosine and a fatty acid and is synthesized in the endoplasmic reticulum. Ceramide is found in high concentrations within the cell membrane and is one of the component lipids that make up sphingomyelin. Ceramide also functions as a signaling molecule. The most well-known functions of ceramides as cellular signals include differentiation, proliferation, and apoptosis. Apoptosis inducers such as TNFα, Fas ligand, and chemotherapeutic agents, regulate one or more enzymes of ceramide metabolism and can result in the accumulation of ceramide (Pettus *et al*., 2002). Ceramide activates endonucleases and c-Jun which stimulate apoptosis. In colon cancer cells, low ceramide levels correlate with a decrease in apoptosis (Lavie *et al*., 1996; Pettus *et al*., 2002).

4.3.2 Butyrate

Butyrate (a short-chain fatty acid) is a colonic bacterial byproduct of fiber metabolism. Butyrate has been shown to upregulate Bax and downregulate Bcl-2 through increased binding of specific transcription factors, thus inducing the mitochondrial pathway of apoptosis (Ruemmelle *et al*., 2003). *In vitro* studies have shown that glycerin tributyrate promotes apoptosis by upregulating the Fas receptor in colon cancer cells (Schroeder *et al*., 2002). *In vitro* studies have demonstrated that sodium butyrate downregulates c-FLIP in colon cancer cell lines and hence sensitizes them to TRAIL-mediated apoptosis (Kim *et al*., 2004).

4.3.3 PPAR

Peroxisome proliferators-activated receptors (PPARs) are nuclear receptors for the metabolites of linoleic and arachidonic acid metabolites. There are three subtypes: PPARα, PPARδ and PPARγ. The ligand of PPARγ has been observed to initiate apoptosis in colon cancer cells and this is associated with a downregulation in c-myc expression and an upregulation of the oncogenes c-jun and gadd153 (Shimada *et al*., 2002). PPARδ also has a prominent role in colon cancer cell apoptosis. 13-S-hydroxyoctadecadienoic acid, the key metabolite of 15-lipoxygenase-1 linoleic metabolism binds and inhibits PPARδ and leads to an increased apoptosis in colon cancer cells (Shureiqi *et al*., 2003).

4.3.4 COX-2

Cyclooxygenase (COX)-2, the inducible form of COX is an essential enzyme in catalyzing the conversion of arachidonic acid to prostaglandins. COX-2 has been observed to be over-expressed in colon cancer cells. (Dimberg *et al*., 2002). Prostaglandin I_2 (PGI_2) is one of the products of COX-2 and has been shown to activate PPARδ in colon cancer cells, thus, by inhibiting COX-2, the activation of PPARδ will be reduced and the induction of apoptosis may occur (Gupta *et al*., 2000). Some studies have indicated that colon cancer patients taking celecoxib, a COX-2 inhibitor over a 6 month period had a 28 % reduction in the number of adenomatous polyps. (Gottlieb., 1998). The COX-2-selective inhibitor NS398 has been shown to induce apoptosis in colon cancer cells via the release of cytochrome c and the subsequent increase in caspase-3 levels (Li *et al*., 2001).

4.3.5 Nitric Oxide

Several studies have shown the significance of nitric oxide in the tumorigenesis of colon cancer. Nitric oxide (NO) is a critical signaling molecule in many physiological processes. A decrease in the concentration of NO has been shown to stimulate the growth of cells and protect numerous types of cells from apoptosis, however a high concentration of NO can hinder cell growth and induce apoptosis (Kim *et al*., 2001).

In vitro studies on various colon cancer cell lines have shown that nitric oxide induces the expression of cyclooxygenase II in the colon cancer cell lines and at higher concentrations it inhibited cell growth and induced apoptosis (Liu *et al*., 2003).

5. Novel nucleoside analogues and imidazo[1,2-a]pyridine compounds

In recent times, the use of multi-component chemical reactions in the synthesis of compounds displaying anti-cancer properties has been an area of prolific research (Adams *et al*., 2010; Bonate *et al*., 2006). Two classes of compounds that are accessible by multi-component chemical reactions are the nucleoside analogues and the imidazo[1,2-a]pyridines.

5.1 Nucleoside analogues

Nucleoside analogues are one of the major classes of chemotherapeutic agents used in the treatment of cancer. (Ewald *et al*., 2008). Examples include 5-fluorouracil (5-FU), cytarabine, gemcitabine, thioguanine and mercaptopurine. Most nucleoside analogues exert their cytotoxic effects by interfering with DNA replication whereas some analogues have several different actions leading to a significant decrease in cell viability (Ewald *et al*., 2008; Chabner *et al*., 2011).

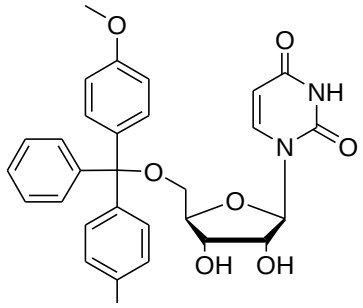


Figure 1.6 Backbone structure of one of the novel nucleoside analogues

5.2 Imidazo[1,2-a]pyridines

The imidazo[1,2-a]pyridine group of compounds have received considerable attention in the recent years for their various antimicrobial, analgesic, anti-inflammatory and anticancer properties (Gudmundsson *et al*., 2003; Lacerda *et al*., 2009; Byth *et al*., 2006).

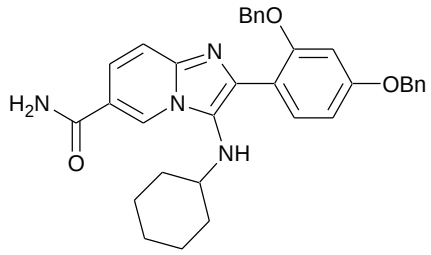


Figure 1.7 Backbone structure of one of the novel imidazo[1,2-a]pyridines

6. Motivation and Aims

Despite the vast improvement of our understanding of the molecular mechanisms of cancer and the application of novel drug therapy, cancer continues to be a major health problem around the world. Colon cancer is associated with a poor prognosis and conventional chemotherapy has limited effectiveness. The management of diagnosed patients places a huge burden on medical facilities and financial resources.

The search for novel drugs to be used in the treatment of colon cancer is essential in combating this life-threatening disease. Large-scale screening of compounds with potential anticancer activity is used to evaluate a broad range of pharmaceutical compounds, including both naturally occurring and synthesized chemical compounds. The primary aim of *in vitro* screening programmes is to identify biologically active compounds showing selective activity against certain tumour cell lines.

Secondly, the identification of inducers of apoptosis presents a strong basis for the development of potential anti-cancer agents and through the induction of apoptosis, these novel compounds can be developed into chemotherapeutic drugs for the treatment of different types of cancer.

Using HT-29 and Caco-2 colon cancer cell lines, the aims of this study were to determine:

- the effects of the novel compounds on cell viability in HT-29 and Caco-2 cells.
- the mode of cell death
- the underlying pathways of apoptosis in the colon cancer cell lines in response to the selected compounds.
- if the compounds induce differentiation in the HT-29 cells

II. Materials and Methods

2.1 Gastrointestinal cell lines

Cell lines are used to study cell biology as an alternative to cultured tissue explants. Under certain *in vitro* conditions, a differentiated phenotype may be shown by colon cancer cell lines. Colon carcinoma cell lines have been used to study cell structure, brush border morphogenesis, electrolyte transport, amino acid/protein uptake, cell death and differentiation as a model of the disease (Bolte *et al*., 1998).

2.1.1 The HT-29 colon adenocarcinoma cell line

The HT-29 cell line is derived from a human colorectal adenocarcinoma and was originally isolated in 1975 (Fogh *et al*., 1977). Under standard culture conditions, the HT-29 cell line exists as a single layer of small, undifferentiated, epithelial cells. When exposed to inducers of differentiation such as acetoacetate and butyrate, the cells differentiate into mature enterocytes which are characterized by flattened cells with the formation of brush borders on apical surfaces and tight junctions between adjacent cells (Jamie *et al*., 2001).

2.1.2 The Caco-2 cell line

The Caco-2 cell line originates from a well differentiated colon adenocarcinoma (Fogh *et al*., 1977; Bolte *et al*., 1998). In culture, they spontaneously differentiate after confluence (when contact inhibition slows proliferation), both structurally and functionally, into cells resembling mature enterocytes (Pinto *et al*., 1983). Caco-2 monolayers have been used in several studies to determine the transcellular movement of various substances including drugs, amino acids and metal ions (Bolte *et al*., 1998).

The use of Caco-2 is favoured as opposed to HT-29 as a model for intestinal epithelium, since Caco-2 cultures are relatively free of multilayers. Many enzyme markers that are normally characteristic of enterocytes such as sucrase-isomaltase, lactase, aminopeptidase and alkaline phosphatase are also expressed by Caco-2 cells (Bolte *et al*., 1998). These are all brush border membrane enzymes that are differentiation-specific markers for small intestinal enterocytes (Hidalgo *et al*., 1989).

This study made use of both HT-29 and Caco-2 cell lines. Since the differentiation in the HT-29 cells is inducible, this cell line was used to measure the effects of the test compounds on the induction of differentiation.

2.2 Cell culture

2.2.1 Maintenance of cell cultures

The HT-29 and Caco-2 cell lines were obtained from Highveld Biologicals, South Africa (Ethics Clearance Waiver Number M070519; Appendix G). Both cell lines were cultured in Dulbecco's Modified Eagles Medium (DMEM) (Highveld Biologicals, South Africa). All solutions used in this study, including the culture medium were prepared with Milli-Q water. (MilliQ Millipore, USA) The culture medium was sterile filtered and supplemented with heat-inactivated foetal bovine serum (FBS) (5 % v/v) and 0.4 % v/v of a 100 mM sodium pyruvate solution. One milliliter of a solution containing 10 kU penicillin G and 10 mg streptomycin sulphate (Highveld Biologicals, South Africa) was added to 500 ml of sterile culture media. The FBS was heat-inactivated at 56˚C for 60 minutes in order to inactivate substances in the serum that inhibit cell proliferation (Freshney., 1994). The cells were maintained at 37°C, in a humidified incubator containing 5 % CO_2 (Afrox, Johannesburg). Spent medium was discarded and replaced 3-4 times per week. Stock cultures were maintained in 25 cm^3 and 75 cm^3 flat-bottomed flasks (Nunc, Denmark).

2.2.2 Subculture and cell viability

The cells were subcultured using trypsin by adding 250 µl of a 0.25 % trypsin solution containing 0.1% EDTA (Highveld Biological, South-Africa) and 250 µl of phosphate buffered saline (PBS) (3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl and 135 mM NaCl, pH 7.4). The PBS was autoclaved and stored at 4°C. The trypsin and EDTA act by preventing the cell-to-surface and cell-to-cell interactions, respectively. The cells were resuspended in 10 ml fresh medium and centrifuged at 120 x g for 5 minutes. Following centrifugation, the supernatant was discarded and the cell pellet resuspended in 5 ml fresh culture medium.

The cell number and viability was determined by counting cells in a haemocytometer using 0.4 % trypan blue dye (Sigma, Germany) in the trypan blue exclusion test. Trypan blue is a negatively charged chromopore and does not interact with the cell unless the membrane is damaged. Hence, the viable cells have a faint yellow colour whereas the non-viable cells stained blue (Freshney., 1987 pg 117).

Cultured cells used for experiments were plated (180 µl/well) in 96-well microtitre plates (Nunc, Denmark) at a cell density of approximately 30 000 cells/well. A background control, containing 180 µl of complete culture medium with the cells, was included in each experiment. Camptothecin, a DNA topoisomerase I inhibitor and a strong inducer of apoptosis (Liu *et al*., 2000) was used as a positive control at a concentration of 100 µM as per the protocol used for the cytotoxicity assessment in the National Cancer Institute(NCI) anticancer screening program (Monks *et al*., 1991). The plated cells were incubated at 37°C for 24 hours before exposure to the compounds in order for the cells to attach to the surface of the plates and to prevent the compounds from interfering with cell attachment.

2.2.3 Cryopreservation

Cryopreservation of cells was carried out according to Freshney (1987) with some modifications. The freezing solution consisted of 60 % complete culture medium, 30 % FBS and 10 % dimethyl sulphoxide (DMSO).

Cultures that were in the log phase of growth were trypsinised and centrifuged for 5 minutes. The supernatant was discarded and the cells were resuspended in the freezing solution at $5x10^6$ cells/ml. Aliquots of 0.8 ml were transferred into 1 ml plastic cryotubes (Nunc, Denmark). The cultures were cooled for 10 minutes at 4^oC, then placed in a $-20^{\circ C}$freezer for 3 hours and thereafter at $-70^{\circ C}$overnight. After freezing, the cryotubes were stored in liquid nitrogen.

2.3 Exposure of cultured cells to the synthetic compounds

The novel, synthetic compounds used in this study were obtained from the Department of Chemistry, University of the Witwatersrand. All compounds were characterized by NMR spectroscopy. The groups of compounds were arbitrarily called CDKs, CLs or JLPs after the initials of the manufacturing chemist**.** Stock

solutions of 1 mM were prepared in 0.1 % DMSO in water. The active compounds are tabulated in Tables 2.1 and Table 2.2. The non-active compounds that were screened are shown in Appendix A; Table A1, Table A2 and Table A3.

The cells were exposed to the different classes of synthetic compounds at a final concentration of 100 µM at 37°C for 24 hours. Compounds showing cytotoxicity at a concentration of 100 µM were tested further to obtain IC_{50} values. The IC_{50} value is the concentration of the compound where the cell viability of the cultured cells is reduced by 50 %. All tests were performed in triplicate.

Table 2.1 Chemical structures and names of the active novel nucleoside analogues

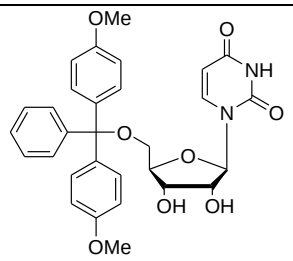
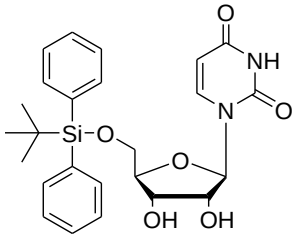
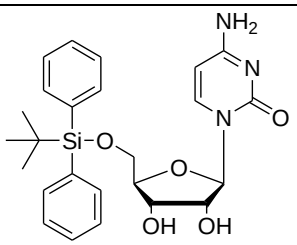
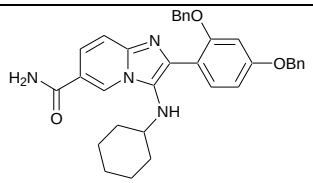
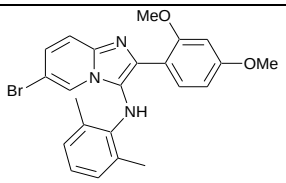
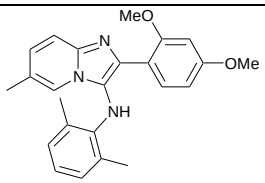
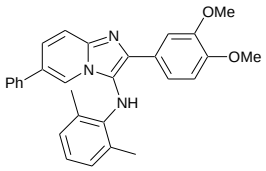
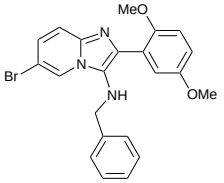
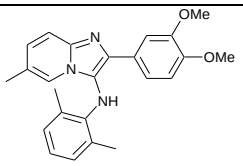
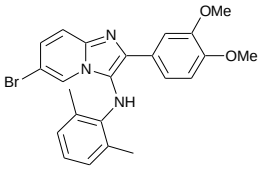
Group	Designated Name	Chemical Name	Structure
Nucleoside analogues	JLP 26.5	5'-(4,4-dimethoxytrityl)-uridine	 Mw: 546.200
	JLP 27.2	5'-tert-butyldiphenylsilyl uridine	 Mw: 482.187
	JLP 38.2	5'-tert-butyldiphenylsilyl cytidine	 Mw: 481.203

Table 2.2 Chemical structures and names of the active novel imidazo[1,2-a]pyridines

Imidazo[1,2-a]pyridines	CDK 3	2-(2,4-Bis-benzyloxyl-phenyl)-3-cyclohexylamino-imidazo[1,2-a]pyridine-6-carboxylic acid amide	 Mw: 546.66
	CDK 6	6-Bromo-2-(2,4-dimethoxy-phenyl)--imidazo[1,2a]pyridin-3-yl]-(2,6-dimethyl-phenyl)-amine	 Mw: 452.34
	CDK 8	[2-(2,4-Dimethoxy-phenyl)- -imidazo[1,2-a]pyridin-3-yl]-(2,6-dimethyl-phenyl)-amine	 Mw: 387.47
	CL 3	[2-(3,4-Dimethoxy-phenyl)-6-phenyl-imidazo[1,2-a]pyridin-3-yl]-(2,6-dimethyl-phenyl)-amine	 Mw: 449.54
	CL 4	Benzyl-[6-bromo-2-2,5 -dimethoxy-phenyl)-imidazo[1,2-a]pyridin-3-yl]-amine	 Mw: 438.32
	CL 5	[2-(3,4-Dimethoxy-phenyl)-6-methyl-imidazo[1,2-a]pyridin-3-yl]-(2,6-dimethyl-phenyl)-amine	 Mw: 387.47
	CL 6	6-Bromo-2-(3,4-dimethoxy-phenyl)-imidazo[1,2-a]pyridin-3-yl]-(2,6-dimethyl-phenyl)-amine	 Mw: 452.34

2.4 Measurement of cell viability

2.4.1 Cell proliferation assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT)

Many disease-orientated, *in vitro* screening procedures use a broad variety of human tumour cell lines and assess cytotoxicity through the use of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). The aim of the screen is to identify compounds showing activity against different tumour cell lines. (Carmichael, 1987).

The MTT cell proliferation assay is a quantitative, colorimetric assay for the measurement of cellular proliferation, viability and cytotoxicity (Berridge and Tan, 1993). The assay is based on cleavage of the yellow tetrazolium salt, MTT, which forms water-insoluble purple formazan crystals. This cleavage is catalysed by the mitochondrial enzyme, succinate-dehydrogenase that only occurs in viable cells with intact mitochondria (Mosmann, 1983). The water-insoluble formazan is then solubilised with an organic solvent such as dimethylsulfoxide (DMSO). The optical density is measured spectrophotometrically at 540 nm, yielding absorbance as a function of concentration of converted dye, which correlates to the number of metabolically active cells in the test sample.

The MTT assay was performed according to Carmichael *et al.* (1987) with some modifications. The cultured HT-29 and Caco-2 cells were plated in 96-well microtitre plates (Nunc, Denmark) at a density of approximately 30 000 cells in a volume of 180 µl/well and incubated for 24 hours before exposure to the compounds. The cells were exposed to 100 µM of the synthetic compounds for 24 hours. Fifty microlitres of 5mg/ml MTT (w/v) prepared in PBS, was added to each well after the 24 hours. The plates were incubated for a further 2 hours at 37 °C and centrifuged at 1107 x g for 5 minutes to collect the formazan crystals. Following centrifugation, 90 % of the medium was carefully aspirated and the formazan crystals were dissolved in 200 µl DMSO. The absorbance was then determined at 540 nm using the Labsystems iEMS Multiskan plate reader. The negative control used in the assay consisted of medium alone with cells, a blank with media only and a positive control was cells and camptothecin.

Since the metabolic activity between different cell lines varies, a calibration curve has to be performed for each cell line to determine the number of cells that will give spectrophotometric absorbances in the linear range (Carmichael *et al.*, 1987). Four calibration curves were determined for each cell line before performing the cytotoxicity assay (A representative calibration curve for each cell line is shown in Appendix B). Cell suspensions of 100 000, 75 000, 50 000, 25 000, 12 500 and 6125 cells/ml were prepared. The cells were processed for the MTT assay as described above.

The IC_{50} values for the novel compounds were obtained for the compounds showing more than a 50 % inhibition of cell viability at a concentration of 100 µM. The MTT assay was performed in triplicate and each concentration was tested in quadruplicate.

2.4.2 Analysis of results

The percentage cell viability in reference to the growth of the control sample (cells only, no test compound) of the various compounds was calculated as follows:

Equation 1:

$$\% \text{ Cell viability} = \frac{A_{540} \text{ test sample} - \text{Mean } A_{540} \text{ blank}}{\text{Mean } A_{540} \text{ negative control} - \text{Mean } A_{540} \text{ blank}} \times 100$$

Statistically significant differences between the control and experimental samples as well as between the different groups of compounds were determined using a Student's t-test with the significance set at 0.05. The values are expressed as mean ± standard error of the mean.

2.5 Leucocyte viability assay

The MTT assay was carried out on leucocytes exposed to the novel compounds for 24 hours in order to determine potential cytotoxic effects on these cells as opposed to the cancer cells. Drug-free, human peripheral blood was obtained from healthy volunteers at the University of the Witwatersrand Medical School. The isolation of human

peripheral white blood cells (WBC) was adapted from the Versagene Blood DNA Kit according to the manufacturer's protocol (Gentra Systems).

Six millilitres of whole blood collected in an anti-clotting factor- K_3EDTA containing vacutainer tube was transferred to a 15-ml centrifuge tube and diluted 1:3 times with Red Blood Cell (RBC) lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2-7.4). The tube was incubated for 5 minutes at room temperature and during this time the tube was inverted 5 times. After centrifugation at 2000 x g, for 10 minutes, the supernatant was decanted, leaving behind the visible WBC pellet. RBC lysis solution (500 µl) was then added to resuspend the human WBCs, and this step was repeated at least 5 times to lyse all RBCs. After centrifugation, the cells were suspended in 50 ml of RPMI-1640 (Gibco, Belgium) containing 2 mM L-glutamine, and supplemented with 5 % heat-inactivated fetal bovine serum (FBS) and 1 ml of a solution containing 10 kU penicillin G and 10 mg streptomycin sulphate.

The number of cells were counted in a haemocytometer using the trypan blue exclusion test as described in section 2.2.2. The cells were then plated in 96-well microtitre plates (Nunc, Denmark) at a density of approximately 30 000 cells in a volume of 180 µl/well. The MTT assay was carried out as described in section 2.4.1. A background control, containing 180 µl of complete culture medium with the cells, was included in each experiment. Camptothecin was used as a positive control at a final concentration of 100 µM.

2.6 Assessment of apoptosis

Numerous experimental techniques have been developed for the detection of apoptosis. There are a great number of factors involved in the apoptotic cascades. The caspases are believed to be the common executors for apoptosis. In this study, the activity of caspase-8 and caspase-3 was measured via a colorimetric assay. Changes to the mitochondrial membrane potential were detected using a cationic dye and analysed via flow cytometry. Cytochrome c levels in the mitochondria and cytosol were quantified via a human cytochrome c Elisa assay. A frequently used method to detect apoptosis relies on the detection of phosphatidylserine (PS) on the surface of the plasma membrane which is an early event in apoptosis. In this study, the exposure

of PS on the cell surface was detected with annexin V, a calcium-dependent PS-binding protein and was analysed by flow cytometry.

2.6.1 Colorimetric assay for caspase detection

The activation of caspases initiate apoptosis in mammalian cells. Caspase-3 cleaves a variety of proteins that contain the amino acid sequence Asp-Glu-Val-Asp (DEVD) whereas caspase-8 cleaves those proteins that contain the amino acid sequence Ile-Glu-Thr-Asp (IETD). Cells that were treated with the test compounds were lysed and the cell lysate was then tested for protease activity by the addition of a caspase-specific peptide that is conjugated to the chromophore p-nitroabilide (pNA) for the detection of caspase-3 and caspase-8 respectively. The cleavage of the peptide by the caspase releases the chromophore that is detected at an absorbance of 405 nm. The level of caspase enzymatic activity in the cell lysate is directly proportional to the absorbance at 405 nm. Caspase-3 and Caspase-8 were detected using the Caspase 3/CPP32 and Caspase-8/FLICE colorimetric Assay kits, respectively. The following procedure (obtained from Biovision research products) was performed.

The HT-29 and Caco-2 cells were plated in 24-well microtitre plates (Nunc, Denmark) at a density of 1.2 x 10^6 cells per well. A control containing complete culture medium with untreated cells was included in each experiment and camptothecin was used as a positive control at a final concentration of 100 µM. The cells were incubated at 37°C for 24 hours to allow the cells to adhere to the plate, and then treated with the test compounds for another 24 hours. The treated cells were then trypsinised, washed with PBS and transferred into sterile 1.5 ml eppendorf tubes.

The HT-29 and Caco-2 cells were resuspended in 50 µl of chilled cell lysis buffer (50 mM of an organic buffer, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 100 mM NaCl, 0.1 % of an anionic detergent, CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate), 1 mM of a reducing agent, DTT (dithiothreitol) and 100 mM EDTA, pH 7.4) and incubated on ice for 10 minutes. The lysed cells were transferred to 1.5 ml Eppendorf tubes, centrifuged for 1 minute (10000 x g) and the supernatant (cytosolic extract) was transferred to a fresh tube and placed on ice. The protein concentration of the cytosolic extract was assayed

using the Bradford assay as described in section 2.6.2. Fifty microlitres of a 2 x Reaction buffer (proprietary composition) containing 10 mM DTT was added to each sample. Respectively, 5 µl of 4 mM IETD-*p*NA substrate was added for the detection of Caspase-8 and 5 µl of 4 mM DEVD-*p*NA substrate was added for the detection of Caspase-3. The samples were incubated at 37°Cfor 1-2 hours and the absorbance was read at 405 nm in the Labsystems Multiskan iEMS MF plate reader. Time dependent studies were performed for caspase activity by harvesting cells at 2, 4, 8, 12, and 24 hours after initiation of treatment with the test compounds.

2.6.2 Protein estimation of the cytoplasmic and mitochondrial fractions

Measurement of the protein concentration was performed according to the Bradford Assay (BIO-RAD). This is a dye-binding assay based on the colour change of the dye in response to different concentrations of protein. The maximum absorbance for the acidic Coomasie brilliant Blue-G250 changes from a wavelength of 465 nm to 595 nm upon its binding to arginine and aromatic residues in proteins (Bradford., 1976). Standardized bovine serum albumin (BSA) (BIO-RAD) was used to construct a standard curve with concentrations of 0 µg, 0.6 µg, 1.25 µg, 2.5 µg, 5 µg and 10 µg. Dye reagent (0.2 ml) was added to each sample and the absorbance was measured at a wavelength of 595 nm. A standard curve was constructed for each protein determination (Example in Appendix B; Figure B4). The protein concentration of the cytoplasmic and mitochondrial fraction samples was determined from the standard curve.

2.6.3 Mitochondrial effects of test compounds

2.6.3.1 Mitochondrial membrane potential

When energy is released during the oxidation reactions in the mitochondrial respiratory chain, it is stored as a negative electrochemical gradient across the mitochondrial membrane. Under these situations, the mitochondrial membrane potential ($\Delta\psi m$) is polarized. The collapse of the $\Delta\psi m$ during the early stages of apoptosis was noticed in several studies and it has thus been suggested that the depolarisation of the mitochondria is one of the first events which occurs in apoptosis and could be a prerequisite for the release of cytochrome c (Shimuzu *et al*., 1999).

The mitochondrial-specific cationic dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazol-carbocyanine iodide (JC-1) was used to measure the electrochemical gradient across the mitochondrial membrane (Salvioli *et al*., 1997).

The HT-29 and Caco-2 cells were plated in 24-well microtitre plates (Nunc, Denmark) at a density of 1.2 x 10^6 cells per well. A control, containing complete culture medium with untreated cells was included in each experiment and camptothecin was used as a positive control at a final concentration of 100 µM. The cells were incubated at 37°C for 24 hours to allow the cells to adhere to the plate, and then treated with the test compounds for another 24 hours. The treated cells were then trypsinised, washed with PBS and transferred into sterile 1.5 ml Eppendorf tubes.

The cells were centrifuged at 400 x g for 5 minutes and the supernatant was discarded. Half a millilitre of freshly prepared JC-1 dye (2 µM) working solution (125 µl JC-1, 12.375 ml prewarmed 1 x assay buffer (proprietary composition) was added to each pellet (0.5 ml of the JC-1 working solution was required for each sample). The cells were resuspended in the JC-1 working solution and then incubated for 15 min at 37°C in a CO_2 incubator. The cells were washed twice in 1x assay buffer and centrifuged at 400 x g for 5 minutes after each washing step. Each cell pellet was resuspended in 0.5 ml of assay buffer and the cells were analysed on a BD FACS Calibur flow cytometer using excitation/emission filters of 485/540 nm (green/FL-1); 540/590 nm (red/FL-2). The ratio of red/green fluorescence was calculated. The JC-1 dye accumulates in the mitochondria of healthy cells as fluorescent red aggregates. At the start of apoptosis, the mitochondrial membrane potential collapses and the JC-1 dye can no longer accumulate in the mitochondria and remains in the cytoplasm in a monomeric form with green flourescence. The mitochondria containing red JC-1 aggregates in healthy cells were detected in the FL 2 channel (R2 area in the scattergrams), whereas green JC-1 monomers in apoptotic cells were detected in the FL 1 channel (R3 area in the scattergrams) (Reers *et al*., 1995).

2.6.3.2 Measurement of Cytochrome c

Cytochrome c is an electron transport protein which is located between the inner and outer mitochondrial membrane. Cytochrome c is released from the mitochondria to

the cytoplasm in response to pro-apoptotic stimuli. The release of cytochrome c is part of the cascade of cellular events that leads to apoptosis. In the cytoplasm, the binding of cytochrome c to Apaf-1 (apoptotic protease activating factor-1), results in the activation of the procaspase-9. Subsequently, the active caspase-9 triggers the downstream effector caspases such as caspase-3 which leads to apoptosis (Elmore., 2007).

An amount of cytochrome c in the cell lysates was quantified by a human cytochrome c Elisa assay. The kit (Titerzyme EIA, Assay Designs Inc) uses a monoclonal antibody specific for cytochrome c, a biotinylated detecting antibody and an alkaline phosphatase-conjugated Streptavidin to provide a colorimetric detection that enables a quantitative determination of the cytochrome c in the cell lysates. The treated HT-29 and Caco-2 cells were harvested and rinsed with ice-cold phosphate buffered saline. The cytosolic and mitochondrial proteins were isolated respectively according to the protocol provided by the Mitochondria Isolation Kit (Assay Designs, Inc).

The HT-29 or Caco-2 cells were used at a density of 1.7 million cells/ml. The cells were harvested with 1 ml 0.25 % trypsin containing 0.1 % EDTA (ethylene diamine tetra-acetic acid) and 1 ml of a 1 x PBS solution. The cells were centrifuged at 800 x g for 5 minutes and the supernatant was discarded. The resulting cell pellet was resuspended in 5 ml PBS solution. The cells were collected by centrifugation at 1000 x g for 5 minutes and the supernatant was discarded. The cell pellet was resuspended with 100 µl of a cell permeabilization buffer (250 mM sucrose, 137 mM NaCl, 70 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM K_2HPO_4 , 0.2 mg/ml Digitonin and 0.1 % Hydorol M) vortexed and incubated on ice for 5 minutes at 4ºC. Cells were then centrifuged at 1000 x g for 5 minutes at 4ºC. The supernatants were frozen since they contained the cytosolic fraction of cytochrome c and the remaining pellet was resuspended with Radio Immunoprecipitation Assay (RIPA) cell lysis buffer 2 (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1mM EDTA, 1mM EGTA, 1 % Triton X-100, 1 % sodium deoxylate and 0.1 % sodium dodecyl sulfate (SDS)), vortexed and incubated on ice for 15 minutes. The lysate was vortexed and centrifuged at 10 000XG for 10 minutes at 4ºC. The protein concentration from each fraction was determined by the Bio-Rad protein assay. The cytosolic and mitochondrial fractions were used in the Elisa assay.

A human cytochrome c standard was provided in the kit in order to create a standard curve. The capture antibody was immobilized on the plate. The standard was solubilised in the provided Assay Buffer (proprietary composition) to create a range of cytochrome c standard dilutions with final concentrations of 900, 450, 225, 112.5, 56.25 and 28.13 pg/ml. The assay buffer, standards and samples (100 µl) were pipetted into the appropriate wells on the provided 96 well microtitre plate. The plate was sealed and incubated at room temperature on a plate shaker for 1 hour at 500 rpm to allow for binding of the cytochrome c specific monoclonal antibody in the plates to the cytochrome c in the lysates. Following this, the contents of the wells were carefully emptied and 400 µl of wash solution (proprietary composition) was added to each well. The wash solution is used to remove any unbound proteins. The wash was repeated 3 times for a total of 4 washes. After the final wash, 100 µl of a solution containing a biotinylated monoclonal antibody to cytochrome c, was pipetted into each well except the blank. The plate was sealed and incubated at room temperature on a plate shaker for 1 hour at 500 rpm to allow for binding of the cytochrome c that is captured in the plate. The contents of the wells were removed and the wash step was repeated. One hundred microlitres of a solution of streptavidin conjugated to alkaline phosphatase was added to each well except the blank and the plate was sealed and incubated at room temperature on a plate shaker for 30 minutes at 500 rpm. This step allowed for binding of the cytochrome c that is captured in the plate (See Figure 2.1 for a flow diagram of the methodology and Figure 2.2 for a schematic representation of the Elisa Plate).

The content of the well was removed and the wash step was repeated. Substrate solution (100 µl) containing p-nitrophenyl phosphate (*p*Npp) was added to each well and incubated at room temperature on a plate shaker for 45 minutes at 500 rpm. A stop solution (25 µl) consisting of trisodium phosphate (proprietary concentration) in sterile water was added to each well and the optical density was measured at 405nm (Figure 2.3).

The absorbances of the standard dilutions at a wavelength of 405 nm minus the blank were used to create a standard curve (Appendix B; Figure B5) of Absorbance (405 nm) versus cytochrome c concentration (pg/ml). The cytosolic and mitochondrial cytochrome c concentrations of the treated cells were determined from the standard curve.

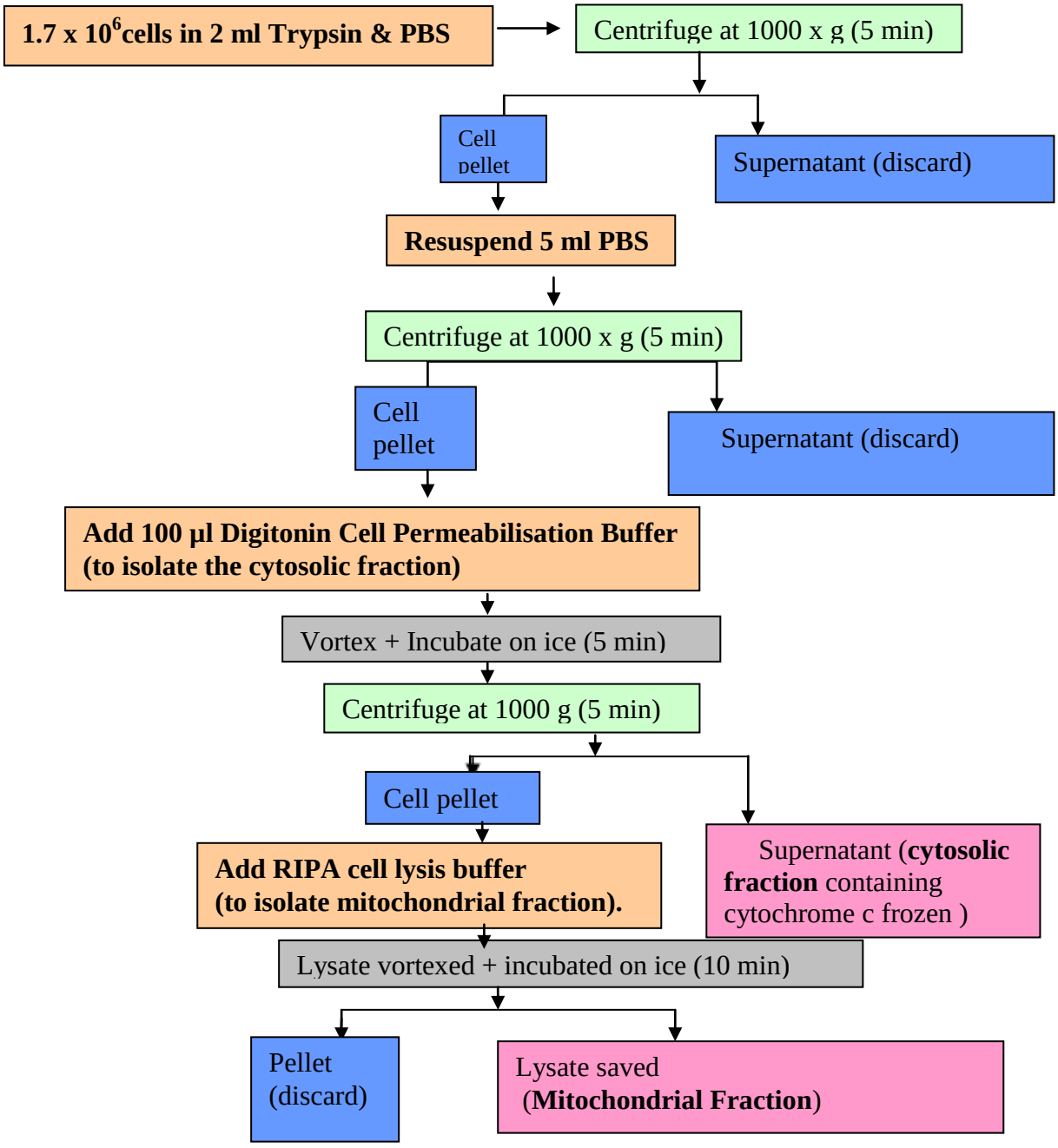


Figure 2.1 **Flow diagram showing the preparation of the cytosolic and mitochondrial fraction**

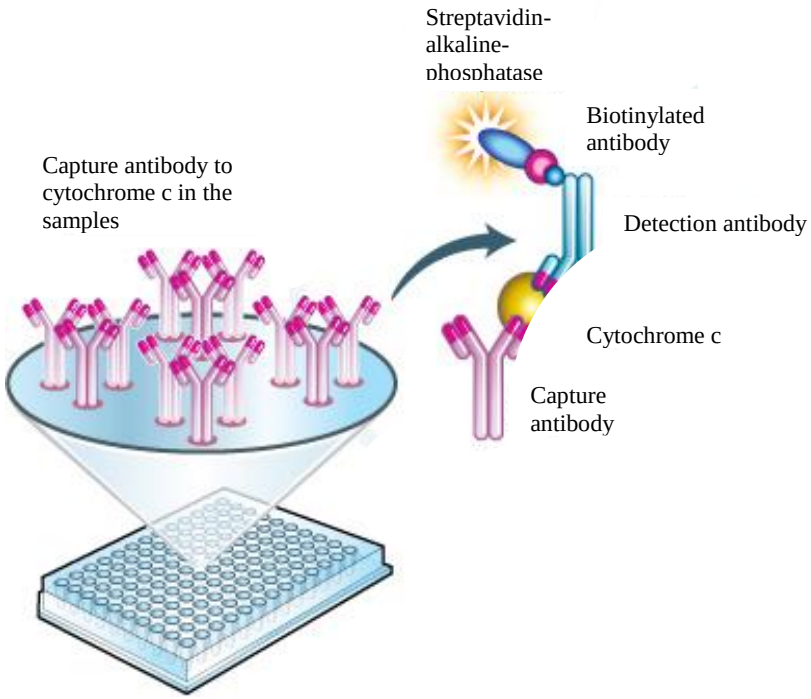


Figure 2.2 **Schematic representation of the Elisa Plate** (modified from http://www.abcam.com/index.html?pageconfig=resource&rid=13855)

ELISA

Incubate 100 µl Cytochrome c standard or sample in plate for 1 hour at RT (Plate Shaker – 500 rpm) to allow for binding of the cytochrome c specific monoclonal antibody in the plates

Aspirate and Wash 4 x

Incubate 100 µl Biotinylated monoclonal antibody in plate for 1 hour at RT (Plate Shaker – 500 rpm) to allow for binding of the cytochrome c that is captured in the plate

Aspirate and Wash 4 x

Incubate 100 µl Streptavidin conjugated to alkaline phosphatase in plate for 30 min at RT
(Plate Shaker – 500 rpm) to allow for binding of the biotinylated monoclonal antibody

Aspirate and Wash 4 x

Incubate 100 µl Substrate solution containing p-nitrophenyl phosphate for 45 minutes at RT

Add 25 µl Stop Solution and Read at 405nm

Figure 2.3 **Schematic diagram of the procedural steps of the ELISA**

2.6.4 Annexin V

One of the most important stages of apoptosis involves the surface changes by dying cells that would eventually result in the uptake of these cells by phagocytes. Many studies have shown that cells undergoing apoptosis break up the phospholipid symmetry of their plasma cell membrane and expose phosphatidylserine (PS), which is translocated to the outer layer of the membrane.

Annexin V, a cellular protein in the annexin group, is a useful tool in detecting apoptotic cells since it binds preferentially to negatively charged phospholipids such as PS. The translocation of PS occurs in both necrosis and apoptosis; hence Annexin V was combined with propidium iodide (PI). Propidium iodide is a fluorescent dye that binds to DNA of non-viable cells. Propidium iodide does not cross the plasma membrane of viable cells or cells that are in the early stages of apoptosis. Cells that are in the late stages of apoptosis or are already dead have lost their plasma membrane integrity and are more permeable to PI. The cell staining was assessed using fluorescein isothiocyanate (FITC)-labelled Annexin V (green fluorescence) as well as dye exclusion of propidium iodide (PI) (negative for red fluorescence). It is possible to detect and quantitate the apoptotic cells on a single-cell basis by flow cytometry and to identify the intact cells (FITC-PI-), early apoptotic (FITC+PI-), late apoptotic or necrotic cells (FITC+PI+) (Vermes *et al*., 1995).

The method as described by Vermes *et al*, (1995) was used. The treated cells were trypsinised and washed with a 1 x PBS solution (pH 7.4). FITC-Annexin V was diluted to a concentration of 1 mg/ml in binding buffer (proprietary composition) and the cells were resuspended in 1 ml of this solution. The binding buffer contains a 2.5 mM concentration of $CaCl_2$ which is required for the annexin V dye to bind to the phosphotidylserine (PS) on the cell surface. Thereafter, the cells were incubated for 10 minutes in the dark at room temperature. Propidium iodide was added to the cell suspension to give a final concentration of 1 mg/ml. The cells were analysed by flow cytometry and the data was displayed as a two-color dot plot with FITC-Annexin V (green fluorescence, X axis) vs. PI (red fluorescence, Y axis).

2.7 Measurement of cell differentiation – the alkaline-phosphatase assay

The most commonly used marker for small intestinal differentiation is alkaline phosphatase (Palmer., 1995 pg 10). Intestinal alkaline phosphatase belongs to the family of metalloenzymes which are anchored to the membrane via glyco-phosphatidylinositol. It catalyzes the hydrolysis of monophosphate esters from nucleotides, proteins and alkaloids in an alkaline medium and results in the formation of an organic radical and inorganic phosphate.

Low levels of this enzyme have been found in undifferentiated HT-29 cells, whereas an increased level has been observed in differentiated HT-29 cells (Pintu *et al*., 1982). Determination of activity was based on the use of *p*-nitrophenylphosphate disodium salt (*p*NPP) (Merck, Germany) as a substrate. During the enzymatic reaction, *p*NPP reacts with alkaline phosphatase and is converted to a yellow substance *p*-nitrophenol, which absorbs light at a wavelength of 412 nm (Bergmeyer, 1983 pp 269-270).

$$\text{p-Nitrophenylphosphate} + H_2O \xrightarrow{\text{alkaline phosphatase}} \text{p-Nitrophenol} + P_i$$

The alkaline phosphatase assay by Bergmeyer (1974) was used in this study. The HT-29 and Caco-2 cells were seeded at a density of 5000 cells/well. A background control, containing 180 µl of complete culture medium with the cells, was included in each experiment. The cells were incubated, at 37°C, in the presence of 5 mM acetoacetate (a known inducer of cell differentiation) as a positive control and 100 µM of the test compounds. A concentration of 5 mM acetoacetate is similar to the concentration that is normally found in the colon. Studies by Rogers (1983) showed that after three days *in situ*, intestinal cells are still dividing. In the artificial environment produced within the tissue culture flasks, chromosomal changes begin in preparation for differentiation at day 4; hence the choice of day 4 assays for HT-29 cells. On day 1 (24 hours post addition of compounds and acetoacetate), days 4 and 8 for HT-29 cells and days 1, 3 and 8 for the Caco-2 cells, cell counts were performed using trypan blue and the MTT assay. These counts were used in order to calculate the activity which is expressed per 5000 cells. The culture medium was not replaced during the 8 day incubation period and the surviving cells were corrected for each assay.

On days 1, 4 and 8 for HT-29 cells and days 1, 3 and 8 for the Caco-2 cells, the cells were washed twice with deionised water. No refeeding occurred during the 8 days. To each well 26 µl of 1 mM $MgCl_2$ and 200 µl of glycine buffer (0.1 M, pH 10.5, 1mM $MgCl_2$, 0.1 mM $ZnCl_2$) were added. At zero time, 40 µl of 0.03 M *p*NPP was added to each well. The reaction was left at room temperature for 5 minutes, after which the absorbance was read at 412 nm, using the Labsystems Multiskan iEMS MF plate reader against a blank consisting of reagent mixture without cells. Commercial alkaline phosphatase was used to construct a standard curve (Appendix B, Figure B6).

Activity was calculated as follows: the concentration in mU/ml of alkaline phosphatase was calculated using a standard curve. Since a linear relationship exists between the standard curve for alkaline phosphatase (mU/ml) and *p*-nitrophenyl (µmol/l), the levels could be expressed as µmol/l of *p*-nitrophenyl/min. One unit of alkaline phosphatase will hydrolyze 1.0 µmole of *p*-nitrophenyl phosphate per minute at pH 8.8 at 37°C. Before assaying, the surviving cells were counted in order to express the alkaline phosphatase activity per 5000 cells.

III. Results

3.1 The effect of the novel compounds on the survival of the HT-29 and Caco-2 cells.

3.1.1 IC_{50} values of the CDK group of imidazo[1,2-a]pyridines

Cell viability was determined by the MTT assay after the HT-29 and Caco-2 cells were exposed to the novel compounds for 24 hours at a concentration of 100 µM. Camptothecin was used as a positive control for cytotoxicity. IC_{50} values (calculated from log dose response curves) were obtained for the compounds showing more than 50% inhibition of growth at 100 µM. The results are shown from Figure 3.1.1 to Figure 3.1.6.

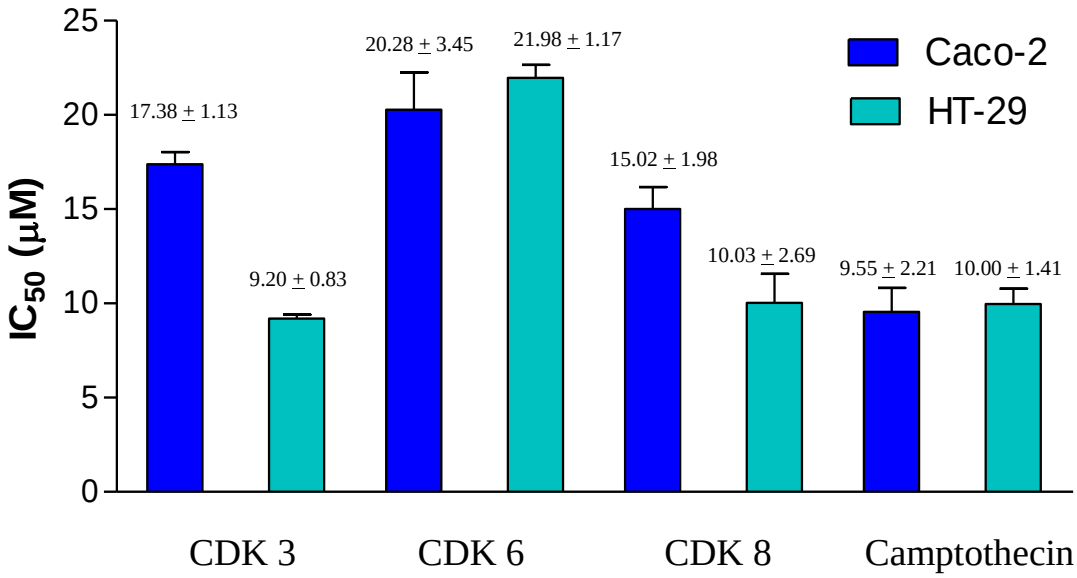


Figure 3.1.1 IC_{50} values for the CDK derivatives and the positive control camptothecin on the Caco-2 and HT-29 cell line. The bars represent the mean ± SEM of the IC_{50} values from three MTT assays carried out for each derivative. The HT-29 and Caco-2 cells were exposed to CDK concentrations ranging from 5 µM to 100 µM for 24 hours.

3.1.2 Sigmoidal dose response curves of the CDK group of imidazo[1,2-a]pyridines

Sigmoidal dose response curves were obtained from the MTT assays that were used to calculate the IC_{50} values of the CDK group of imidazo[1,2-a]pyridines on the HT-29 and Caco-2 cells. Each curve is a representative of at least three dose response curves.

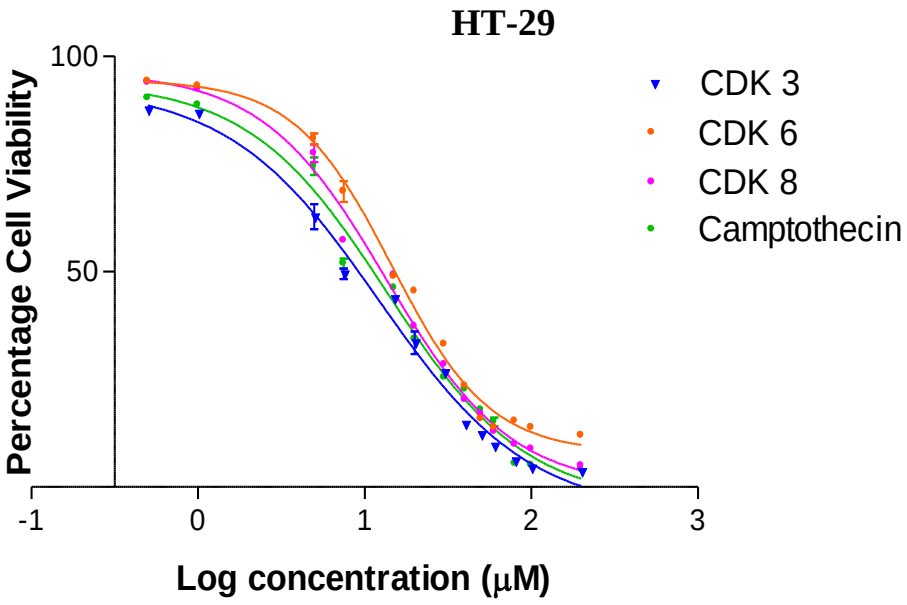


Figure 3.1.2 Dose-response curves of the CDK imidazo[1,2-a]pyridine compounds and camptothecin in the HT-29 cell line. IC_{50} values of these curves are: CDK 3, 9.20 ± 0.83 μM; CDK 6, 21.98 ± 1.17 μM; CDK 8, 10.03 ± 2.69 and camptothecin 10.00 ± 0.41 μM . Each point represents the mean and standard error of the triplicate values of the HT-29 cell viability for each CDK concentration.

In the HT-29 cell line, no significant difference ($p > 0.05$, $p = 0.665$) was detected between the test compounds (CDK 3 and CDK 8) and camptothecin indicating that the test compounds have a similar effect to camptothecin. The HT-29 cells were the least sensitive to CDK 6 with an IC_{50} value of 21.98 μM (Figure 3.1.2).

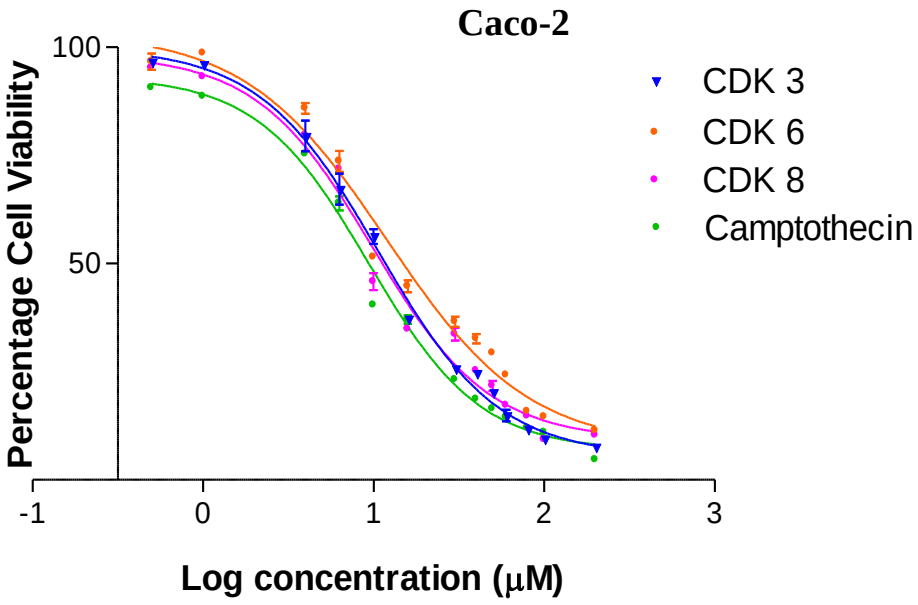


Figure 3.1.3 Dose-response curves of the CDK imidazo[1,2-a]pyridine compounds and camptothecin on the Caco-2 cell line. IC_{50} values of these curves are: CDK 3, 17.38 ± 1.13 μM; CDK 6, 20.28 ± 3.45 μM; CDK 8, 15.02 ± 1.98 and camptothecin 9.55 ± 0.21 μM. Each point represents the mean and standard error of the triplicate values of the Caco-2 cell viability for each CDK concentration.

After a 24 hour treatment with the active compounds, the IC_{50} values determined for the CDK compounds on the Caco-2 cell line were significantly higher ($p < 0.05$, p = 0.001) than that of camptothecin (9.55 ± 2.21 μM) indicating a lower cytotoxic activity. The Caco-2 cells were most sensitive to CDK 8 (IC_{50} = 15.02 ± 2.69 μM) and least sensitive to CDK 6 (IC_{50} = 20.28 ± 3.45). The IC_{50} of CDK 3 was 17.38 ± 1.13 μM. In comparison with the HT-29 cells, the Caco-2 cells were less sensitive to the CDK compounds with the exception of CDK 6 which was similar to the effect on the HT-29 cells. The IC_{50} of CDK 6 was more than double that of camptothecin (Figure 3.1.3).

3.1.3 IC_{50} values of the CL group of imidazo[1,2-a]pyridines

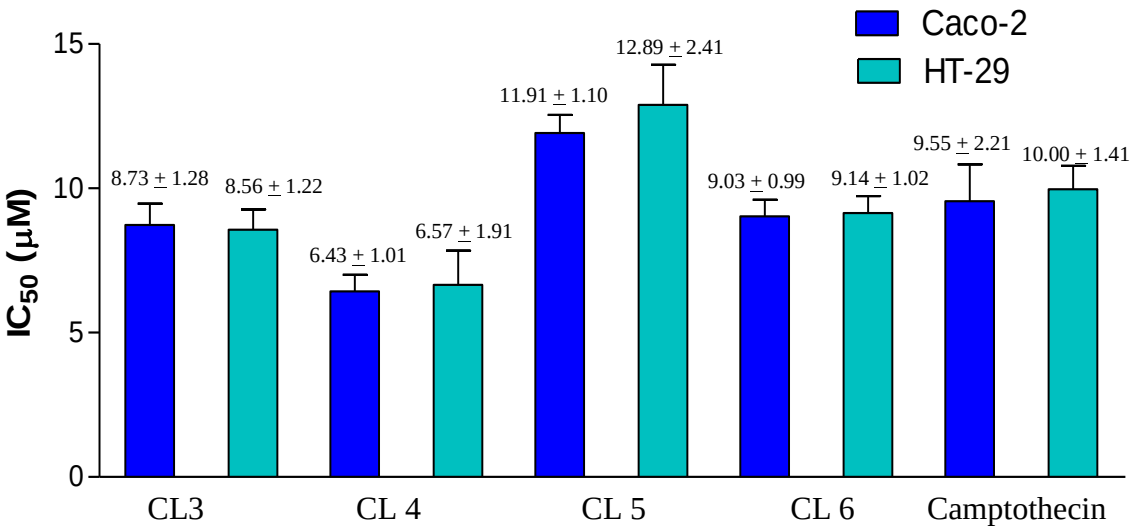


Figure 3.1.4 IC_{50} values for the CL derivatives and the positive control camptothecin on the Caco-2 and HT-29 cell line. The bars represent the mean ± SEM of the IC_{50} values from three MTT assays carried out for each derivative. The HT-29 and Caco-2 cells were exposed to CL concentrations ranging from 5 µM to 100 µM for 24 hours.

Figure 3.1.4 summarizes the IC_{50} values obtained for the CL group of imidazo[1,2-a]pyridines and the positive control camptothecin on both cell lines. CL 4 was a more effective cytotoxic agent than camptothecin and the other CL group of compounds in reducing HT-29 cell viability (Figure 3.1.5 and Figure 3.1.6). There was no significant difference ($p > 0.05$, $p = 0.107$) between the IC_{50} values of CL 3, CL 6 and camptothecin. The HT-29 cells were least sensitive to CL 5 ($IC_{50} = 12.89 \pm 2.41$ µM).

The Caco-2 cells were the most sensitive to CL 4 compared to the other CL compounds (Figure 3.1.6). The compound CL 5 was the least effective on the Caco-2 cells ($IC_{50} = 11.91 \pm 1.10$ µM). There was no significant difference between the IC_{50} values of CL 3, CL 4, CL 6 and camptothecin ($p > 0.05$).

3.1.4 Sigmoidal dose response curves of the CL group of compounds

Figure 3.1.5 and Figure 3.1.6, show the dose response curves of the CL compounds on the HT-29 and the Caco-2 cell line

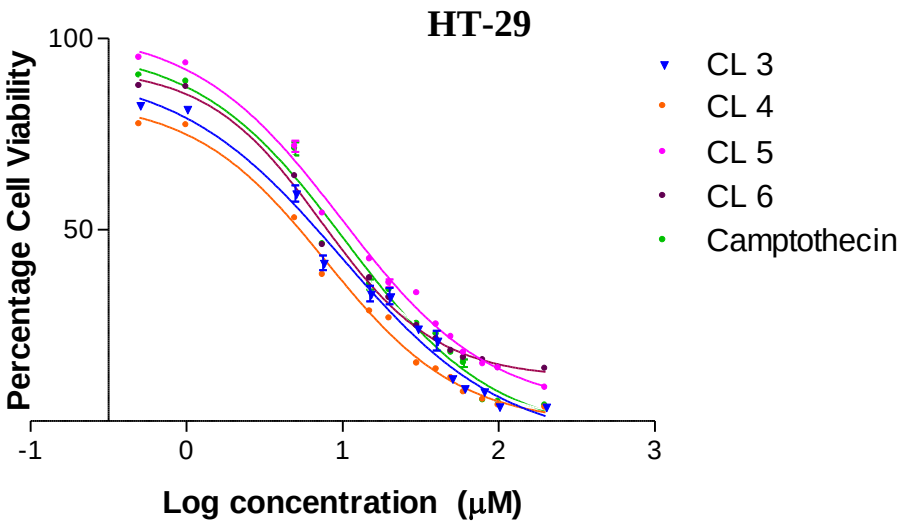


Figure 3.1.5 Dose-response curves of the CL imidazo[1,2-a]pyridine compounds on the HT-29 cell line. IC_{50} values of these curves are: CL 3, 8.56 ± 1.22 µM; CL 4, 6.57 ± 1.91 µM; CL 5, 12.89 ± 2.41 CL 6, 9.14 ± 1.02 µM and camptothecin 10.00 ± 0.41 µM. Each point represents the mean and standard error of the triplicate values of the HT-29 cell viability for each CL concentration.

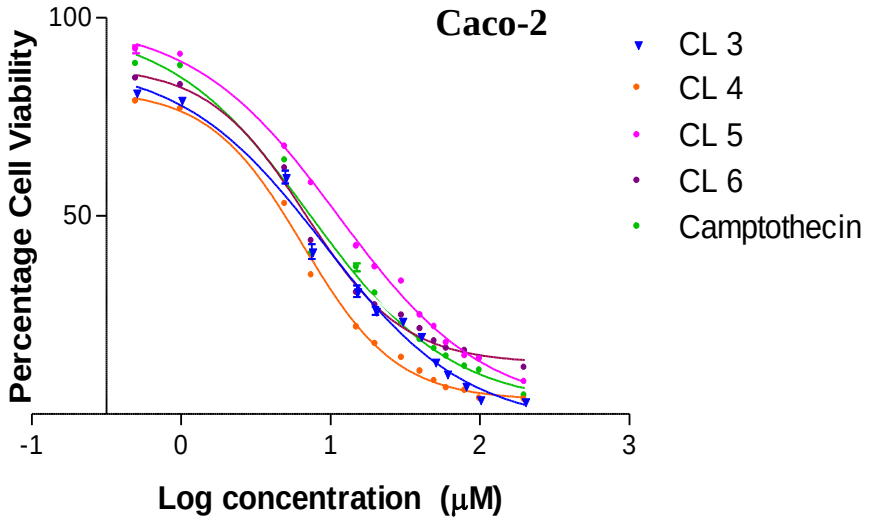


Figure 3.1.6 Dose-response curves of the four CL imidazo[1,2-a]pyridine compounds on the Caco-2 cell line. IC_{50} values of these curves are: CL 3, 8.73 ± 1.28 µM; CL 4, 6.43 ± 1.01 µM; CL 5, 11.91 ± 1.10 CL 6, 9.03 ± 0.99 µM and camptothecin 9.55 ± 0.1 µM. Each point represents the mean and standard error of the triplicate values of the Caco-2 cell viability for each CL concentration.

3.1.5 IC_{50} values of the JLP nucleoside analogues

Figure 3.1.7 summarizes the IC_{50} values obtained for the JLP compounds and the positive control camptothecin. The IC_{50} values obtained for the HT-29 cells were lower than those obtained for the Caco-2 cells indicating that the HT-29 cells were more sensitive to this group of compounds but they were less effective than camptothecin.

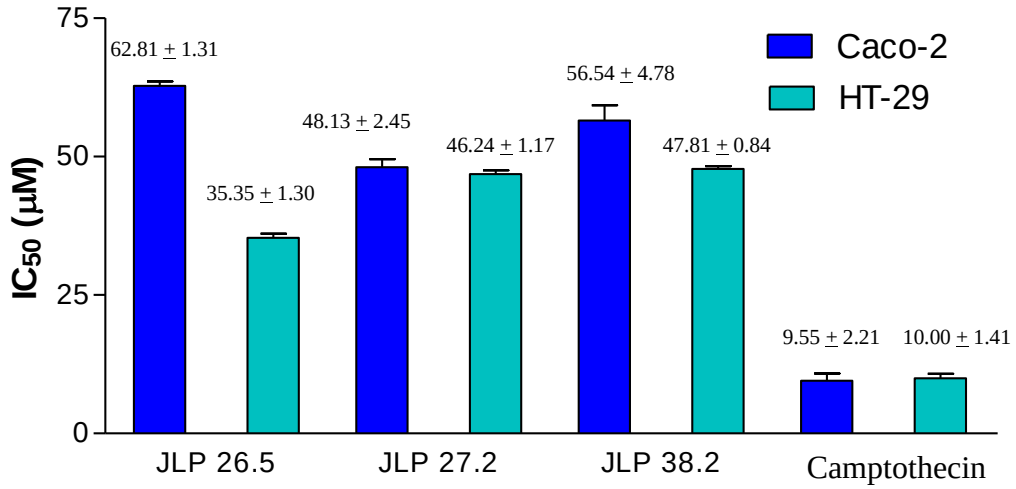


Figure 3.1.7 IC_{50} values for the JLP nucleoside analogues and the positive control camptothecin on the Caco-2 and HT-29 cell lines. The bars represent the mean ± SEM of the IC_{50} values from three MTT assays carried out for each derivative. The HT-29 and Caco-2 cells were exposed to JLP concentrations ranging from 5 µM to 100 µM.

In the HT-29 cells, treatment with JLP 26.5 caused more inhibition of cell viability when compared to JLP 27.2 and JLP 38.2 (Figure 3.1.7 and 3.1.8). However, camptothecin was between 3.5 and 6 times more active than any of the JLP compounds ($p < 0.05$, $p = 0.00002$). There was no significant difference between the IC_{50} values of JLP 27.2 and JLP 38.2 ($p > 0.05$, $p = 0.139$).

In the Caco-2 cells, treatment with JLP 27.2 caused a greater inhibition of cell viability when compared to JLP 26.5 and JLP 38.2 (Figure 3.1.5 and 3.1.5.2). Camptothecin was more effective than all the JLP compounds. There was no significant difference between the IC_{50} values of JLP 26.5 and JLP 38.2 ($p > 0.05$, $p = 0.143$). The JLP compounds were less effective than camptothecin in both Caco-2 and HT-29 cells.

3.1.6 Dose response curves of the JLP compounds

The sigmoidal dose response curves of the JLP compounds are shown in Figure 3.1.8 and Figure 3.1.9. The JLP nucleoside analogues were between 2.5 and 4 times less effective in inhibiting cell growth of the HT-29 and Caco-2 cells compared to camptothecin.

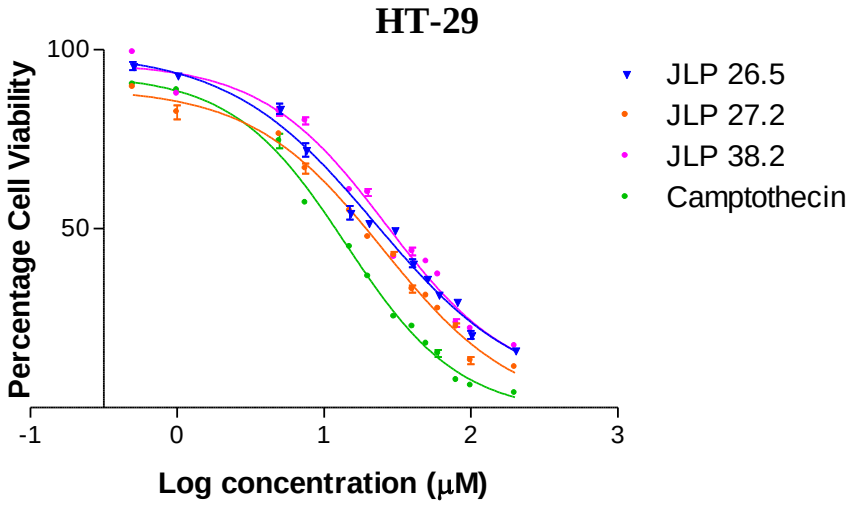


Figure 3.1.8 Dose-response curves of the JLP nucleoside analogues and camptothecin on the HT-29 cell line. IC_{50} values of these curves are: JLP 26.5, 35.35 ± 1.30 μM; JLP 27.2, 46.24 ± 1.17 μM; JLP 38.2, 47.81 ± 0.84. Each point represents the mean and standard error of the triplicate values of the HT-29 cell viability for each JLP concentration.

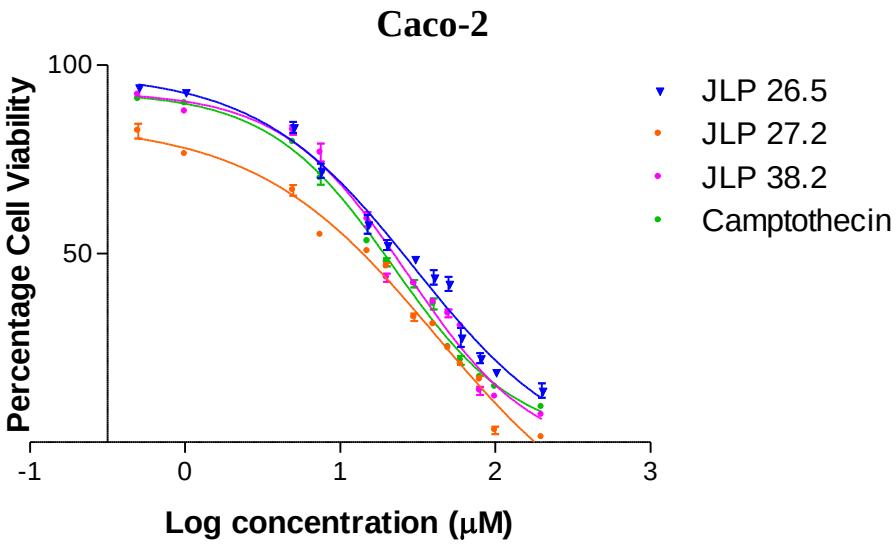


Figure 3.1.9 Dose-response curves of the JLP nucleoside analogues on the Caco-2 cell line. IC_{50} values of these curves are: JLP 26.5, 62.81 ± 1.31 μM; JLP 27.2, 48.13 ± 2.45 μM; JLP 38.2, 65.64 ± 4.78. Each point represents the mean and standard deviation of the triplicate values of the Caco-2 cell viability for each JLP concentration.

3.2 The effect of the novel compounds on leucocyte viability

The effect of the novel compounds on leucocyte viability was performed to determine whether the novel compounds exhibit any cytotoxic activity to normal, unstimulated, white blood cells as opposed to the cancer cells. Viability was determined by the MTT assay after the leucocytes were exposed to the novel compounds for 24 hours at a final concentration of 100 µM. Camptothecin was used as a positive control for measuring cytotoxicity. The assay was done in triplicate and the results obtained are summarized in Figure 3.2.

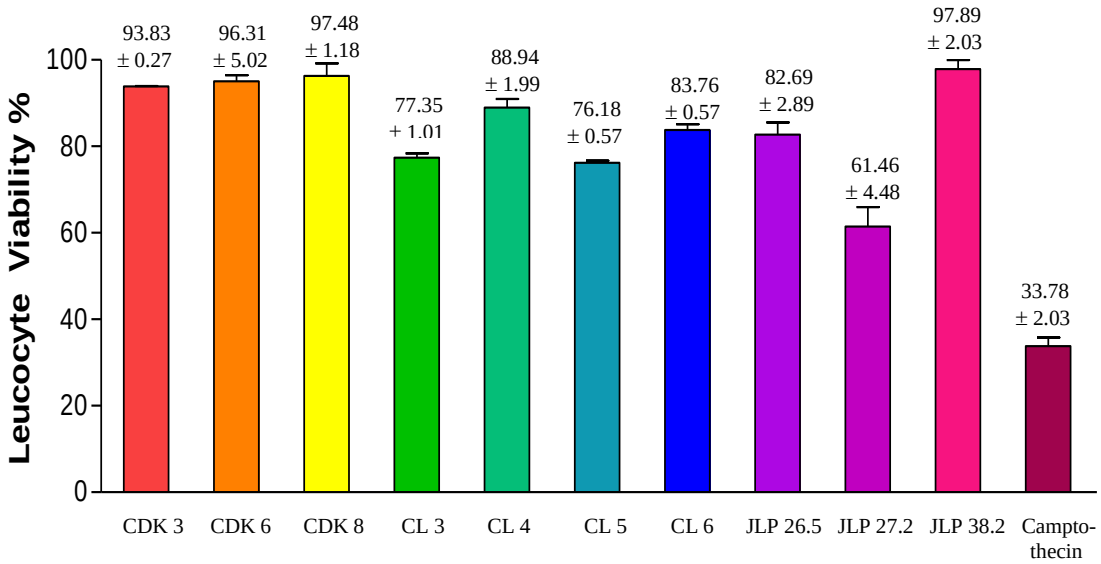


Figure 3.2 Percentage cell survival of leucocytes exposed to the test compounds and camptothecin. The bars represent the mean ± SEM of the % leucocyte survival from three MTT assays carried out for each compound. The leucocytes were exposed to the test compounds at a concentration of 100 µM for 24 hours.

Camptothecin was observed to be significantly ($p < 0.05$) more toxic to the leucocytes than all the compounds tested (Table 3.2.1). After 24 hours, treatment with camptothecin at 100 µM, led to the greatest decrease in cell viability with only 33.782 ± 2.031 % of leucocytes surviving. Of all the tested compounds, JLP 27.2 caused the greatest inhibitory effect of leucocyte viability (61.456 ± 4.481 %). The CL group of imidazo[1,2-a]pyridines were more toxic than the CDK group although these effects were lower than the effects of camptothecin.

3.3 Induction of caspase-8 and caspase-3 activity by the novel compounds

Caspases are proteolytic enzymes and their activation is a specific feature of apoptosis. Caspase-8, an initiator caspase, cleaves and activates the effector caspase-3 which cleaves proteins in apoptotic cells (Kawai *et al*., 2004). For this reason, the caspase-8 activity is always referred to first, followed by the caspase-3 activity. The activity of caspase-8 and caspase-3 was determined in cells treated with 100 µM of the active test compounds since this was the concentration where a definite cytotoxic effect was observed after 24 hours. Caspase activity was measured over a period of 24 hours with samples taken at 2 hours, 4 hours, 8 hours, 12 hours and 24 hours.

Caspase-8 and caspase-3 activity was measured as described in the Materials and Methods section 2.6.1. Caspase activity in the cell lysate is directly proportional to the absorbance of p-nitroabilide (pNA). Results of caspase activity are presented as graphs showing absorbance versus time (Figure 3.3.1 – Figure 3.3.6) and in tables showing the percentage increase relative to the untreated cells (Table 3.3.1 – Table 3.3.6). The effect of camptothecin on the caspases is shown in Figure 3.3.1d for the HT-29 cells and figure 3.3.2d for the Caco-2 cells.

3.3.1 Caspase activity in HT-29 cells

3.3.1.1 Effect of the CDK imidazo[1,2-a]pyridine compounds on caspase activity in HT-29 cells.

Figure 3.3.1 illustrates the induction of caspase-8 and caspase-3 activity in the HT-29 cells after treatment with 100 µM of the CDK imidazo[1,2-a]pyridines and camptothecin. After two hours, all compounds showed an increase in caspase-8 activity relative to the control with CDK 6 being the highest (220.8 %). However, the activity was not as high as after treatment with camptothecin (233.3 %). At 24 hours, after treatment with CDK 3 and CDK 8, caspase-8 levels returned to that of the control. In contrast, after treatment with CDK 6, caspase-8 levels remained elevated (119 %) above that of the untreated cells and camptothecin (Table 3.3.1a).

CDK 6 caused the greatest increase in caspase-3 activity (122 %) above that of the control at 2 hours when compared to CDK 3 (50.7 %) as well as CDK 8 (-9.4 %). However, the caspase-3 activity was not as high after treatment with camptothecin which caused a 172 % increase above the control.

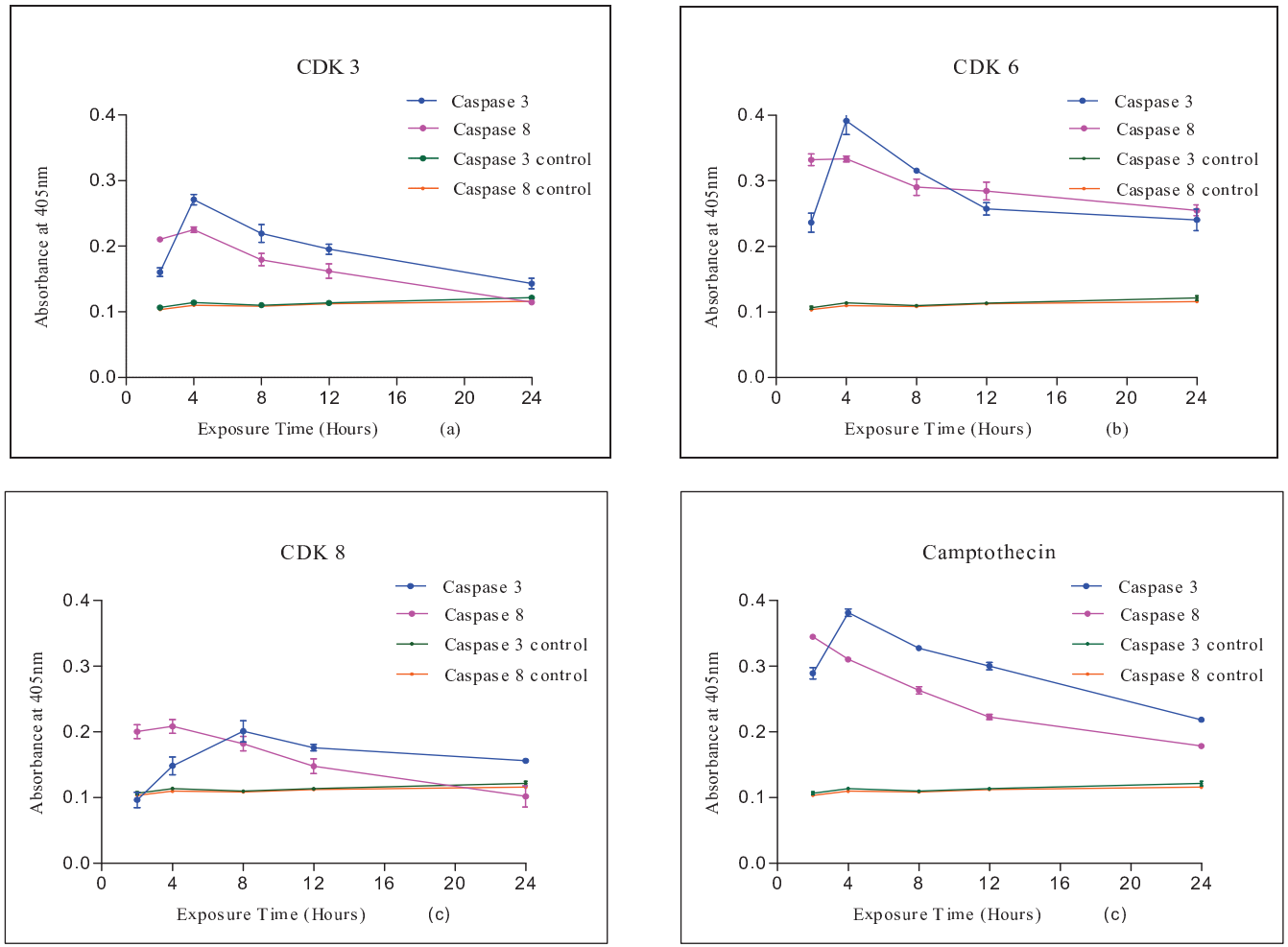
The results show that CDK 8 was the weakest inducer of caspase-8 and caspase-3 activity whereas CDK 6 was the strongest inducer (Table 3.3.1a and 3.3.1b) and maintained the elevated caspase levels at 24 hours. CDK 6 induction of caspase-8 activity compares well to that of camptothecin although the caspase-8 activity remained elevated for longer than camptothecin and CDK 6 induction of caspase-3 activity was very similar to camptothecin at all time periods.

Table 3.3.1a **Percentage increase in caspase-8 activity relative to the control in the HT-29 cells after cell exposure to the CDK group of imidazo[1,2-a]pyridines**

Compound	**% Increase in caspase-8 activity relative to the control**				
	2 hours	4 hours	8 hours	12 hours	24 hours
CDK 3	103.4	104.6	65.4	44.0	-1.3
CDK 6	220.8	203.2	167.7	152.9	119.8
CDK 8	93.7	89.6	67.7	31.6	-12.1
Camptothecin	233.3	182.3	142.9	98.2	53.9

Table 3.3.1b **Percentage increase in caspase-3 activity relative to the control in the HT-29 cells after cell exposure to the CDK group of imidazo[1,2-a]pyridines**

Compound	**% Increase in caspase-3 activity relative to the control**				
	2 hours	4 hours	8 hours	12 hours	24 hours
CDK 3	50.7	137.7	99.6	72.3	17.7
CDK 6	122.1	243.3	186.8	126.9	97.9
CDK 8	-9.4	30.3	82.7	55.1	28.4
Camptothecin	171.8	234.7	197.7	164.8	79.8



<u>Figure 3.3.1</u> Time-dependent induction of caspase 8 and caspase 3 activity by 100 µM (a) CDK 3, (b) CDK 6, (c) CDK 8 and (d) camptothecin in HT-29 cells. Each point represents the mean and standard deviation of the triplicate values of caspase 3 and caspase 8 activity after cell exposure to 100 µM of the CDK derivatives and camptothecin over 2 hours, 4 hours, 8 hours, 12 hours and 24 hours.

3.3.1.2 Effect of the CL imidazo[1,2-a]pyridine compounds on caspase activity in the HT-29 cells.

Figure 3.3.2 illustrates the induction of caspase-8 and caspase-3 activity in the HT-29 cells after treatment with 100 µM of the CL group of imidazo[1,2-a]pyridines and camptothecin. After two hours, all compounds caused an increase in caspase-8 activity relative to the control with CL 6 being the highest (174.6 %), however, the activity was not as high as after treatment with camptothecin (233.3 %). At 24 hours, after treatment with CL 4 and CL 5, caspase-8 levels returned to that of the control. At 24 hours, after treatment with CL 3, caspase-8 levels were lower than that of the control. In contrast, after treatment with CL 6, caspase-8 levels remained elevated at 86.8 % above the control value (Table 3.3.2a) and when compared to treatment with camptothecin.

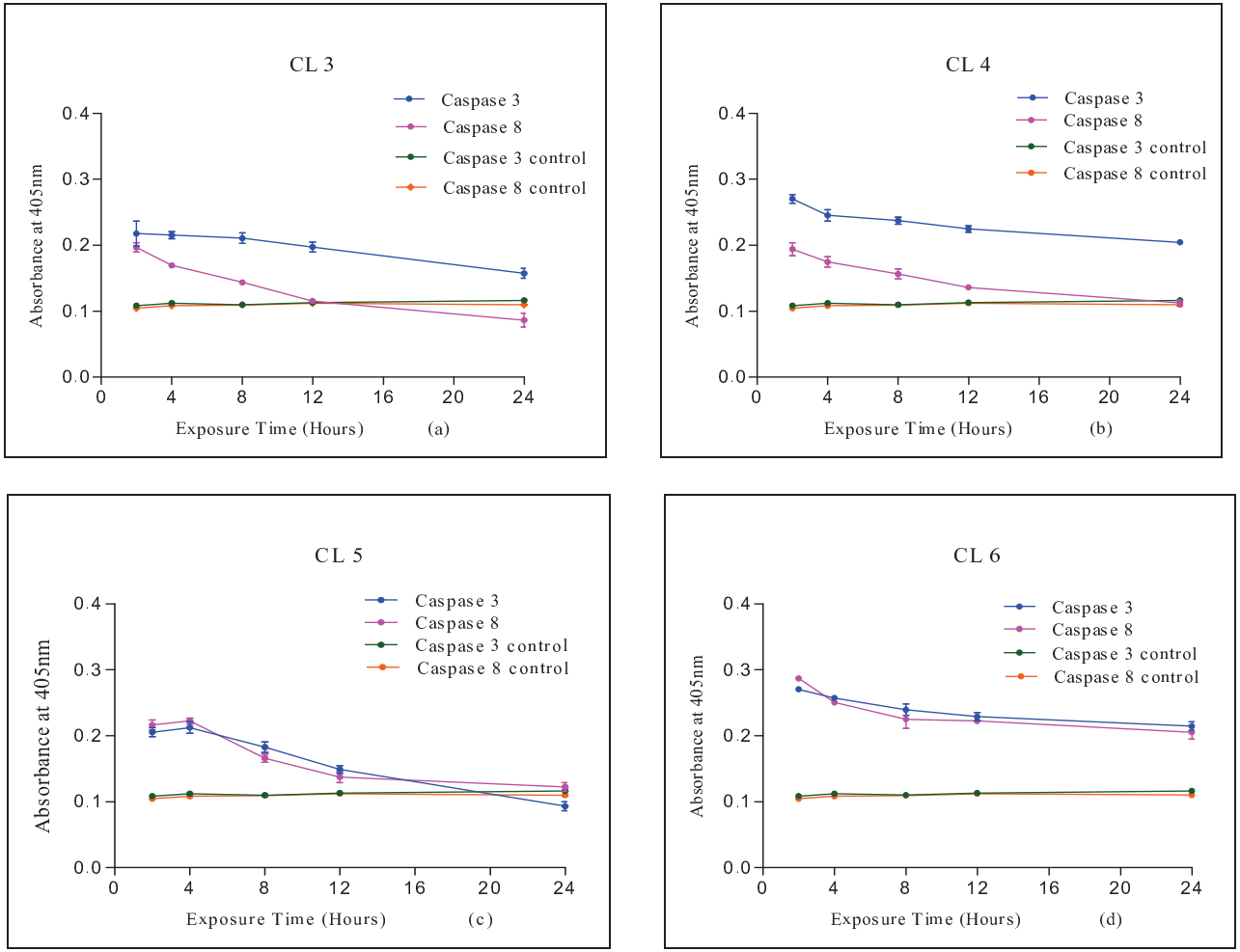
Table 3.3.2a Percentage increase in caspase-8 activity relative to the control in the HT-29 cells after cell exposure to the CL group of imidazo[1,2-a]pyridines

Compound	% Increase in caspase-8 activity relative to the control				
	2 hours	4 hours	8 hours	12 hours	24 hours
CL 3	88.5	56.9	31.5	3.1	-21.4
CL 4	85.7	62.0	42.9	21.4	2.3
CL 5	107.2	106.0	51.6	22.8	11.4
CL 6	174.6	131.9	105.5	98.7	86.8
Camptothecin	233.3	182.3	142.9	98.2	53.9

Table 3.3.2b Percentage increase in caspase-3 activity relative to the control in the HT-29 cells after cell exposure to the CL group of imidazo[1,2-a]pyridines

Compound	% Increase in caspase-3 activity relative to the control				
	2 hours	4 hours	8 hours	12 hours	24 hours
CL 3	100.9	92.4	91.8	74.8	35.2
CL 4	150.0	119.2	115.9	99.1	75.5
CL 5	89.4	89.7	66.4	31.9	-20.2
CL 6	149.3	129.9	117.7	102.7	84.1
Camptothecin	171.8	234.7	197.7	164.8	79.8

HT-29 cells



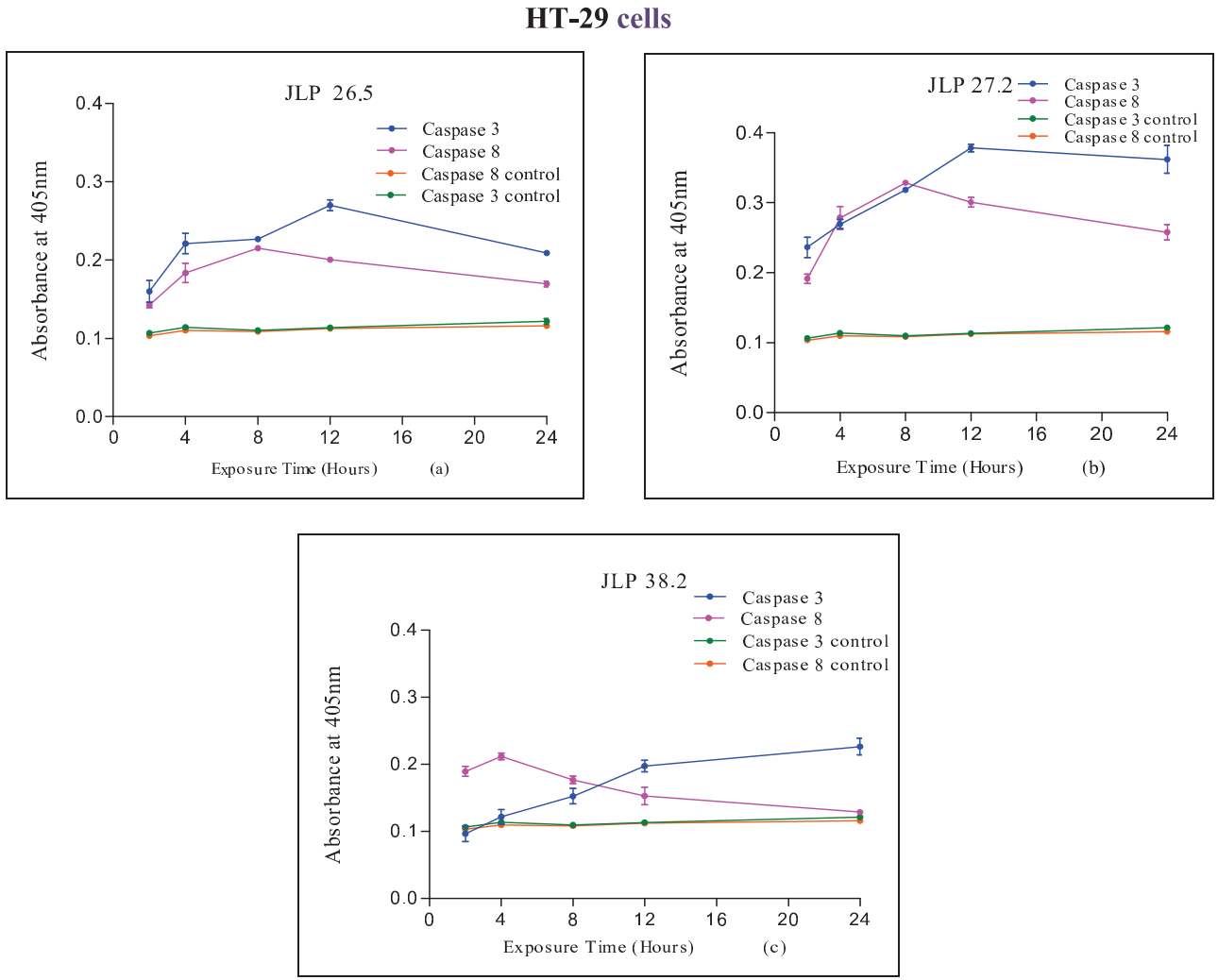
<u>Figure 3.3.2</u> Time-dependent induction of caspase 8 and caspase 3 activity by 100 µM (a) CL 3, (b) CL4, (c) CL 5 and (d) CL 6 in HT-29 cells. Each point represents the mean and standard deviation of the triplicate values of caspase 3 and caspase 8 activity after cell exposure to the CL derivatives over 2 hours, 4 hours, 8 hours, 12 hours and 24 hours. The camptothecin control is shown in Figure 3.3.1.

Figure 3.3.2 shows that caspase-3 activity was increased above that of the control for all four CL compounds. CL 4 caused the greatest increase in activity at 2 hours with a 150 % increase above the control but not as high as camptothecin with a 171 % increase. Caspase-3 activity peaks at 2 hours after treatment with CL 3, CL 4 and CL 6. However, after treatment with CL 6, the caspase-3 activity at 4 hours was higher than after treatment with camptothecin and remained elevated at 24 hours in comparison to camptothecin, CL 3, CL 4 and CL 5 (Table 3.3.2b). CL 6 was the strongest inducer (Table 3.3.1a and 3.3.1b) of caspase-8 and caspase-3 activity and maintained the high induction levels at 24 hours.

3.3.1.3 Effect of the JLP nucleoside analogues on caspase activity in HT-29 cells

Figure 3.3.3 illustrates the induction of caspase-8 and caspase-3 activity in the HT-29 cells after treatment with 100 μM of the JLP nucleoside analogues and camptothecin. After two hours, all compounds caused an increase in caspase-8 activity relative to the control with JLP 27.2 being the highest (85 %), however, the activity was not as high as after treatment with camptothecin (233.3 %). After an 8 hour treatment with JLP 27.2, the increase in caspase-8 activity was higher relative to the control (202.8 %) and camptothecin (142.9 %). At 24 hours, after treatment with all 3 JLP compounds, caspase-8 levels remained elevated relative to the control and camptothecin with JLP 27.2 being the highest at 122.4 % compared to the control value (Table 3.3.3a). Induction of caspase-8 activity for all three JLP compounds was slower than that of camptothecin.

Caspase-3 activity was also increased above that of the control for JLP 26.5 and JLP 27.2 at 2 hours. However the activity was not as high as after treatment with camptothecin. JLP 27.2 caused the greatest increase in activity at 2 hours with a 122.1 % increase above the control but not as high as camptothecin with a 171 % increase. JLP 27.2 activity peaks at 12 hours (233.4 %) where it is higher than camptothecin and remains elevated up to 24 hours in comparison to camptothecin, JLP 26.5 and JLP 38.2 (Table 3.3.3a and Table 3.3.3b). JLP 27.2 induced caspase activity in the HT-29 cells to a similar level as camptothecin but took longer to achieve the maximum activity.



<u>Figure 3.3.3</u> Time-dependent induction of caspase 8 and caspase 3 activity by 100 µM (a) JLP 26.5, (b) JLP 27.2 and (c) JLP 38.2 in HT-29 cells. Each point represents the mean and standard deviation of the triplicate values of caspase 3 and caspase 8 activity after cell exposure to the JLP derivatives over 2 hours, 4 hours, 8 hours, 12 hours and 24 hours. The camptothecin control is shown in Figure 3.3.1.

Table 3.3.3a Percentage increase in caspase-8 activity relative to the control in the HT-29 cells after cell exposure to the JLP nucleoside analogues

Compound	% Increase in caspase-8 activity relative to the control				
	2 hours	4 hours	8 hours	12 hours	24 hours
JLP 26.5	37.7	66.8	98.2	78.2	46.1
JLP 27.2	85.0	153.2	202.8	167.6	122.4
JLP 38.2	83.1	92.7	63.1	36.0	11.1
Camptothecin	233.3	182.3	142.9	98.2	53.9

Table 3.3.3b Percentage increase in caspase-3 activity relative to the control in the HT-29 cells after cell exposure to the JLP nucleoside analogues

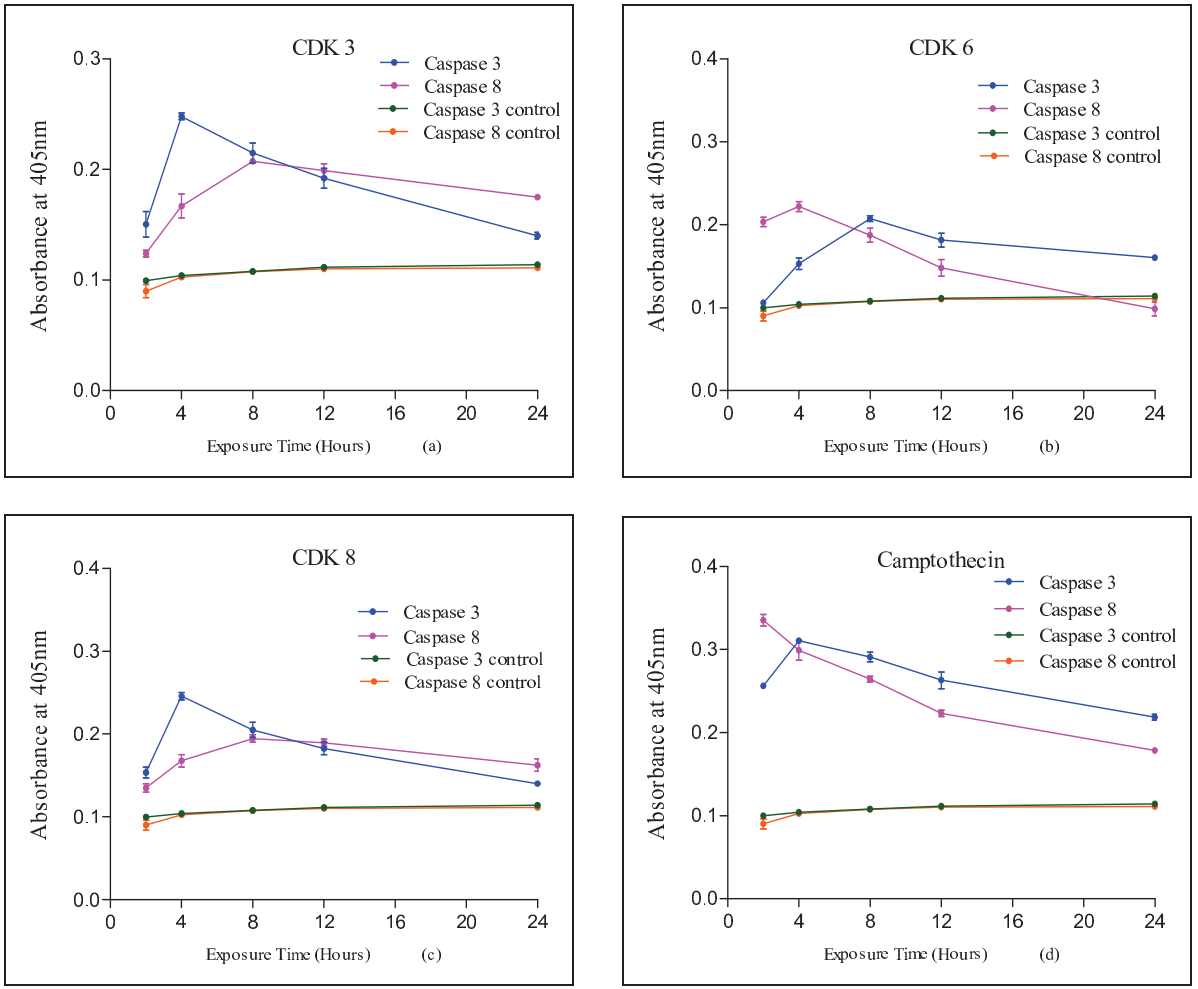
Compound	% Increase in caspase-3 activity relative to the control				
	2 hours	4 hours	8 hours	12 hours	24 hours
JLP 26.5	50.2	93.9	106.4	137.9	72.0
JLP 27.2	122.1	136.4	189.6	233.4	197.9
JLP 38.2	-9.4	7.0	38.6	74.0	86.4
Camptothecin	171.8	234.7	197.7	164.8	79.8

3.3.2 Caspase activity in Caco-2 cells

3.3.2.1 Effect of the CDK imidazo[1,2-a]pyridine compounds on caspase activity in Caco-2 cells.

Figure 3.3.4 illustrates the induction of caspase-8 and caspase-3 activity in the Caco-2 cells after treatment with 100 µM of the CDK group of imidazo[1,2-a]pyridines and camptothecin. After two hours, all compounds caused an increase in caspase-8 activity relative to the control with CDK 6 being the highest (126.1%), however, the activity was not as high as after treatment with camptothecin (272.2 %). The cells treated with CDK 3 and CDK 8 showed maximum caspase-8 activity at 8 hours after which the activity slowly declined whereas after treatment with CDK 6, maximum levels were reached at 2 hours. At 24 hours, after treatment with CDK 3 and CDK 8, caspase-8 levels remained elevated at 57.7 % and 46.4 % of the control value. In contrast, after treatment with CDK 6, caspase-8 levels returned to that of the control. The effect of camptothecin is shown in Figure 3.3.4d. Compared to camptothecin, the CDK compounds were inferior in their ability to induce caspase-8 and caspase-3 activity.

Caco-2 cells



<u>Figure 3.3.4</u> Time-dependent induction of caspase 8 and caspase 3 activity by 100 µM (a) CDK 3, (b) CDK 6, (c) CDK 8 and (d) camptothecin in Caco-2 cells. Each point represents the mean and standard deviation of the triplicate values of caspase 3 and caspase 8 activity after cell exposure to the CDK derivatives over 2 hours, 4 hours, 8 hours, 12 hours and 24 hours.

Table 3.3.4a Percentage increase in caspase-8 activity relative to the control in the Caco-2 cells after exposure to the CDK group of imidazo[1,2-a]pyridines

Compound	% Increase in caspase-8 activity relative to the control				
	2 hours	4 hours	8 hours	12 hours	24 hours
CDK 3	37.8	62.9	93.0	80.9	57.7
CDK 6	126.1	116.6	74.4	34.6	-11.3
CDK 8	50.0	63.3	80.9	72.3	46.4
Camptothecin	272.2	191.8	146.1	102.7	60.8

Table 3.3.4b Percentage increase in caspase-3 activity relative to the control in the Caco-2 cells after exposure to the CDK group of imidazo[1,2-a]pyridines

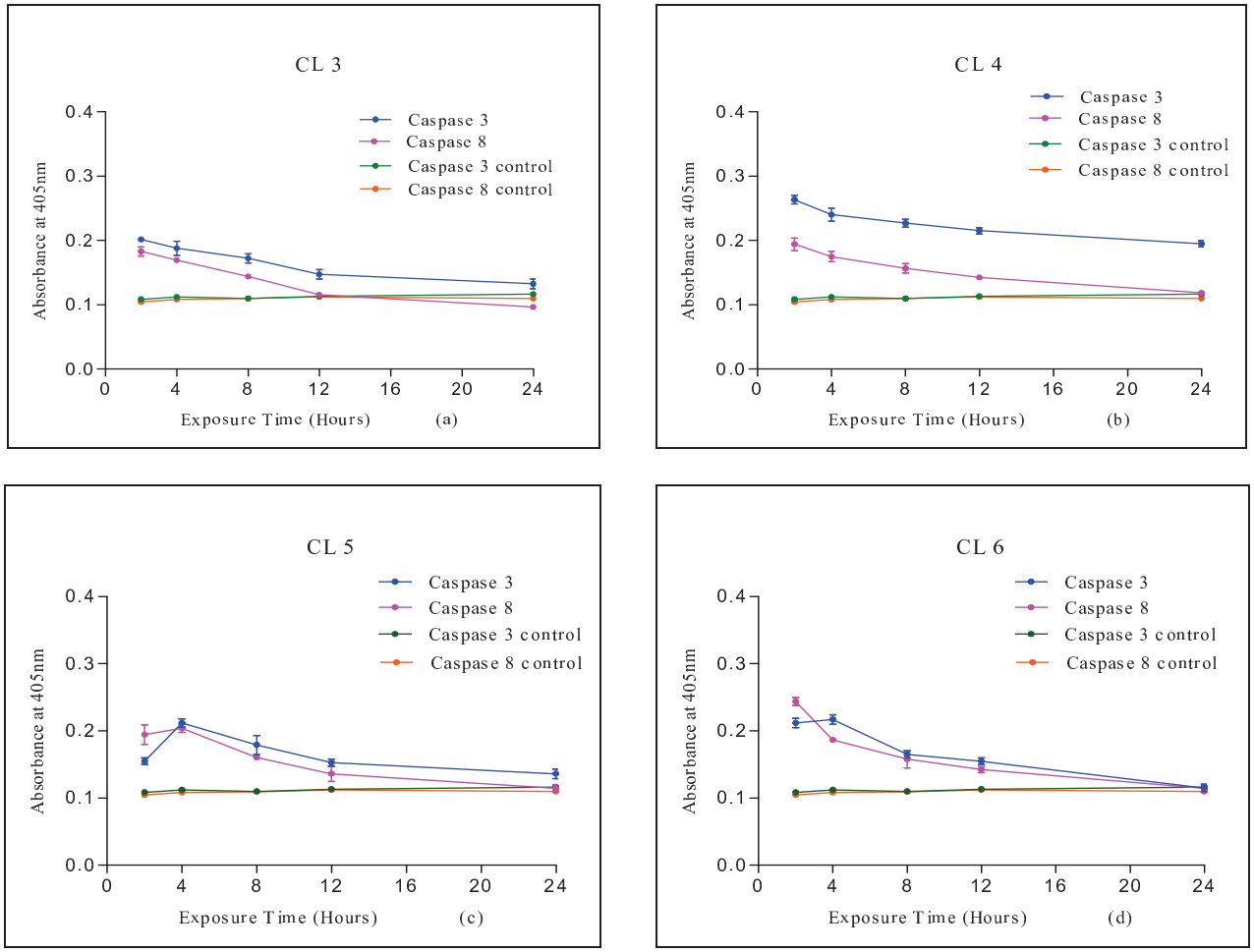
Compound	% Increase in caspase-3 activity relative to the control				
	2 hours	4 hours	8 hours	12 hours	24 hours
CDK 3	52.3	138.5	99.1	71.2	22.9
CDK 6	6.5	47.1	92.1	62.8	40.8
CDK 8	54.3	136.1	89.8	63.7	22.9
Camptothecin	157.3	198.6	169.4	135.9	91.7

Caspase-3 activity was also increased above that of the control for the CDK test compounds at 2 hours. CDK 8 caused the greatest increase in activity at 2 hours with a 54.3 % increase above the control but not as high as camptothecin with a 157.3 % increase. The cells treated with CDK 3 and CDK 8 showed maximum caspase-3 activity at 4 hours whereas after treatment with compound CDK 6, maximum caspase-3 activity was observed after 8 hours. The activity of camptothecin remained higher until 24 hours in comparison to the CDK test compounds (Table 3.3.4b).

3.3.2.2 Effect of the CL group of imidazo[1,2-a]pyridine compounds on caspase activity in Caco-2 cells.

Figure 3.3.5 illustrates the induction of caspase-8 and caspase-3 activity in the Caco-2 cells after treatment with 100 µM of the CL group of imidazo[1,2-a]pyridines and camptothecin. After two hours, all compounds caused an increase in caspase-8 activity relative to the control with CL 6 being the highest (133.4 %), however, the activity was not as high as after treatment with camptothecin (272.2 %). At 24 hours, after treatment with CL 3, caspase-8 levels returned to that of the control. Camptothecin caused a sustained elevation of caspase-8 activity throughout the 24 hours in comparison to the CL test compounds (Table 3.3.5a).

Caco-2 cells



<u>Figure 3.3.5</u> Time-dependent induction of caspase 8 and caspase 3 activity by 100 µM (a) CL 3, (b) CL4, (c) CL 5 and (d) CL 6 in Caco-2 cells. Each point represents the mean and standard deviation of the triplicate values of caspase 3 and caspase 8 activity after cell exposure to the CL derivatives over 2 hours, 4 hours, 8 hours, 12 hours and 24 hours. The camptothecin control is shown in Figure 3.3.4.

As shown in Figure 3.3.5, caspase-3 activity was increased above that of the control for all 4 CL compounds. CL 4 caused the greatest increase in activity at 2 hours with a 142.9 % increase above the control but the activity was not as high as that of camptothecin with a 157.3 % increase. The activity of CL 3, CL 4 and CL 6 peaks at 2 hours though it is lower than camptothecin and remains lower until 24 hours. At 24 hours, after treatment with CL 6, caspase-3 levels returned to that of the control.

Table 3.3.5a Percentage increase in caspase-8 activity relative to the control in the Caco-2 cells after exposure to the CL group of imidazo[1,2-a]pyridines

Compound	% Increase in caspase-8 activity relative to the control				
	2 hours	4 hours	8 hours	12 hours	24 hours
CL 3	75.1	56.9	31.5	3.1	-12.3
CL 4	85.7	62.0	42.9	27.2	7.7
CL 5	86.1	88.9	46.6	21.4	4.1
CL 6	133.4	72.7	44.3	27.2	4.1
Camptothecin	272.2	191.8	146.1	102.7	60.8

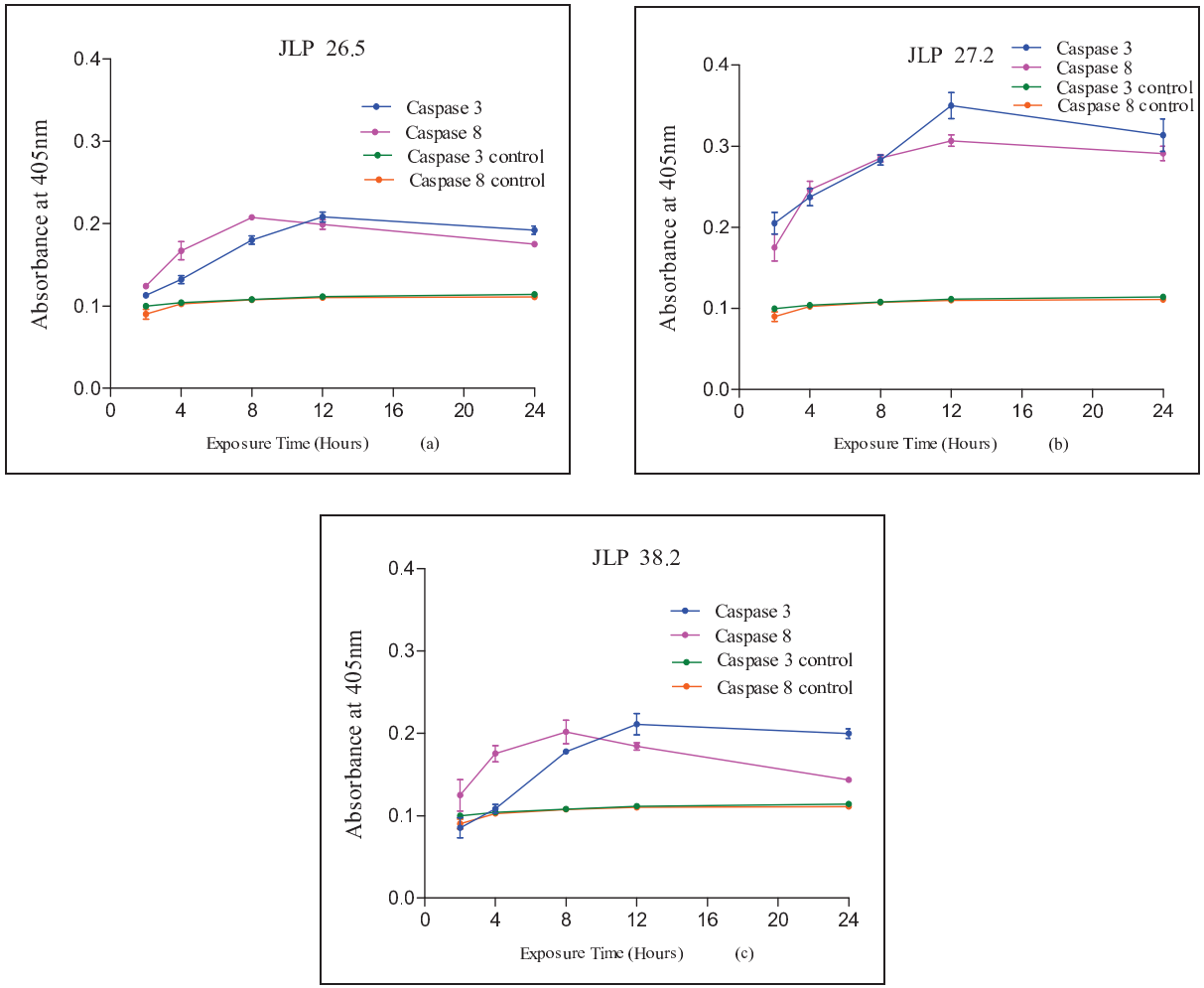
Table 3.3.5b Percentage increase in caspase-3 activity relative to the control in the Caco-2 cells after exposure to the CL group of imidazo[1,2-a]pyridines

Compound	% Increase in caspase-3 activity relative to the control				
	2 hours	4 hours	8 hours	12 hours	24 hours
CL 3	85.7	67.9	56.4	30.5	13.7
CL 4	142.9	114.3	106.4	90.3	67.4
CL 5	42.9	89.3	62.7	35.4	16.7
CL 6	95.4	93.8	50.0	37.2	-1.3
Camptothecin	157.3	198.6	169.4	135.9	91.7

3.3.2.3 Effect of the JLP nucleoside analogues on caspase activity in Caco-2 cells.

Figure 3.3.6 illustrates the time-dependent induction of caspase-8 and caspase-3 activity in the Caco-2 cells after treatment with 100 µM of the JLP nucleoside analogues and camptothecin. After two hours, all the JLP compounds caused an increase in caspase-8 activity relative to the control with JLP 27.2 being the highest (94.6 %), however, the activity was not as high as after treatment with camptothecin (272.2 %). At 24 hours, after treatment with the 3 JLP compounds and camptothecin, caspase-8 levels remained elevated relative to the control with JLP 27.2 being the highest (162.3 %).

Caco-2 cells



<u>Figure 3.3.6</u> Time-dependent induction of caspase 8 and caspase 3 activity by 100 µM (a) JLP 26.5, (b) JLP 27.2 and (c) JLP 38.2 in Caco-2 cells. Each point represents the mean and standard deviation of the triplicate values of caspase 3 and caspase 8 activity after cell exposure to the JLP derivatives over 2 hours, 4 hours, 8 hours, 12 hours and 24 hours. The camptothecin control is shown in Figure 3.3.4.

Caspase-3 activity was increased above that of the control for JLP 26.5 and JLP 27.2 at 2 hours. JLP 27.2 caused the greatest increase in caspase-3 activity at 2 hours with a 106.3 % increase above control but not as high as camptothecin with a 157.3 % increase. JLP 27.2 induced caspase-3 activity peaks at 12 hours where it is higher than camptothecin and remains elevated until 24 hours in comparison to camptothecin, JLP 26.5 and JLP 38.2 (Table 3.3.6b)

Table 3.3.6a Percentage increase in caspase-8 activity relative to the control in the Caco-2 cells after cell exposure to the JLP nucleoside analogues

Compound	% Increase in caspase-8 activity relative to the control				
	2 hours	4 hours	8 hours	12 hours	24 hours
JLP 26.5	35.8	62.9	93.0	80.9	57.7
JLP 27.2	94.6	140.3	165.5	178.9	162.3
JLP 38.2	38.5	71.1	87.6	67.5	29.1
Camptothecin	272.2	191.8	146.1	102.7	60.8

Table 3.3.6b Percentage increase in caspase-3 activity relative to the control in the Caco-2 cells after cell exposure to the JLP nucleoside analogues

Compound	% Increase in caspase-3 activity relative to the control				
	2 hours	4 hours	8 hours	12 hours	24 hours
JLP 26.5	13.6	26.9	66.7	86.6	68.4
JLP 27.2	106.3	128.1	161.9	214.1	175.1
JLP 38.2	-14.3	4.3	64.6	89.4	75.2
Camptothecin	157.3	198.6	169.4	135.9	91.7

These findings show that the test compounds are able to induce caspase-8 and caspase-3 activity at a concentration of 100 µM in the HT-29 and Caco-2 cells. From the CDK group of imidazo[1,2-a]pyridines, CDK 6 induction of caspase-8 activity compares well to that of camptothecin although the activity remained elevated for longer than camptothecin and CDK 6 induction of caspase-3 activity was very similar to camptothecin at all time periods in the HT-29 cells. As shown in Fig. 3.3.2, after treatment with the CL group of imidazo[1,2-a]pyridines, maximal caspase-8 activity was observed 2 hours after exposure to CL 3, CL 4 and CL 6 in comparison to the untreated control cells. Caspase-3 activity

was detected as early as 2 hours after treatment with imidazo[1,2-a]pyridines CL 3, CL 4 and CL 6 in the HT-29 and Caco-2 cells and persisted through 24 hours. From the CL group of imidazo[1,2-a]pyridines, CL 6 was the strongest inducer (Table 3.3.1a and 3.3.1b) of caspase-8 activity in both cell lines.

From the nucleoside analogues, JLP 27.2 induction of caspase-8 activity was similar to camptothecin in the HT-29 cells although the activity was not as high whereas JLP 27.2 induction of caspase-3 activity was similar to camptothecin but only reached maximal caspase-3 activity after 12 hours versus after 4 hours with camptothecin. In the Caco-2 cells, JLP 27.2 induction of caspase-8 activity was similar to camptothecin in the HT-29 cells although the activity was not as high and maximal activity was only reached after 12 hours versus after 2 hours of treatment with camptothecin. Nucleoside analogue JLP 27.2 induction of caspase-3 activity was higher than camptothecin but only reached maximal caspase-3 activity after 12 hours versus after a 4 hour treatment with camptothecin.

Since caspase-3 is the main effector caspase in the apoptotic cycle, increased levels of this enzyme indicates the induction of early apoptosis after exposure to the test compounds and camptothecin. There was no significant change ($p > 0.05$, $p = 0.107$) in caspase-3 and caspase-8 activity in the untreated cells after 24 hours for both cell lines. All the active compounds tested induced caspase-8 and caspase-3 activity, however, the overall caspase activity induced was less than that of camptothecin with the exception of JLP 27.2 on caspase-3 activity in the HT-29 and Caco-2 cells.

3.4 The effects of the novel compounds on the mitochondrial membrane potential in the HT-29 and Caco-2 cells

The energy generated during cellular respiration accumulates in the mitochondrial transmembrane space as an electron gradient called the mitochondrial membrane potential or $\Delta\psi m$. Mitochondrial dysfunction has been shown to participate in the induction of apoptosis, and is central to the intrinsic apoptotic pathway. Assessment of the $\Delta\psi m$ in the HT-29 and Caco-2 cells was performed 24 hours after treatment with the novel compounds at a concentration of 100 μM. Flow cytometric analysis using JC-1 staining was used to evaluate the status of the mitochondrial membrane potential as described in the Materials and Methods section 2.6.3.1.

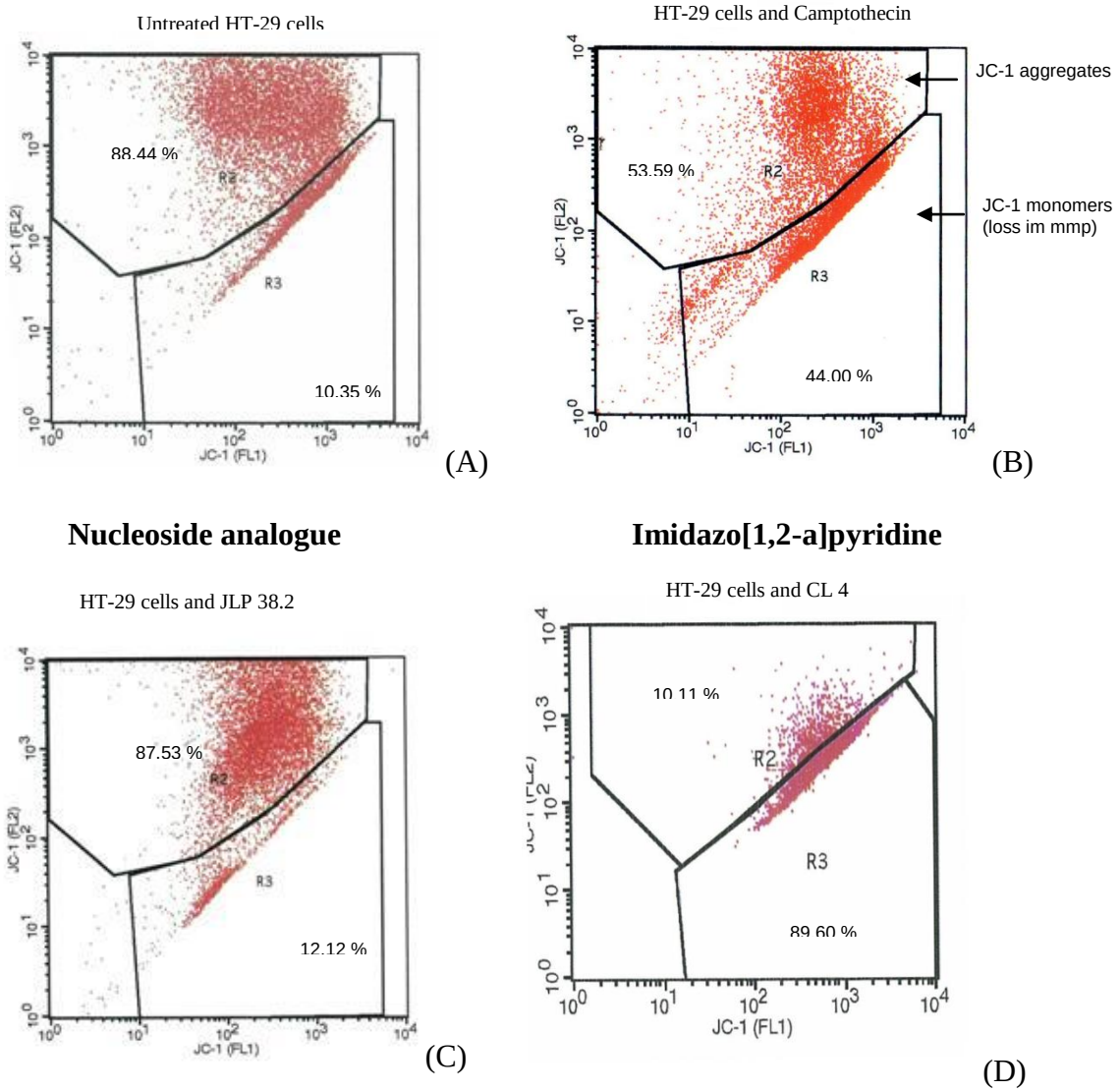
In the scattergraphs, regions of high mitochondrial polarization are indicated by the red fluorescence due to JC-1 aggregate formation by the concentrated dye in the R2 region. Depolarized regions (due to a loss in $\Delta\psi m$) are indicated by the JC-1 monomers in the R3 region of the scattergraphs. The scattergraphs showing the least and most $\Delta\psi m$ changes are shown in Figures 3.4.1 and 3.4.2. The percentages of mitochondrial membrane depolarization induced by the compounds in the HT-29 and Caco-2 cells are shown in Tables 3.4.1 and 3.4.2. The remaining scattergraphs are shown in Appendix C.

Results of the flow cytometric analyses show JC-1 fluorescence in both the R2 and R3 regions in the untreated HT-29 cells (Figure 3.4.1A) with the highest percentage (88.44 %) being in the R2 region meaning there was no loss in the mitochondrial membrane potential. A small percentage of cells show fluorescence in the R3 region indicating a loss in the mitochondrial membrane potential.

There was a significant increase in the number of cells with JC-1 monomer fluorescence indicative of a change in the $\Delta\psi m$ after treatment with the novel compounds. Compound CL 4 was shown to cause the greatest loss in $\Delta\psi m$ of the HT-29 cells (Figure 3.4.1D), with 89.60 % of the cells undergoing a change in the $\Delta\psi m$ in comparison to 10.11 % of cells with an unchanged $\Delta\psi m$ remaining after treatment. The $\Delta\psi m$ effects of both the CDK and the CL group of imidazo[1,2-a]pyridines were greater than those of camptothecin. Treatment with JLP 38.2 caused the least change in $\Delta\psi m$ with only 12.12 % of cells

undergoing a change in the Δψm (Figure 3.4.1C and Table 3.4.1), similar to the untreated cells. The effects of the JLP compounds on the Δψm were comparable to that of the untreated control (Figure 3.4.1C and Table 3.4.1), meaning the mitochondrial membrane potential was maintained.

3.4.1 The effects of the novel compounds on the mitochondrial membrane potential in the HT -29 cells



<u>Figure 3.4.1</u> Flow cytometric analyses of JC-1 staining in HT-29 cells after a 24 hour treatment with 100 µM camptothecin, JLP 38.2 and CL 4. The scattergraphs represent the changes in the mitochondrial membrane potential (mmp) after treatment with the selected compounds for 24 hours and stained with JC-1 (A) Untreated HT-29 cells (B) HT-29 treated with camptothecin, (C) JLP 38.2 and (D) CL 4.

Table 3.4.1: Analysis of mitochondrial membrane depolarisation induced by the novel compounds in the HT-29 colon cancer cell line

Compound	% cells with unchanged Δψ	% cells with depolarized Δψ.
Untreated HT-29 cells	88.44	10.35
CDK 3	14.49	83.43
CDK 6	34.54	65.30
CDK 8	20.33	79.62
CL 3	15.19	84.29
CL 4	10.11	89.60
CL 5	13.86	85.92
CL 6	35.60	63.09
JLP 26.5	79.55	14.39
JLP 27.2	87.02	12.74
JLP 38.2	87.53	12.12
Camptothecin	53.59	44.00

Table 3.4.1 shows the analyses of the mitochondrial membrane depolarisation in the HT-29 cells after treatment with the novel imidazo[1,2-a]pyridines and the novel nucleoside analogues. The untreated HT-29 cells maintained their Δψm as observed by the presence of few events associated with a lower FL-1 fluorescence (R3 region) being 10.35 %. The imidazo[1,2-a]pyridines had the most effect on the mitochondrial membrane potential with between 63 and 86 % of cell mitochondria becoming depolarised which is greater than the mitochondrial effects of camptothecin. The JLP nucleoside analogues had the least effect on the mitochondrial membrane potential with between 12 and 15 % of cell mitochondria becoming depolarised which is comparable to that of the untreated control.

Treatment with the CDK group of imidazo[1,2-a]pyridines caused between 65 and 93 % of cells to lose their Δψm as shown by the increased percentage of cells with a higher FL-1 fluorescence (R3 region) as compared to the untreated control cells and camptothecin. Of the CDK compounds, treatment with CDK 3 was associated with the greatest loss in the Δψm with 83.43 % of cells staining in the R3 region.

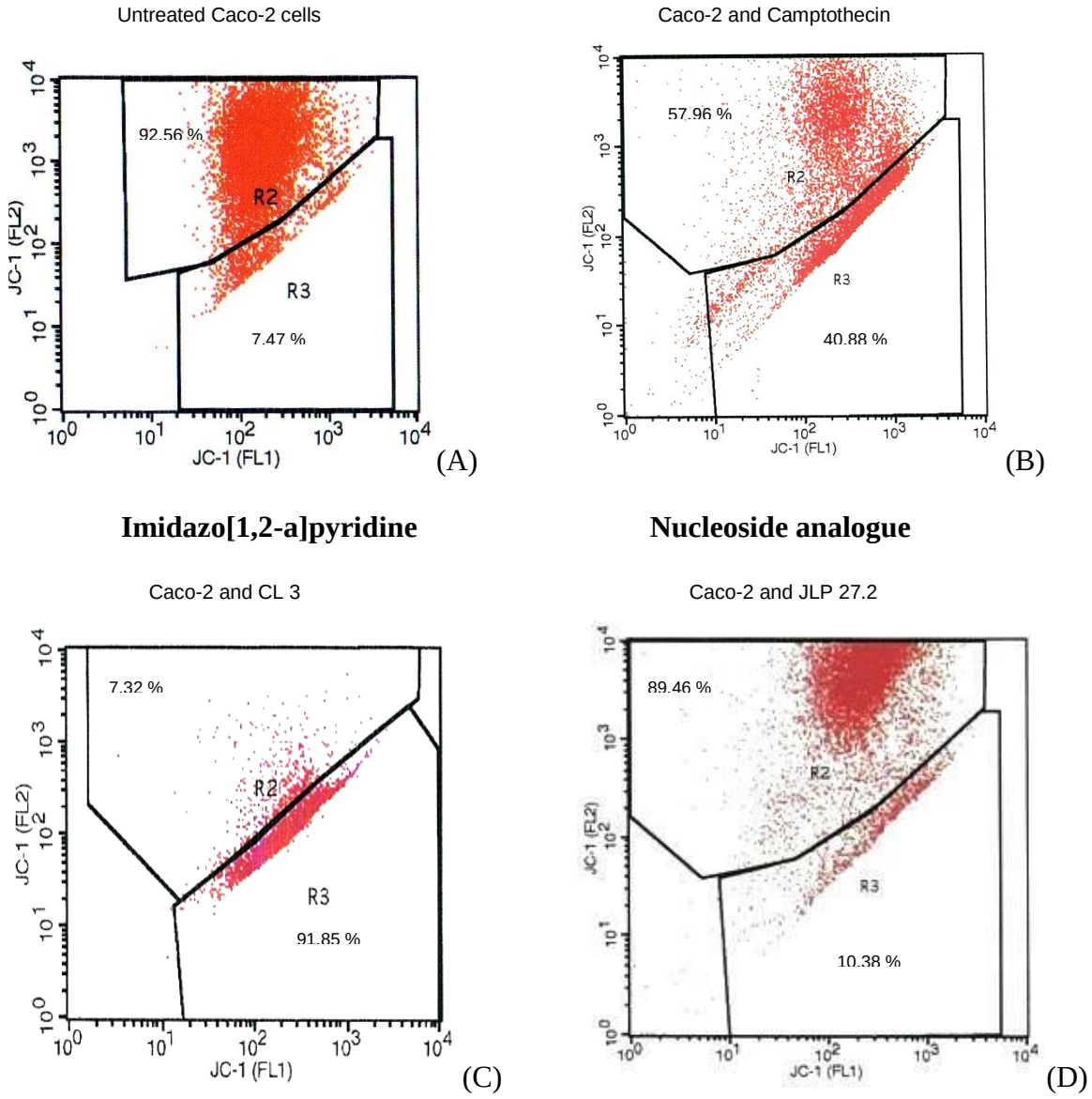
Treatment with the CL group of imidazo[1,2-a]pyridines caused between 63 and 90 % of cells to lose their Δψm as shown by the increased percentage of cells with a higher FL-1 fluorescence (R3 region) as compared to the untreated cells and camptothecin. Apart from CL 6, the CL compounds caused a greater loss in Δψm when compared to the CDK imidazo[1,2-a]pyridines and the JLP nucleoside analogues. The effects of CL 6 on the Δψm was comparable to that of CDK 6 with 63.09 % cells staining in the R3 region. Of all the CL compounds, treatment with CL 4 was associated with the greatest loss in the ΔΨm with 89.60 % of cells staining in the R3 region.

3.4.2 The effects of the novel compounds on the mitochondrial membrane potential in the Caco-2 cells

Figure 3.4.2 represents a flow cytometric analysis of the mitochondrial membrane potential (Δψm) in Caco-2 cells exposed for 24 hours to two of the novel compounds and compared to a negative and positive control. There was a significant increase in the number of cells with JC-1 monomer fluorescence indicative of a change in the Δψm after treatment with the novel compounds. Results of the flow cytometric analyses shows that after treatment with camptothecin for 24 hours, 40.88 % of Caco-2 cells were observed to undergo changes in the Δψm (Figure 3.4.2B).

Imidazo[1,2-a]pyridine treatment of cells caused between 76 and 92 % of cells to lose their mitochondrial membrane potential. The Δψm effects of the imidazo[1,2-a]pyridines were greater than those of camptothecin and the control. Compound CL 3 caused the greatest loss in Δψm of the Caco-2 cells (Figure 3.4.2C), with 91.85 % of the cells showing a loss of Δψm.

Treatment with the JLP nucleoside analogues caused between 7 and 25 % of cells to lose their mitochondrial membrane potential. The effects of the JLP compounds on the Δψm were comparable to that of the untreated control (Figure 3.4.2C and Table 3.4.2), meaning the mitochondrial membrane potential was maintained. Nucleoside analogue JLP 27.2 caused the least change in Δψm with only 10.38 % of cells undergoing a change in the Δψm (Figure 3.4.2D and Table 3.4.2), similar to the untreated control.



<u>Figure 3.4.2</u> Flow cytometric analyses of JC-1 staining in Caco-2 cells after a 24 hour treatment with 100 µM Camptothecin, CL 3 and JLP 27.2. The histograms represent the changes in mitochondrial membrane potential (mmp) after cell treatment with the selected compounds for 24 hours and stained with JC-1 (A) Untreated Caco-2 cells (B) Caco-2 cells treated with Camptothecin, (C) CL 3 and (D) JLP 27.2.

Table 3.4.2: Analysis of mitochondrial membrane depolarisation induced by the novel compounds in the Caco-2 colon cancer cell line

Compound	% Caco-2 cells with unchanged Δψ	% Caco-2 cells with depolarized Δψ.
Untreated Caco-2 cells	92.58	7.47
CDK 3	18.07	81.91
CDK 6	21.10	78.00
CDK 8	20.81	79.17
CL 3	7.32	91.85
CL 4	16.54	83.43
CL 5	14.62	85.12
CL 6	23.23	76.10
JLP 26.5	75.64	24.33
JLP 27.2	89.46	10.38
JLP 38.2	75.68	23.96
Camptothecin	57.96	40.88

Table 3.4.2 shows the analyses of the mitochondrial membrane depolarisation in the Caco-2 cells after treatment with the novel imidazo[1,2-a]pyridines and the novel nucleoside analogues. The untreated Caco-2 cells maintained their Δψm as observed by the presence of few events associated with a lower FL-1 fluorescence (R3 region) being 7.47 %.

Treatment with the CDK group of imidazo[1,2-a]pyridines was associated with mitochondrial depolarization in the HT-29 cells as shown by the increased percentage of cells with a higher FL-1 fluorescence (R3 region) as compared to the untreated cells and camptothecin. Of the CDK compounds, treatment with CDK 3 was associated with the greatest loss in the Δψm with 81.91 % of cells staining in the R3 region.

Treatment with the CL group of imidazo[1,2-a]pyridines was also associated with mitochondrial depolarization in the HT-29 cells as shown by the increased percentage of cells with a higher FL-1 fluorescence (R3 region) as compared to the untreated cells and camptothecin. The CL compounds caused a greater loss in Δψm when compared to the JLP

nucleoside analogues. The effects of CL 6 on the Δψm was comparable to that of the CDK group of imidazo[1,2-a]pyridines with 76.10 % cells staining in the R3 region indication a loss in the Δψm. Of all the CL compounds, treatment with CL 3 was associated with the greatest loss in the Δψm with 91.80 % of cells staining for JC-1 in the R3 region.

The effects of the JLP nucleoside analogues on the mitochondrial membrane potential are comparable to that of the untreated control with JLP 27.2 causing the greatest increase in the mitochondrial depolarisation with 10.38 % JC-1 staining in the R3 region.

For both cell lines tested, the results indicate that the CDK and CL compounds induced a greater change in the Δψm as opposed to the changes after addition of the JLP nucleoside analogues. These findings suggest that cell death induced by the novel imidazo[1,2-a]pyridine derivatives is associated with depolarisation of the mitochondrial membrane which could lead to apoptosis.

3.5 The effect of the novel compounds on cytochrome c protein levels in the HT-29 and Caco-2 cell line

Cytochrome c has been shown to translocate from the mitochondria to the cytosol during the early phase of apoptosis (Jiang and Wang., 2004). Cytochrome c levels were determined by a human cytochrome c protein Elisa assay as described in the Materials and Methods section 2.6.3.2. All cells were treated with 100 µM of the test compounds for 24 hrs. Results are presented as a percentage subcellular distribution of cytochrome c. A table showing the corresponding cytochrome c concentrations is shown in Appendix D.

3.5.1 Cytochrome c subcellular distribution in the HT-29 cells

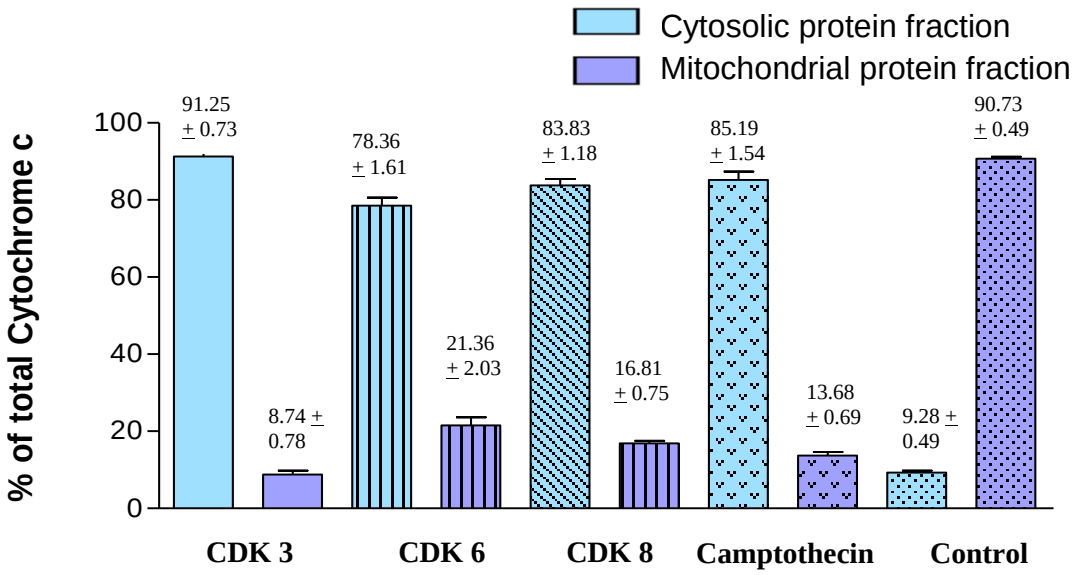


Figure 3.5.1 Percentage cytochrome c protein in the cytosolic and mitochondrial protein fractions of HT-29 cells after treatment with 100 µM CDK 3, CDK 6, CDK 8 and camptothecin for 24 hours. The bars represent the mean ± SEM from two Elisa assays carried out for each compound.

Figure 3.5.1 shows the effect of the CDK group of imidazo[1,2-a]pyridines on the subcellular distribution of cytochrome c in the HT-29 cells. The untreated cells show that 90.73 ± 0.49 % of the cytochrome c protein was found in the mitochondrial fraction. In contrast, camptothecin treated cells have 85.19 ± 1.5 % of cytochrome c in the cytosolic fraction. The effects of the CDK group of imidazo[1,2-a]pyridines on the subcellular

distribution of cytochrome c in the HT-29 cells are similar to those of camptothecin with between 78 and 92 percent of cytochrome c in the cytosolic fraction as opposed to between 8 and 22 percent of cytochrome c in the mitochondrial fraction. This indicates that all three CDK compounds elicit the release of cytochrome c from the mitochondria. Compound CDK 3 was more effective than camptothecin (91.25 ± 0.73 % vs 85.19 ± 1.54 %). The results indicate that treatment with the CDK group of imidazo[1,2-a]pyridines results in decreased levels of mitochondrial cytochrome c and increased levels of cytosolic cytochrome c which supports the evidence for apoptosis.

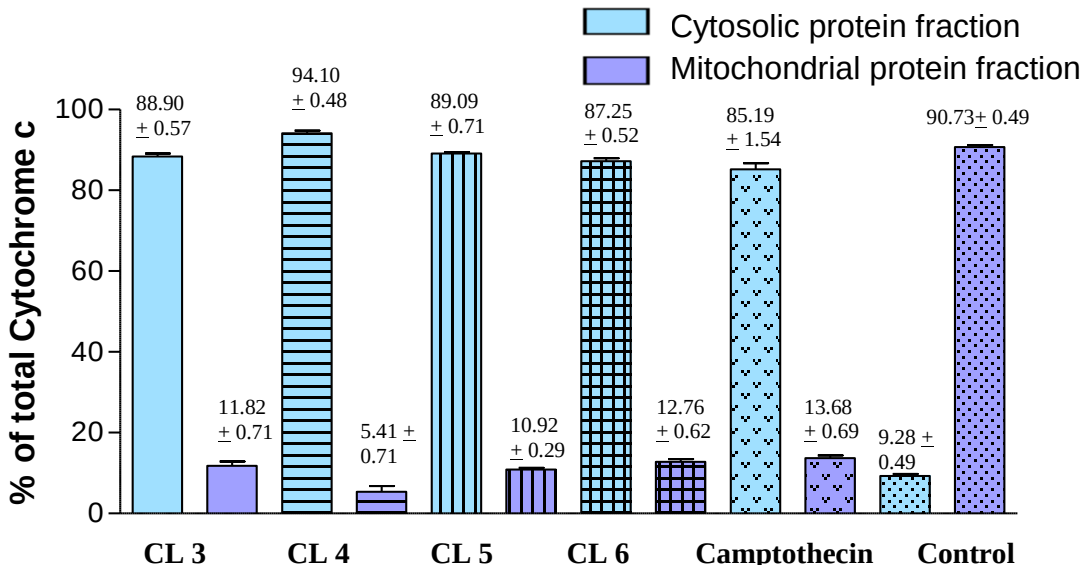


Figure 3.5.2 Percentage cytochrome c protein in the cytosolic and mitochondrial protein fractions of HT-29 cells after treatment with 100 µM CL 3, CL 4, CL 5, CL 6 and camptothecin for 24 hours. The bars represent the mean ± SEM from two Elisa assays carried out for each compound.

The four CL imidazo[1,2-a]pyridines had a similar effect to the CDK imidazo[1,2-a]pyridines on the HT-29 cells (Figure 3.5.2). The percentages of cytochrome c in the cytosolic fractions are as follows: CL 3 = 88.90 ± 0.57 %, CL4 = 94.10 ± 0.48 %, CL 5 = 89.09 ± 0.71 %, CL 6 = 87.25 ± 0.52 %. The CL compounds were more effective than camptothecin in causing the release of cytochrome c from the mitochondria. The results indicate that treatment with the CL group of imidazo[1,2-a]pyridines causes decreased levels of mitochondrial cytochrome c and increased levels of cytosolic cytochrome c which supports the evidence for apoptosis.

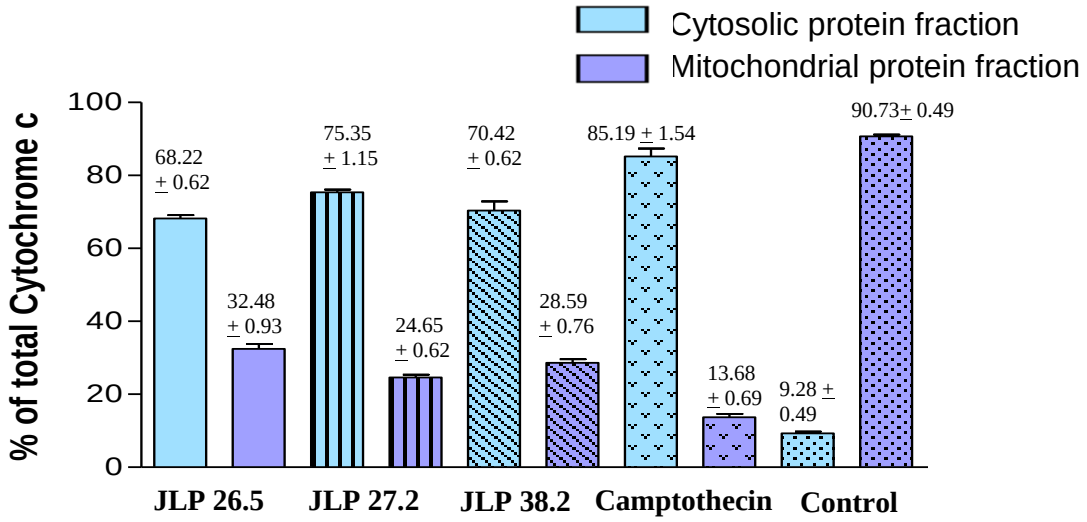


Figure 3.5.3 Percentage cytochrome c protein in the cytosolic and mitochondrial protein fractions of HT-29 cells after treatment with 100 µM JLP 26.5, JLP 27.2, JLP 38.2 and camptothecin for 24 hours. The bars represent the mean ± SEM from two Elisa assays carried out for each compound.

Figure 3.5.3 shows that the JLP nucleoside analogues also increase the cytosolic cytochrome c fraction but to a lesser extent than camptothecin and the imidazo[1,2-a]pyridines. The cytosolic cytochrome c fraction ranged between 68 and 76 percent. JLP 27.2 had the highest cytosolic fraction of cytochrome c (75.35 ± 1.15 %) which is considerably lower than that of camptothecin (85.19 ± 15.54 %). The results thus indicate that treatment with the JLP nucleoside analogues causes a decrease in mitochondrial cytochrome c and an increase in cytosolic cytochrome c which supports the evidence for apoptosis.

3.5.2 Cytochrome c subcellular distribution in the Caco-2 cells

Figure 3.5.4 shows the effect of the CDK group of imidazo[1,2-a]pyridines on the subcellular distribution of cytochrome c in the Caco-2 cells. The untreated cells show that 90.23 ± 1.01 % of the cytochrome c protein is found in the mitochondrial fraction. In contrast, camptothecin treated cells have 86.94 ± 1.72 % of cytochrome c in the cytosolic fraction. The CDK group of compounds are similar to camptothecin with between 80 and 92 percent of cytochrome c in the cytosolic fraction as opposed to between 11 and 19 percent of cytochrome c in the mitochondrial fraction. Compound CDK 8 was more effective than camptothecin (89.13 ± 0.97 % vs 86.94 ± 1.72 %). This indicates that all

three CDK compounds cause a release of cytochrome c from the mitochondria to the cytosol, which could lead to apoptosis.

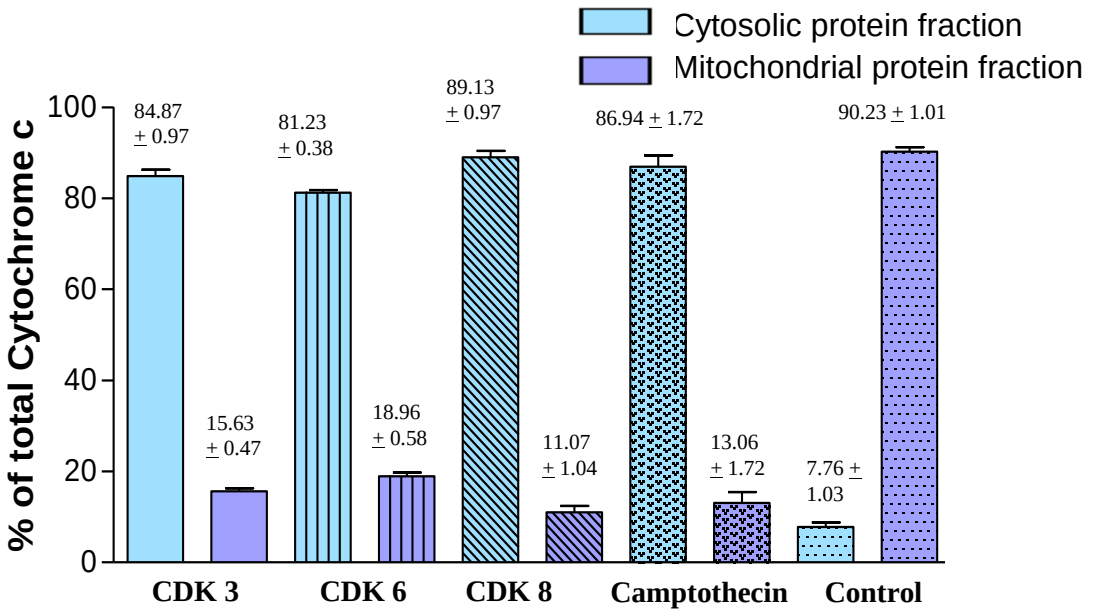


Figure 3.5.4 Percentage cytochrome c protein in the cytosolic and mitochondrial protein fractions of Caco-2 cells after treatment with 100 µM CDK 3, CDK 6, CDK 8 and camptothecin for 24 hours. The bars represent the mean ± SEM from two Elisa assays carried out for each compound.

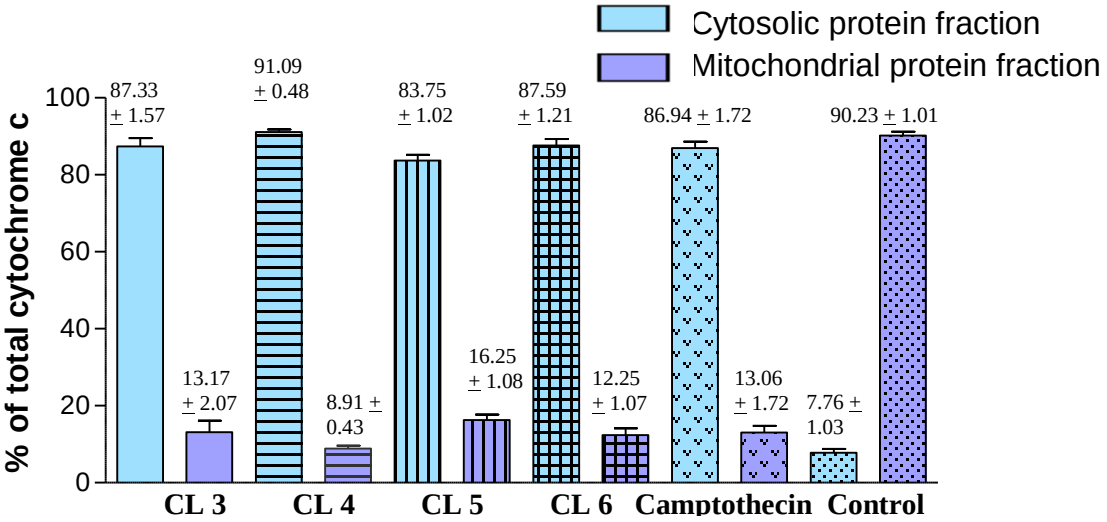


Figure 3.5.5 Percentage cytochrome c protein percentage in the cytosolic and mitochondrial protein fractions of Caco-2 cells after treatment with 100 µM CL 3, CL 4, CL 5, CL 6 and Camptothecin for 24 hours. The bars represent the mean ± SEM from two Elisa assays carried out for each compound.

As shown in Figure 3.5.5, the four CL imidazo[1,2-a]pyridines had a similar effect to the CDK imidazo[1,2-a]pyridines on the Caco-2 cells. The percentages of cytochrome c in the cytosolic fractions are as follows: CL 3 = 87.33 + 1.57 %, CL4 = 91.09 ± 0.48 %, CL5 = 83.75 ± 1.02 %, CL6 = 87.59 ± 1.21 %. This compares well to the effect of camptothecin where the cytochrome c in the cytosolic fraction was 86.94 ± 1.72 %. The CL compounds were more effective than camptothecin in causing the release of cytochrome c from the mitochondria. The results indicate that the CL group of imidazo[1,2-a]pyridines cause a release of cytochrome c from the mitochondria to the cytosol which supports the evidence for apoptosis.

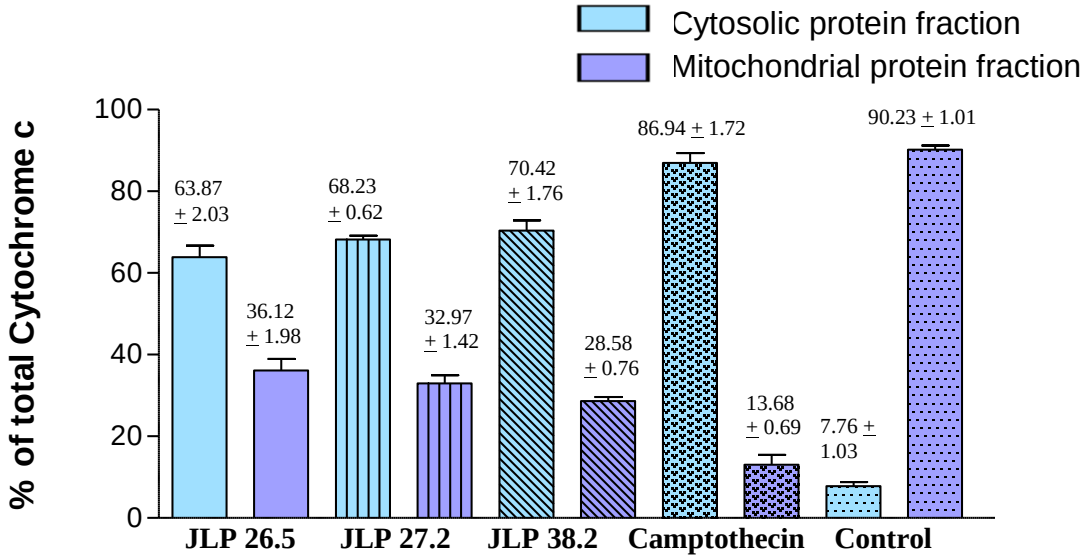


Figure 3.5.6 Percentage cytochrome c protein in the cytosolic and mitochondrial protein fractions of Caco-2 cells after treatment with 100 µM JLP 26.5, JLP 27.2, JLP 38.2 and camptothecin for 24 hours. The bars represent the mean ± SEM from two Elisa assays carried out for each compound.

Figure 3.5.6 shows that the JLP nucleoside analogues also increase the cytosolic cytochrome c fraction but to a lesser extent than camptothecin and the imidazo[1,2-a]pyridine group of compounds. The cytosolic cytochrome c fraction ranged between 61 and 72 percent. Nucleoside analogue JLP 38.2 had the highest cytosolic fraction of cytochrome c (70.42 ± 1.76 %) percent which is lower than that of camptothecin (86.94 ± 1.72 %). This indicates that the three JLP compounds cause a release of cytochrome c from

the mitochondria which provide evidence that the compounds are inducing apoptosis in the Caco-2 cells.

In summary, in the HT-29 cells, the cytochrome c protein fractions of the total cytosolic protein component were significantly increased ($p < 0.05$; $p = 0.00001$) compared to the mitochondrial protein fraction after treatment with the novel nucleoside compounds and novel imidazo[1,2-a]pyridines. The cytochrome c protein fraction in the cytosol of cells treated with camptothecin was evidently more than in the cytosol of cells treated with the JLP nucleoside analogues (Figure 3.5.3) but less in the cells that were treated with either the CDK or CL imidazo[1,2-a]pyridines (Figure 3.5.1 and Figure 3.5.2). Treatment with CL 4 caused the greatest efflux of cytochrome c protein from the mitochondria to the cytosol with 94.10 $\pm$ 0.48 % of the total cytochrome c protein located in the cytosolic protein fraction and 5.41 $\pm$ 0.71 % in the mitochondrial protein fraction. Treatment with JLP 26.5 caused the lowest efflux of cytochrome c protein with 68.22 % in the cytosolic portion and 32 % remaining in the mitochondrial portion.

In the Caco-2 cells, the percentage cytochrome c protein was significantly increased ($p < 0.05$; $p = 0.00001$) in the cytosolic protein fraction compared to the mitochondrial protein fractions after treatment with the novel compounds for 24 hours. Treatment with CL 4 caused the greatest leakage of cytochrome c protein with 86.34 % of the total cytochrome c protein located in the cytosolic protein fraction and 14.66 % in the mitochondrial protein fraction. Treatment with JLP 26.5 caused the lowest efflux of cytochrome c protein with 63.87 $\pm$ 2.03 % in the cytosolic portion and 36.12 $\pm$ 1.98% remaining in the mitochondrial portion.

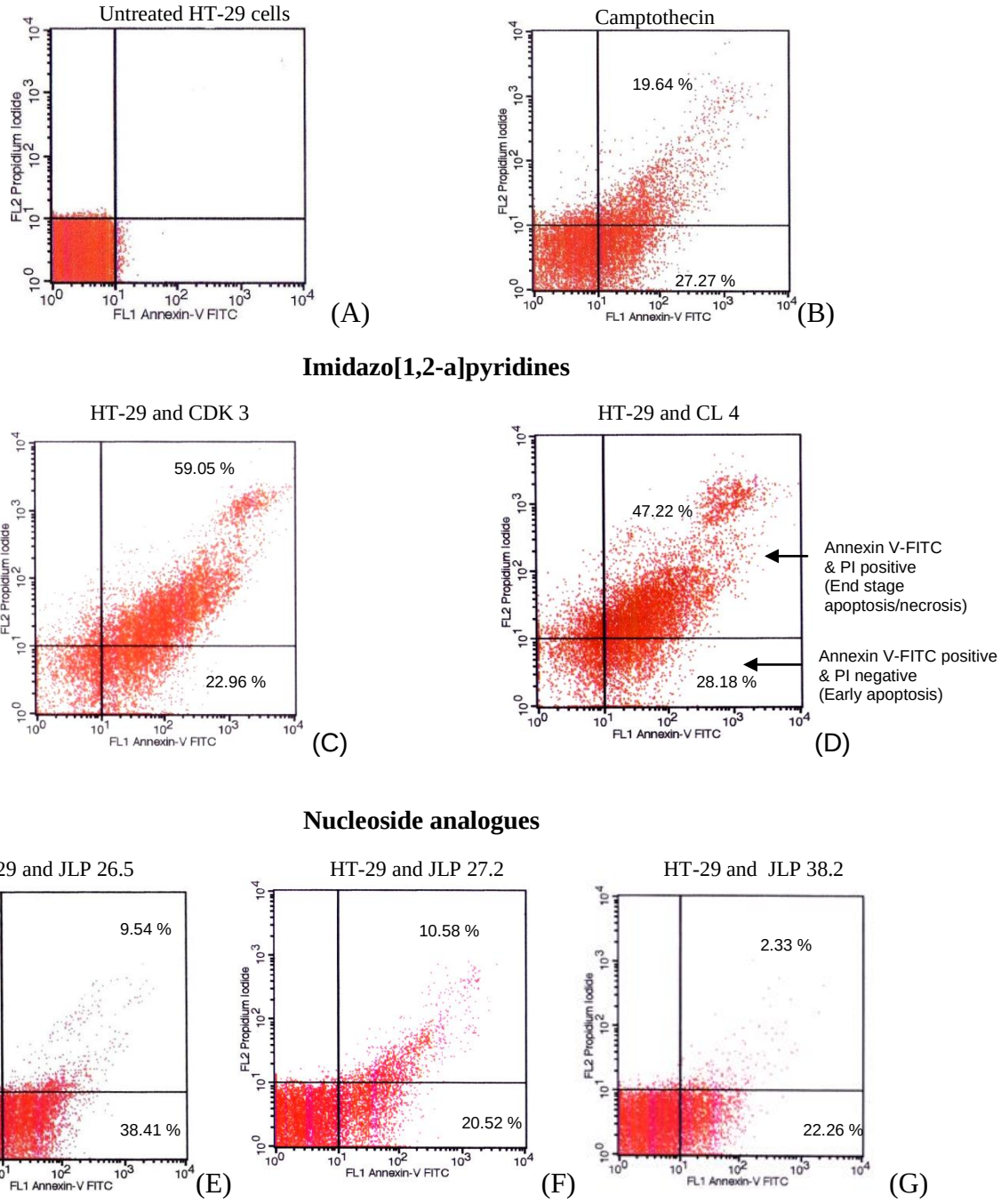
In the HT-29 and Caco-2 cells, treatment with a 100 µM concentration of the JLP nucleoside analogues caused less leakage of the cytochrome c protein to the cytosol compared to treatment with camptothecin and the imidazo[1,2-a]pyridines. After treatment with a 100 µM concentration of the imidazo[1,2-a]pyridine group of compounds, the cytochrome c protein in the cytosolic protein fraction were comparable to that of camptothecin. These results confirm the data obtained on the mitochondrial membrane potential.

3.6 Analysis of the cell membrane integrity in the HT-29 and Caco- 2 cells after treatment with the test compounds

In apoptotic cells, phosphatidylserine (PS), a membrane phospholipid, is translocated from the inner to the outer leaflet of the plasma membrane, hence it is exposed to the external cellular environment. Annexin V is a phospholipid-binding protein with a high affinity for PS, and binds to cells with exposed PS. FACS analysis using Annexin V-FITC staining and PI accumulation was used to differentiate early apoptotic cells (Annexin V+ and PI-) from late apoptotic/necrotic cells (Annexin V+ and PI+). Results are presented as scatter graphs in Figure 3.6.1 and Figure 3.6.2. The lower left quadrant of each panel shows the viable cells that are negative for Annexin V-FITC and PI binding ($FITC^-/PI^-$). The upper right quadrant represents the non-viable, late apoptotic or necrotic cells, positive for Annexin V-FITC binding and for PI uptake (FITC+/PI+). The lower right quadrant represents the cells undergoing early apoptosis, positive for Annexin V-FITC and negative for PI ($FITC+/PI^-$).

3.6.1 Flow cytometric analyses of Annexin V-FITC staining in HT-29 cells

Figure 3.6.1 represents a flow cytometric analysis of HT-29 cells exposed for 24 hours to a 100 µM concentration of CDK 3, CL 4, JLP 26.5, JLP 27.2 and JLP 38.2 compared to untreated cells and camptothecin. The remaining scattergraphs are shown in Appendix E, Figure E2. The results of the test compounds are also tabulated in Table 3.6.1. Results of the flow cytometric analyses shows that after treatment with camptothecin for 24 hours, 27.7% of the HT-29 cells stained FITC+/PI- (early apoptosis) and 19.61 % of cells stained positive for FITC+/PI+ (late apoptosis/necrosis). As shown in Figure 3.6.1C, CDK 3 treatment results in 22.96 % of cells that are positive for early apoptosis and an increased percentage (59.05 %) of late apoptosis/necrosis staining. After treatment with CL 4 (Figure 3.6.1D) for 24 hours, 28.18 % of HT-29 cells were in early apoptosis and 47.22 % of HT-29 cells were in late apoptosis/necrosis.



<u>Figure 3.6.1</u>: Flow cytometric analyses of Annexin V-FITC staining in HT-29 cells after a 24 hour treatment with 100 µM Camptothecin, CDK 3, CL 4, JLP 26.5, JLP 27.2 and JLP 38.2. The scattergraphs represent typical apoptotic and necrotic cell populations detected by annexin V-FITC and PI staining. (A) Untreated HT-29 cells. (B) HT-29 cells treated with camptothecin, (C) CDK 3 (D) CL 4 (E) JLP 26.5, (F) JLP 27.2 and (G) JLP 38.2.

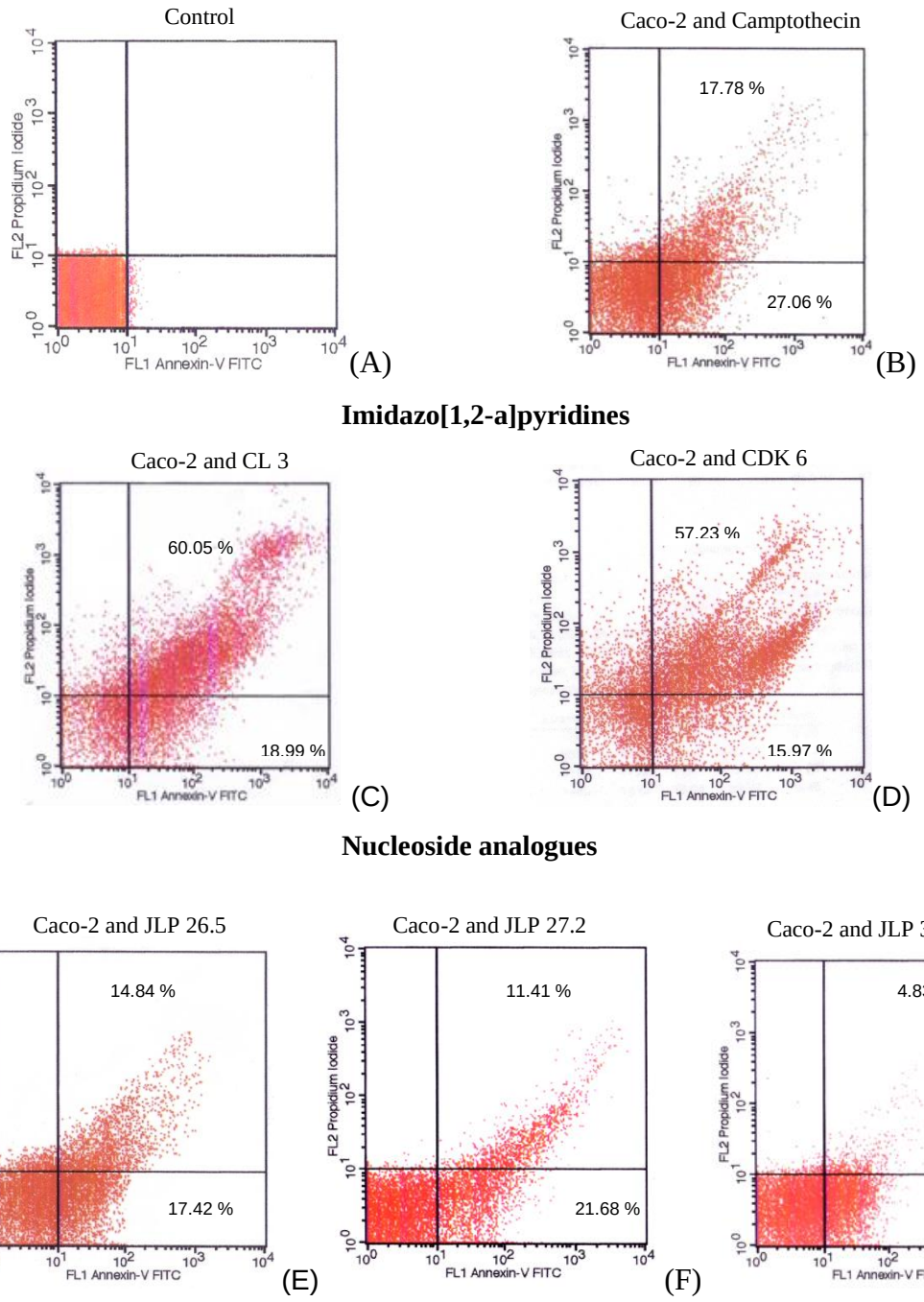
After treatment with the nucleoside analogue JLP 26.5 (Figure 3.6.1E) for 24 hours, 38.41 % of the HT-29 cells stained for early apoptosis and a small percentage (9.54 %) of cells stained for late apoptosis/necrosis. When compared to camptothecin and the other two nucleoside analogues, treatment with JLP 26.5 led to an increased fraction of cells binding FITC/PI- (early apoptosis) with 38.41 % staining as compared to camptothecin and the other two nucleoside analogues, JLP 27.2 and JLP 38.2 (Table 3.6.1). As shown in Figure 3.6.1G and Table 3.6.1, compound JLP 38.2 was observed to result in the smallest percentage of late apoptosis (2.33 %) staining.

Table 3.6.1 **Analysis of cell death induced by the novel compounds in the HT-29 colon cancer cell line**

Compound	Viable cells %	Early apoptosis %	Late apoptosis
CDK 3	14.48	22.96	59.05
CDK 6	38.57	42.71	15.75
CDK 8	18.51	43.28	30.8
CL 3	50.18	27.06	17.78
CL 4	16.53	28.18	47.22
CL 5	12.53	18.11	60.98
CL 6	12.11	18.29	66.54
JLP 26.5	55.08	38.41	5.94
JLP 27.2	68.39	20.52	10.58
JLP 38.2	74.53	22.26	2.33
Camptothecin	47.95	27.27	19.64

In comparison with the untreated control, treatment of HT-29 cells with 100 μM of CDK 8 resulted in increased staining for early apoptosis compared to the other test compounds. After a 24 hour treatment with the JLP nucleoside analogues, an overall decrease in staining for late apoptosis/necrosis was observed as compared to treatment with the imidazo[1,2-a]pyridine compounds as well as camptothecin (Table 3.6.1). The results show that the nucleoside analogues induced cell death at a slower rate when compared to camptothecin and the imidazo[1,2-a]pyridines in the HT-29 cells.

3.6.2 Flow cytometric analyses of Annexin V-FITC staining in Caco-2 cells



<u>Figure 3.6.2</u> Flow cytometric analyses of Annexin V-FITC staining in Caco-2 cells after a 24 hour treatment with 100 µM Camptothecin, CL 3, CDK 6, JLP 26.5, JLP 27.2 and JLP 38.2. The scattergraphs represent typical apoptotic and necrotic cell populations detected by Annexin V-FITC and PI staining. (A) Untreated Caco-2 cells. (B) Caco-2 cells treated with camptothecin, (C) CL 4, (D) CDK 6, (E) JLP 26.5, (F) JLP 27.2 and (G) JLP 38.2.

Figure 3.6.2 represents a flow cytometric analysis of Caco-2 cells exposed for 24 hours to CL 4, CDK 6, JLP 26.5, JLP 27.2 and JLP 38.2 compared to untreated cells and camptothecin. The results of the remaining test compounds are tabulated in Table 3.6.2. Results of the flow cytometric analyses shows that after treatment with camptothecin for 24 hours, 27.06 % of the Caco-2 cells stained FITC+/PI^- (early apoptosis) and 17.78 % of cells stained for FITC+/PI+ (late apoptosis or necrosis). As shown in Figure 3.6.2D, CDK 6 results in an increase of FITC+/PI+ staining of 57.23 % with a smaller percentage (15.97 %) of cells that are only positive for FITC+/PI^-. After treatment with CL 4 for 24 hours, 25.87 % of Caco-2 cells stained for early apoptosis and 44.87 % of cells stained for late apoptosis/necrosis. Of all the imidazo[1,2-a]pyridines, treatment with CL 3 caused the most staining (60.05 %) for late apoptosis (Figure 3.6.2C and Table 3.6.3).

After treatment with JLP 38.2 for 24 hours (Figure 3.6.2G), 30.95 % of the Caco-2 cells stained for early apoptosis and a small percentage (4.83 %) of cells stained for late apoptosis or necrosis. When compared to camptothecin, treatment with JLP 38.2 showed more cells staining for early apoptosis and a fewer cells staining for late apoptosis/necrosis. Treatment with JLP 26.5 (Figure 3.6.1E, Table 3.6.2) led to more staining for late apoptosis/necrosis (14.84 %) and less staining for early apoptosis (17.42 %). The JLP group had the highest percentage of viable cells of the three classes of compounds.

The results obtained from the annexin-V assay support the cell viability results obtained from the MTT assay (Section 3.1 pp 48-54). The results indicate that the JLP nucleoside analogues induced cell death at a slower rate when compared to camptothecin and the CDK and CL imidazo[1,2-a]pyridines (Table 3.6.2) in the Caco-2 cells.

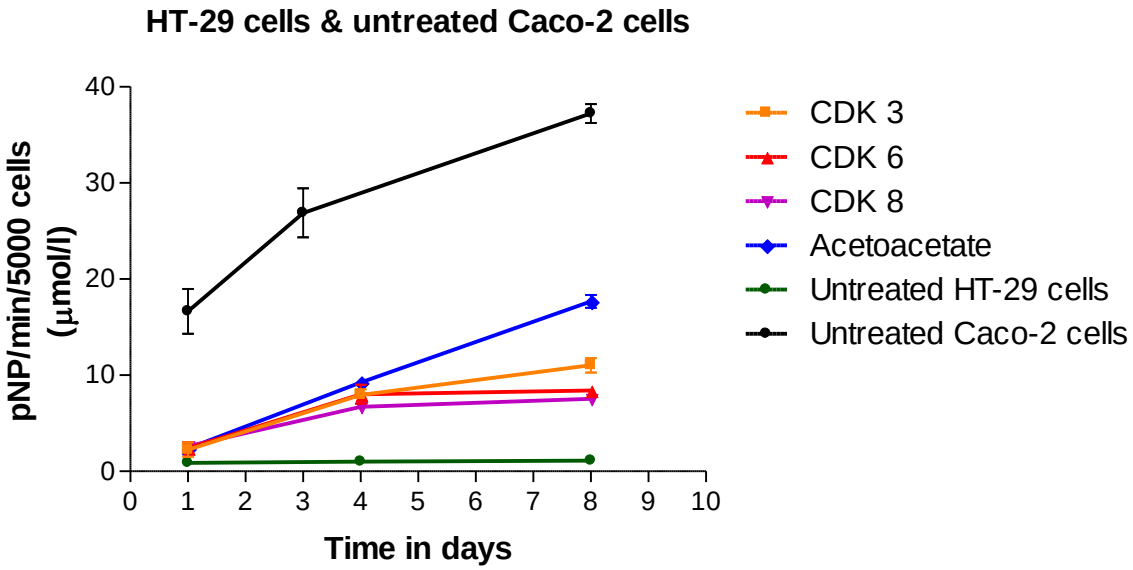
Table 3.6.2 **Analysis of cell death induced by the novel compounds in the Caco-2 colon cancer cell line**

Compound	Viable cells %	Early apoptosis %	Late apoptosis
CDK 3	20.27	18.67	53.8
CDK 6	44.40	15.06	57.23
CDK 8	13.97	19.93	59.81
CL 3	15.55	18.99	60.05
CL 4	23.88	25.87	44.77
CL 5	13.21	21.85	56.31
CL 6	17.47	15.97	57.23
JLP 26.5	61.38	17.42	14.84
JLP 27.2	66.34	21.68	11.41
JLP 38.2	62.59	30.95	4.83
Camptothecin	50.18	27.06	17.78

3.7 The effect of the novel compounds on cellular differentiation in HT-29 cells.

Intestinal alkaline phosphatase, a recognised marker of cellular differentiation, was used to assess induction of differentiation in the HT-29 cells. Low levels of this enzyme has been found in undifferentiated HT-29 cells, whereas a higher level has been observed in differentiated HT-29 and Caco-2 cells (Pintu *et al*., 1982).

As shown in Figure 3.7.1, intestinal alkaline phosphatase activity was significantly higher after treatment with acetoacetate than the CDK compounds for 8 days ($p < 0.05$; $p = 0.0163$). There was no significant difference ($p > 0.05$; $p = 0.785$) in the induction of differentiation between CDK 6, CDK 8 and CDK 3 over the 8 day period. There was no significant increase ($p > 0.05$; $p = 0.0656$) in the intestinal alkaline phosphatase expression levels of the untreated control cells over the 8 days.



<u>Figure 3.7.1:</u> Intestinal alkaline phosphatase expression in untreated Caco-2 cells and HT-29 cells treated with the CDK group of imidazo[1,2-a]pyridines and acetoacetate. The cells were incubated at 37ºC in the presence of 100 µM CDK derivatives and 5 mM acetoacetate for 8 days. Activity is expressed as µmol/l of *p*-nitrophenyl/min/5000 cells. Each point represents the mean and standard deviation of the triplicate values of the intestinal alkaline phosphatase activity on day 1, 4 and 8.

When compared to the untreated HT-29 cells, all the CDK compounds caused a significant increase ($p < 0.05$; $p = 0.0097$) in intestinal alkaline phosphatase activity. The untreated Caco-2 cells exhibited significantly more ($p < 0.05$; $p = 0.00058$) intestinal alkaline phosphatase activity as opposed to the untreated HT-29 cells and the HT-29 cells that were treated with the CDK group of imidazo[1,2-a]pyridines for the 8 day period (Figure 3.7.1).

Of all the CL compounds, CL 3 induced the most intestinal alkaline phosphatase activity after 8 days, however, the activity induced was significantly lower ($p < 0.05$; $p = 0.00015$) than that of acetoacetate (Figure 3.7.2). There was no significant difference ($p > 0.05$; $p = 0.0648$) between the expression levels of intestinal alkaline phosphatase by CL 4, CL 5 and CL 6 on day 8. There was no significant ($p > 0.05$; $p = 0.0656$) increase in the intestinal alkaline phosphatase expression levels of the untreated control cells over the 8 days. When compared to the untreated HT-29 cells, all the CL compounds caused a significant increase ($p < 0.05$) in intestinal alkaline phosphatase activity. As seen in Figure 3.7.2 all the HT-29 cells that were treated with the CL compounds resulted in more alkaline phosphatase activity than the untreated cells. The untreated Caco-2 cells exhibited significantly more ($p < 0.05$; $p = 0.00058$) intestinal alkaline phosphatase activity as opposed to the untreated HT-29 cells and the HT-29 cells that were treated with the CL group of imidazo[1,2-a]pyridines for the 8 day period.

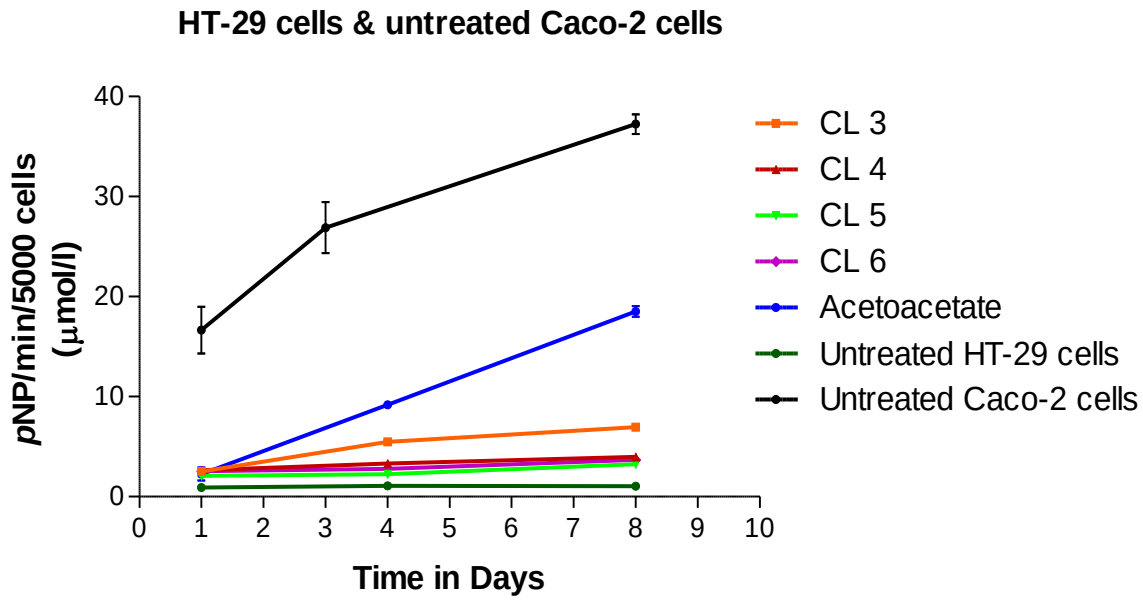


Figure 3.7.2: Intestinal alkaline phosphatase expression in untreated Caco-2 cells and HT-29 cells treated with the CL group of imidazo[1,2-a]pyridines and acetoacetate. The cells were incubated at 37ºC in the presence of 100 µM CL derivatives and 5 mM acetoacetate for 8 days. Activity is expressed as µMol/l of *p*-nitrophenyl/min/5000 cells. Each point represents the mean and standard deviation of the triplicate values of the intestinal alkaline phosphatase activity on day 1, 4 and 8.

Compared to acetoacetate, JLP 26.5 induced a significantly increased ($p = 0.01$) amount of alkaline phosphatase activity, as measured on day 4 and day 8 (Figure 3.7.3). There was no significant ($p > 0.05$; $p = 0.0656$) increase in the intestinal alkaline phosphatase expression levels of the untreated control cells over the 8 days. The HT-29 cells treated with either JLP 27.2 or JLP 38.2 were observed to have significantly higher levels ($p < 0.05$) of alkaline phosphatase activity after the 8 day period when compared to the untreated cells, however these levels were not as high as the cells that were treated with JLP 26.5. The untreated Caco-2 cells exhibited significantly more ($p < 0.05$; $p = 0.00058$) intestinal alkaline phosphatase activity as compared to the untreated HT-29 cells and the HT-29 cells that were treated with the JLP nucleoside analogues for the 8 day period.

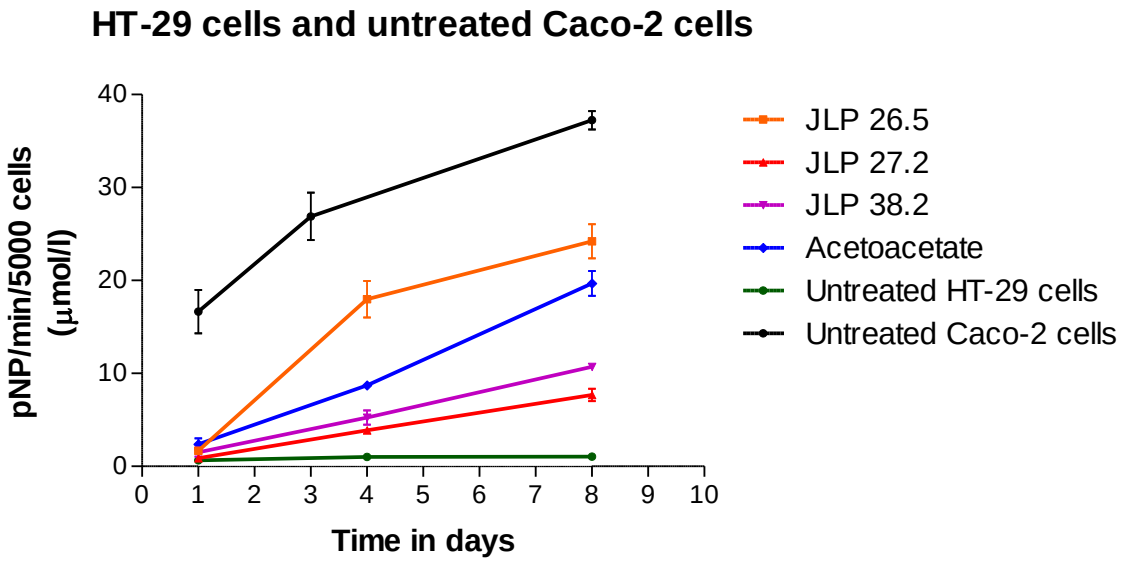


Figure 3.7.3: Intestinal alkaline phosphatase expression in untreated Caco-2 cells and HT-29 cells treated with the JLP nucleoside analogues and acetoacetate. The cells were incubated at 37ºC in the presence of 100 µM JLP compounds and 5 mM acetoacetate for 8 days, after which the reaction was stopped. Activity is expressed as µMol/l of *p*-nitrophenyl/min/5000 cells. Each point represents the mean and standard deviation of the triplicate values of the intestinal alkaline phosphatase activity on day 1, 4 and 8.

IV. Discussion

In the present study, two colon cancer cell lines were exposed to novel nucleoside analogues and novel imidazo[1,2-a]pyridines, aiming to investigate cytotoxic effects on the cells, the mode of cell death, and to explore the pathways by which cell death was induced. Nucleoside analogues form one of the most important classes of chemotherapeutic agents for the treatment of cancer (Heidelberger *et al*., 1983; Galmirini *et al*., 2001). The imidazo[1,2-a]pyridine group of compounds have received considerable attention in the recent years for their various antimicrobial, analgesic, anti-inflammatory and anticancer properties (Gudmundsson KS *et al*., 2003; Lacerda RB *et al*., 2009; Byth *et al*., 2006). The potential cytotoxic effect of the novel imidazo[1,2-a]pyridines and novel nucleoside analogues used in this study have not yet been established on colon cancer cell lines.

Forty compounds were screened for their effects on the HT-29 and Caco-2 cell lines. IC_{50} values were obtained for compounds showing 50 % inhibition of cell growth at a 100 µM concentration. Of the screened compounds, 3 nucleoside analogues and 7 imidazo[1,2-a]pyridines showed cytotoxic effects on the two colon cancer cell lines. The effects of the novel compounds were compared to that of camptothecin, which is a mammalian topoisomerase I inhibitor that acts in the S-phase of the cell cycle and induces apoptosis through both the death receptor pathway and the mitochondrial pathway (Zhang *et al*., 2000b; Wang *et al*., 2008).

4.1 The effects of the imidazo[1,2-a]pyridines and nucleoside analogues on the cell survival of HT-29 cells, Caco-2 cells and leucocytes

Of the eight CDK imidazo[1,2-a]pyridines tested, three CDK compounds caused more than 50 % inhibition of cell viability at 100 µM. As reported in Chapter 3.1, treatment with the active CDK group of imidazo[1,2-a]pyridines showed a significant decrease in the cell viability of the two cancer cell lines (Figure 3.1.1). When the HT-29 and Caco-2 cells were treated with increasing concentrations of the CDK compounds for 24 hours, the percentage of viable cells decreased in a dose-dependent manner. Compounds CDK 3 (IC_{50} = 9.20 ± 0.83 µM) and CDK 8 (IC_{50} = 10.03 ± 2.69 µM) were comparable to camptothecin (IC_{50} = 10.00 ± 1.41 µM) in the HT-29 cells. The Caco-2 cells displayed similar sensitivity to all

three CDK compounds with IC_{50} values as follows: Compound CDK 8 (IC_{50} = 15.02 ± 1.98 µM), CDK 3 (IC_{50} = 17.38 ± 1.13 µM) and CDK 6 (IC_{50} = 20.28 ± 3.45 µM). Camptothecin (IC_{50} = 9.55 ± 2.21 µM) caused more cell inhibition than the CDK imidazo[1,2-a]pyridines in the Caco-2 cells.

Of the six CL imidazo[1,2-a]pyridines tested, four CL compounds caused more than 50 % decrease in cell viability of both cell lines at 100 µM. (Figure 3.1.4). When the HT-29 and Caco-2 cells were treated with the four effective CL compounds for 24 hours, the percentage of viable cells decreased in a dose dependent manner (Figures 3.1.4.1 and Figure 3.1.4.2). Compound CL 4 caused the most cytotoxicity in both cell lines (IC_{50} = 6.57 ± 1.91 µM in the HT-29 cells and IC_{50} = 6.43 ± 1.01µM in the Caco-2 cells). All the CL compounds were comparable to camptothecin in the HT-29 and Caco-2 cells.

Of the 26 JLP nucleoside analogues tested, three JLP compounds caused more than 50 % cell inhibition at 100 µM. As shown in figure 3.1.7, none of the JLP compounds were comparable to camptothecin in their effects on cell viability in the HT-29 and Caco-2 cells. From all the nucleoside analogues, compound JLP 26.5 (IC_{50} = 35.35 ± 1.30 µM) caused the most cell inhibition in the HT-29 cells whereas compound JLP 27.2 (IC_{50} = 48.13 ± 2.45 µM) caused the most cell inhibition in the Caco-2 cells. Treatment with the JLP nucleoside analogues showed less inhibition of cell viability in the HT-29 and Caco-2 cells (IC_{50} between 36 and 63 µM) than the imidazo[1,2-a]pyridine group of compounds (IC_{50} between 5.4 and 23 µM).

Camptothecin is a plant alkaloid that that has been noted to arrest cells during the S phase of mitosis. In literature, the IC_{50} values of Camptothecin in cell culture are reported to range from 7 nm to 13 µM (Tanizawa., 1994: Luzzio *et al.*, 1995). The range in values is due to differences in cell density, different incubation times, solubility of the compound, chemical stability, and any factors affecting the cellular uptake of the compound. In this study, there was no significant difference ($p > 0.05$) between the IC_{50} values of camptothecin on the HT-29 and Caco-2 cell lines after 24 hours.

Leucocytes were exposed to the novel imidazo[1,2-a]pyridines and novel nucleoside analogues to determine whether the compounds exhibit any cytotoxic activity to normal, unstimulated, white blood cells as opposed to the cancer cells. As shown in Table 3.2.1, camptothecin was observed to be significantly ($p < 0.05$) more toxic to the leucocytes than

all the compounds tested. Of all the tested compounds, the nucleoside analogue JLP 27.2 caused the greatest decrease in leucocyte cell viability (61.456 ± 4.481 %). The CL imidazo[1,2-a]pyridines were more toxic than the CDK imidazo[1,2-a]pyridines although these effects were lower than the effects of camptothecin (33.78 ± 2.03 %).

4.2 Caspase-8 and caspase-3 activity

The activity of caspase-8 and caspase-3 was investigated as it gives an indication of the mechanism of cell death induced by the novel compounds in the colon cancer cells. Caspase-8, a member of the caspase family, is one of the molecules that is crucial for the induction of apoptotic cell death. Impaired expression or function of caspase-8 can promote tumor formation, progression and treatment resistance in several types of cancers. Caspase-3, the key executioner of apoptosis, is responsible for the cleavage and breakdown of several cellular components related to DNA repair and regulation. In this study, the effect of the novel compounds on caspase-8 activity was determined. It is expected that caspase-8 will reach maximum activity earlier than caspase-3 after treatment with the novel compounds since it induces the effector caspases (Degterav *et al*., 2003).

As shown in Figure 3.3.1 and Figure 3.3.4, a sequential activation of caspase-8 and caspase-3 was observed in the HT-29 and Caco-2 cells after exposure to the imidazo[1,2-a]pyridines. Caspase-8 activity was detected after 2 hours of exposure with the compounds in the HT-29 cells and Caco-2 cells. Although CDK 6 caused less inhibition of cell viability compared to CDK 3 and CDK 8 in both cell lines, it induced caspase-8 activity to the same level as camptothecin.

Caspase-3 activity was measured after exposing the HT-29 and Caco-2 cell lines to the novel compounds from 2 to 24 hours. Caspase-3 activity was detected as early as 2 hours after treatment with the CDK compounds (Figure 3.3.1 and Figure 3.3.4). CDK 8 caused the greatest increase in activity at 2 hours. Caspase-3 activity after treatment with CDK 3 was shown to be comparable to treatment with camptothecin in the HT-29 cells. The decrease in caspase-3 activity at 24 hours in the tested cell lines could occur due to the cytotoxic effects of the novel compounds resulting in the death of cells with active caspase-3.

Treatment with the selected CL group of imidazo[1,2-a]pyridines at a concentration of 100 μM also caused a sequential activation of caspase-8 and caspase-3 activity as early as 2 hours post treatment of the compounds. The reduction of the caspase-3 activity at 24 hours may be due to the cytotoxicity of the novel CL compounds resulting in the death of cells with active caspase-3. Compound CL 6 was the strongest inducer of both caspase-8 and caspase-3 activity and maintained the high induction levels at 24 hours. Treatment with the selected CDK and CL group of imidazo[1,2-a]pyridines at a concentration of 100 μM for 24 hours, caused an increase in caspase-8 and caspase-3 activity which provides evidence that apoptosis has been induced by the CDK and CL compounds on both cell lines.

Treatment with the selected JLP nucleoside analogues at a concentration of 100 μM for 24 hours also caused a sequential activation of caspase-8 and caspase-3 activity which provides evidence that the cells are entering apoptosis. Compound JLP 27.2 induced the caspases in HT-29 cells to a similar level as camptothecin but at a slower rate (8 hours versus 2 hours for caspase-8 activity and 12 hours versus 4 hours for caspase-3 activity). In the Caco-2 cells, JLP 27.2 sustained increased caspase activity even more so than camptothecin (Table 3.3.6b). Although compound JLP 27.2 induced increased caspase activity relative to the other compounds, it showed less cytotoxic effects in the HT-29 and Caco-2 cells as measured by the MTT assay. This was contrary to the expectation and could be due to lower mitochondrial toxicity compared to the CDK and the CL imidazo[1,2-a]pyridines.

4.3 Mitochondrial membrane potential and the release of cytochrome c

Mitochondria play a central role in the regulation of apoptotic signaling (Godvadse and Orrenius., 2006). Several studies have shown that oxidants activate the mitochondrial permeability transition pore which results in a loss of the inner mitochondrial membrane potential (Kroemer *et al*., 2007). The disruption of the mitochondrial membrane potential ($\Delta\psi m$) is one of the earliest intracellular events that occurs following the induction of apoptosis (Ly *et al*., 2003) and could be a prerequisite for the release of cytochrome c. Cytochrome c is an electron transport protein which is normally located between the inner

and outer mitochondrial membranes. Cytochrome c has been shown to translocate from the mitochondria to the cytosol during the early phase of apoptosis (Jiang and Wang., 2004).

In this study, the imidazo[1,2-a]pyridines caused a significant decrease in mitochondrial membrane potential ($\Delta\psi m$) in the HT-29 and Caco-2 cell lines particularly after treatment with CDK 3 and CL 4 (Table 3.4.1 and Table 3.4.2). As shown in Figure 3.4.1d and Table 3.4.1, treatment with CL 4 caused an increased reduction of the mitochondrial membrane potential in the HT-29 cells whereas CL 3 caused the most reduction of the mitochondrial membrane potential in the Caco-2 cells (Figure 3.4.2c and Table 3.4.2). The loss in $\Delta\psi m$ measured using the CDK and CL group of imidazo[1,2-a]pyridines after a 24 hour treatment was supported by the translocation of cytochrome c from the mitochondria to the cytosol in the HT-29 and Caco-2 cells as well as the increase in caspase-8 and caspase-3 activity. Since cytochrome c was detected after a 24 hour treatment with the compounds, this supports the evidence that the cells were undergoing apoptosis. This together with the results from the MTT assay indicates mitochondrial damage in the cells which supports the activation of the intrinsic mitochondrial-apoptotic pathway.

In this study, treatment with the JLP nucleoside analogues caused a decrease in $\Delta\psi m$ in the HT-29 and Caco-2 cell lines, however these changes were relatively small when compared to the reduction in the $\Delta\psi m$ after treatment with the CDK and CL imidazo[1,2-a]pyridine compounds. Treatment with a 100 µM concentration of the JLP compounds showed a decrease in the levels of cytochrome c release compared to treatment with the imidazo[1,2-a]pyridines (Figure 3.5.3 and Figure 3.5.6). This is consistent with the observed lower cytotoxicity of the JLP compounds in both cell lines. In the Caco-2 cells, compound JLP 38.2 induced higher levels of cytochrome c than the other two nucleoside analogues.

The JLP nucleoside analogues showed less mitochondrial effects than all the tested compounds including camptothecin. The mitochondrial membrane potential was retained in the JLP nucleoside-treated HT-29 and Caco-2 cells. Thus, the release of cytochrome c in the JLP-treated cells appears to occur by a more specific mechanism that does not include mitochondrial depolarization such as through a pore formed by the oligomerization of Bax and/or Bak in the mitochondrial membrane (Degenhardt *et al*., 2002; Guo *et al*., 2004).

4.4 Annexin V-FITC staining

The cytotoxic effect of the novel CL compounds, as analyzed by binding of annexin V-FITC, was evident at the dose of 100 µM after treatment for 24 hours. The annexin-V binding assay demonstrated that phosphatidylserine was translocated to the outer leaflet of the plasma membrane of the imidazo[1.2a]pyridine-treated HT-29 and Caco-2 cells which provides further evidence that the compounds induce apoptosis. The results obtained from the annexin-V assay confirm the IC_{50} values of the CDK and CL imidazo[1.2a]pyridines in the HT-29 and Caco-2 cells as well as the changes in the mitochondrial membrane potential.

The results obtained from the annexin-V assay verify the IC_{50} values of the JLP nucleoside analogues in the HT-29 and Caco-2 cells. Camptothecin caused more cell death in the HT-29 and Caco-2 cells than the JLP compounds. Although JLP 27.2 showed maximal caspase activity, fewer cells stained for early apoptosis when compared to JLP 26.5 and JLP 38.2 yet an increased amount of cells (10.58 %) stained for late apoptosis (Figure 3.4.1F). Since externalization of phosphatidylserine (PS) occurs in the earlier stages of apoptosis, it may be that the JLP nucleoside analogues induce apoptosis at a slower rate than the imidazo[1,2-a]pyridines and camptothecin.

4.5 Intestinal alkaline phosphatase activity

The alkaline phosphatase assay was performed in order to determine if any of the novel compounds induce differentiation in the HT-29 cells. Since cancer cells are generally less differentiated than normal cells, increasing or restoring differentiation in cancer cells may be one method of reducing or removing their replicative or invasive potential (Gagna *et al*., 2001). Drug-induced differentiation has been demonstrated with agents that exert minimal effects on normal cells and is therefore favoured over conventional chemotherapeutic agents (Cohen., 1999; Musch., 2010). The low alkaline phosphatase levels in the HT-29 control sample throughout the 8 day experiment confirms the findings of Pinto *et al*., 1982. Acetoacetate is thought to be the most potent inducer of gastrointestinal differentiation (Graz and Cowley, 1997). Exposure to increased concentrations of acetoacetate would result in an increased expression of alkaline phosphatase, which is shown in the marked increase in alkaline phosphatase expression from day 4 to day 8 in the HT-29 cells.

When compared to the untreated HT-29 cells, all the CDK and CL imidazo[1,2-a]pyridines caused a significant increase ($p < 0.05$; $p = 0.0097$) in intestinal alkaline phosphatase activity. As seen in Figure 3.7.1 and Figure 3.7.2, all the HT-29 cells that were treated with the CDK and CL imidazo[1,2-a]pyridines showed more alkaline phosphatase activity than the untreated HT-29 cells but less alkaline phosphatase activity than the untreated caco-2 cells. Compound CDK 3 was a stronger inducer of alkaline phosphatase activity than the other CDK imidazo[1,2-a]pyridines (Figure 3.7.1) and compound CL 3 was a stronger inducer of alkaline phosphatase activity than the other CL imidazo[1,2-a]pyridines in the HT-29 cell line (Figure 3.7.2).

The JLP nucleoside analogues were more effective than the CDK and CL imidazo[1,2-a]pyridine compounds in inducing intestinal alkaline phosphatase activity with JLP 26.5 showing more alkaline phosphatase activity than acetoacetate (Figure 3.7.3). Compound JLP 26.5 caused more reduction in cell viability than the other test JLP nucleoside analogues but had a decreased effect on the mitochondria in comparison to the imidazo[1,2-a]pyridine compounds as demonstrated by the marginal changes to the $\Delta\psi m$. Hence, the possibility that this compound has an effect on the HT-29 colon cancer cell differentiation should be investigated.

The untreated Caco-2 cells exhibited high levels of alkaline phosphatase expression, very similar to that of acetoacetate in the HT-29 cells. On day 8, the alkaline phosphatase expression levels in the Caco-2 cells (Figure 3.7.1) were significantly higher than those determined for HT-29 cells. This can be explained by the spontaneous differentiation that occurs with contact inhibition (Briske-Anderson *et al*., 1996) in this cell line.

4.6 Apoptotic induction potential of the novel compounds in HT-29 and Caco-2 cells

Numerous studies have confirmed the involvement of cell death receptors such as FAS and DR4, in apoptosis induced by chemotherapeutic agents in a variety of cancer cells. In contrast, other studies have reported that apoptosis could also be independent of the cell death receptor signaling pathways (Debatin and Krammer., 2004). From these studies, it seemed that the cell death signaling system and the mitochondrial pathway participated in triggering apoptosis after drug treatment in this study.

Among the selected imidazo[1,2-a]pyridines that were tested against the HT-29 and Caco-2 cell lines, CDK 3 and CL 4 appeared to be the most cytotoxic (IC_{50} range 6 – 22 µM) compared to the rest of the compounds. The compounds showed a degree of selective cytotoxicity for the cancer cell lines, with less cytotoxicity against unstimulated white blood cells. The proteolytic phase of apoptosis was initiated within 2 hours after treatment with these compounds as observed by the increased caspase-8 and caspase-3 activity and thus suggests the involvement of the death receptor pathway. These compounds were also associated with a marked reduction in the mitochondrial membrane potential ($\Delta\psi m$) as well as causing an increase in cytochrome c levels within the cytosolic fraction of the cells. The electron transport protein, cytochrome c has been identified to bind to the apoptotic protease activating factor-1 (Apaf-1) which forms the apoptosome. This results in the proteolytic activation of procaspase 9. Active caspase-9 can then activate the executioner caspase-3 which results in apoptosis (Nadege *et al*., 2009). Since the translocation of cytochrome c to the cytosol was seen after treatment with the novel compounds, the activation of the intrinsic apoptotic pathway is also a possibility.

Many different types of cancers occur due to changes in the cyclin-dependent kinases, the key regulatory proteins that control the phases of the cell cycle. A previously derived class of imidazo[1,2-a]pyridines has been identified as effective cyclin-dependent kinase inhibitors, and has shown cytostatic and cytotoxic activity on a number of cancer cell lines (Jaramillo *et al*., 2004; Byth *et al*., 2006). Since the novel imidazo[1,2-a]pyridines tested in in this study share the same scaffold as the imidazo[1,2-a]pyridines identified as cyclin-dependent kinase inhibitors, their potential effects on the protein kinase signaling pathways should be investigated in future studies.

Among the selected nucleoside analogues that were tested against the HT-29 and Caco-2 cell lines, JLP 26.5 appeared to be the most cytotoxic (IC_{50}= 35.35 ± 1.30 µM) to the HT-29 cells and JLP 27.2 appeared to be the most cytotoxic to the Caco-2 cells (IC_{50} = 48.13 ± 2.45 µM). In the Caco-2 cells, JLP 27.2 induced increased levels of caspase-8 and caspase-3 activity in comparison to camptothecin although the peak caspase activity was reached of time (4-8 hours versus 2 hours). In comparison to the imidazo[1,2-a]pyridine compounds, the mitochondrial membrane potential was retained in the JLP-treated HT-29 and Caco-2 cells until after the release of cytochrome c and caspase-8 and caspase-3 activation.

It is generally thought that a reduction in the mitochondrial membrane potential precedes the release of cytochrome c from the mitochondria. However, the results from this study for the JLP nucleoside analogues showed that the two events are not interdependent. There are several reports in the literature that confirm that the release of cytochrome c is not always linked to a change in the mitochondrial membrane potential (Ly *et al*., 2003). Bax (Bcl-2-associated X protein) and Bak (Bcl-2-antagonist killer) are two very important pro-apoptotic Bcl-2 proteins (Mikhailov *et al*., 2003). Bax is normally present in the cytosol of living cells and has been shown to translocate to the mitochondria during apoptosis due to the upregulation of p53 (Bedner *et al*., 2000; Feng *et al*., 2000). Once in the mitochondria, bax can form a pore composed of either homo- or hetero-oligomers in the mitochondrial membrane. The release of cytochrome c has been shown to occur through these pores (Shimizu *et al*., 2000).

Nucleoside analogues such as gemcitabine, cytarabine and zidovudine require cellular activation to the triphosphate form before they are incorporated into the DNA (Chabner *et al*., 2011). Once incorporated into the DNA, the resultant faulty DNA activates p53 as the cell cycle proceeds to the G2/Mitosis checkpoint. The accumulation of p53 can activate the transcription of the BH3-only proteins such as PUMA (Feng *et al*., 2000). PUMA interacts with anti-apoptotic Bcl-2 and Bcl-X_L and is dependent on Bax to induce the intrinsic pathway of apoptosis (Ming *et al*., 2006). This process provides a possible explanation for the delay in the cytotoxicity of the novel nucleoside analogues tested in this study.

4.7 Future Studies

Future work should include an additional method to assess cell viability such as the thymidine incorporation assay which directly measures DNA synthesis. The current study can be expanded by performing fluorescent microscopy to determine morphological changes. Novel compounds based on the structure of the active ones in this study should be designed and synthesised, and screened against various cancer lines. Future studies could include investigating combination studies with existing anticancer agents like gemcitabine, etoposide and cisplatin.

The active compounds can also be screened against various other cancer cell lines as well as a primary animal model for *in vivo* studies. Pro-apoptotic and anti-apoptotic genes such as Bax, Bcl-2, BID, PARP, and Apaf-1 could also be investigated for their roles in apoptosis as well as identifying the changes in the expression and activity of the p53 tumour suppressor gene.

V. Conclusion

The results of this study showed that the novel imidazo[1,2-a]pyridines and novel nucleoside analogues illicit cytotoxic effects in the HT-29 and Caco-2 cells with the effects of the imidazo[1,2-a]pyridines being comparable to those of camptothecin. Both groups of compounds induced a caspase-dependent apoptosis as shown by the sequential activation of caspase-8 and caspase-3 activity after 2 hours of treatment with the compounds. The imidazo[1,2-a]pyridines caused a marked reduction in the mitochondrial membrane potential ($\Delta\psi m$) as well as a translocation of cytochrome c from the mitochondria to the cytosol after a 24 hour treatment. This together with the results from the MTT assay supports the involvement of both an extrinsic and an intrinsic apoptotic pathway. The annexin-V binding assay demonstrated that phosphatidylserine was translocated to the outer leaflet of the plasma membrane of the treated HT-29 and Caco-2 cells which provides further evidence that the compounds induce apoptosis.

In contrast to the imidazo[1,2-a]pyridine compounds, the mitochondrial membrane potential was unaffected by the JLP nucleoside analogues. Contrary to expectations, the nucleoside analogues caused an increase in the release of cytochrome c from the mitochondria and an increase in caspase-8 and caspase-3 activity, more so than the imidazo[1,2-a]pyridines. It is possible that cytochrome c was released from the mitochondria to the cytosol, through a protein permeable pore or channel in the mitochondrial membrane. The JLP nucleoside analogues were more effective than the imidazo[1,2-a]pyridine compounds in inducing intestinal alkaline phosphatase activity with JLP 26.5 showing more alkaline phosphatase activity than acetoacetate. It is thus likely that JLP 26.5 has an effect on the HT-29 colon cancer cell differentiation and should be investigated further.

Both groups of novel compounds showed promising antitumor properties in vitro. Since the induction of apoptosis is considered to be the main mechanism underlying the therapeutic efficacy of anticancer drugs, the present results suggest that the novel compounds offer potential to serve as lead compounds for the treatment of colon cancer.

VI. References

Abud HE (2004) Shaping developing tissues by apoptosis. *Cell Death and Differentiation* 11: 797-799.

Adams VR, Deremer DL, Stevich B, Mattingly CA, Gallt B, Subramanian T, Troutman JM and Spielmann HP (2010) Anticancer activity of novel unnatural synthetic isoprenoid. *Anticancer Research* 30 (7): 2505-12.

Alberts B, Johnson A, Lewis J, Raff M, Roberts K and Watson JD (2008a) Chapter 5. The Initation and Completion of DNA Replication In Chromosomes. Molecular Biology of the Cell. 5th Edition. Garland Science, New York. pp 294-295.

Alberts B, Johnson A, Lewis J, Raff M, Roberts K and Watson JD (2008b) Chapter 20. Cancer as A Microevolutionary Process. Molecular Biology of the Cell. 5th Edition. Garland Science, New York. pp 1205-1206.

Bao Q and Shi Y (2007) Apoptosome: a platform for the activation of initiator caspases. *Cell death and differentiation* 1: 56-65.

Barnhart BC, Lee JC, Alappat EC and Peter ME (2003) The death effector domain protein family. *Oncogene* 22 (53): 8634-44.

Bedner E, Li X, Kunicki J and Darzynkiewicz Z (2000) Translocation of Bax to mitochondria during apoptosis measured by laser scanning cytometry. *Cytometry* 41: 83–88.

Bendardaf R (2006) Colorectal cancer: from epidemiology to current treatment. *Libyan Journal of Medicine* 1 (1): 42-59.

Benson AB and Goldberg RM (2003) Optimal use of the combination of irinotecan and 5-fluorouracil. *Seminars in Oncology* 30: 68-77.

Bergmeyer, HU (1983) Methods of Enzymatic Analysis. Verlag Chemie, Weinheim pp. 269–270.

Bingham S and Riboli E (2004) Diet and Cancer, the European Prospective Investigation into Cancer and Nutrition. *Nature Reviews Cancer* 4: 206-215.

Bjelakovic G, Nagorni A, Bjelakovic M, Stamenkovic I, Arsic R and Katic V (2005) Apoptosis: Programmed cell death and its clinical implications. *Facta Universitatis, Series Medicine and Biology* 12: 6-11.

Bolte G, Wolburg H, Beuermann K, Stocker S and Stern M (1998) Specific interaction of food proteins with apical membranes of the human intestinal cell lines Caco-2 and T84. *Clinica Chimica Acta* 270: 151-167

Bonate PL, Arthaud L, Cantrell WR, Stephenson K, Secrist JA and Weitman S (2006) Discovery and development of clofarabine: a nucleoside analogue for treating cancer. Nature Reviews Drug Discovery 5: 855-863.

Boyle P and Leon ME (2002) Epidemiology of colorectal cancer. *British Medical Bulletin* 64 (1): 1-25.

Briske-Anderson MJ, Finley JW and Newman SM, (1997) The influence of culture time and passage number on the morphological and physiological development of Caco-2 cells. *Proceedings of the Society for Experimental Biology and Medicine* 214: 248-257.

Broker LE, Kruyt FA and Giaccone G (2005) Cell death independent of caspases: a review. *Clinical Cancer Research* 11: 3155-3162.

Brosh R and Rotter V (2009) When mutants gain new powers: news from the mutant p53 field. *Nature Reviews Cancer* 9: 701-713.

Byth KF, Geh C, Forder CL, Oakes SE and Thomas AP (2006) The cellular phenotype of AZ703, a novel selective imidazo[1,2-a]pyridine cyclin-dependent kinase inhibitor. *Molecular Cancer Therapeutics* 5: 655–664.

Cao Y and Prescott SM (2002) Many Actions of Cyclo-oxygenase-2 in Cellular Dynamics and in Cancer. *Journal of Cellular Physiology* 190: 279-286.

Carmichael J, DeGraff WG, Gazdar AF, Minna JD and Mitchell JB (1987) Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Research* 47: 936–942.

Castedo M, Ferri K, Roumier T, Me´tivier D, Zamzami N and Kroemer G (2002) Quantitation of mitochondrial alterations associated with apoptosis. *Journal of Immunological Methods* 265: 39– 47.

Chabner BA, Bertino J, Cleary J, Ortiz T, Lane A, Supko JG and Ryan D (2011) Chapter 61. Cytotoxic Agents. In: Brunton LL, Chabner BA & Knollmann BC (Eds). *Goodman & Gilman's The Pharmacological Basis of Therapeutics.* 12th Edition. New York, McGraw-Hill. pp 1695-1700.

Chakrabarty S, Radjendirane V, Appelman H and Varani J (2003) Extracellular calcium and calcium sensing receptor function in human colon carcinomas: promotion of E-cadherin expression and suppression of beta-catenin/TCF activation. *Cancer Research* 63: 67–71.

Chang D, Xing WZ, Pan Y, Algeciras-Schimnich A, Barnhart BC, Yaish-Ohad S, Peter ME, and Yang X (2001) c-FLIP(L) is a dual function regulator for caspase-8 activation and CD95-mediated apoptosis. *European Molecular Biology Organization (EMBO)* 21: 3704–3714.

Chao A, Thun MJ, Connell CJ, McCullough ML, Jacobs EJ, Dana Flanders W, Rodriguez C, Sinha R and Calle EE (2005) Colorectal cancer and long-term meat consumption. *Nature Reviews Gastroenterology and Hepatology* 293 (2): 172-82.

Chibaudel B, Tournigand C, Andre T and de Gramont A (2012) Therapeutic strategy in unresectable metastatic colorectal cancer. *Therapeutic advances in medical oncology* 4 (2)**:** 75-89.

Cho E, Smith-Warner SA, Ritz J, van dan Brandt PA, Colditz GA, Folsom AR, Freudenheim JL, Giovannucci E, Goldbuhm RA, Graham S, Holmberg L, Kim DH, Malila N, Miller AB, Chung KY and Saltz LB (2007) Adjuvant therapy of colon cancer: current status and future directions. *Cancer Journal* 13 (3): 192–197.

Cohen E, Ophir I and Shaul YB (1999) Induced differentiation in HT-29, a human colon adenocarcinoma cell line. *Journal of Cell Science* 112: 2657 – 2666.

Coleman ML and Olson MF (2002) Rho GTPase signalling pathways in the morphological changes associated with apoptosis. *Cell Death and Differentiation* 9: 493–504.

Croce CM and Carlo (2008) Oncogenes and Cancer. *New England Journal Medicine* 358: 502-511.

Cusack JC Jr (2003) Overcoming antiapoptotic responses to promote chemosensitivity in metastatic colorectal cancer to the liver. *Annals of Surgical Oncology* 10: 852-862.

Dabare AA, Nouri M, Cannell H, Moss T, Nigam AK and Oliver RT (1999) Profile of placental alkaline phosphatase expression in human malignancies: effect of tumor cell activation on alkaline phosphatase expression. *International Journal of Urology* 63: 168–174.

Debatin KM and Krammer PH (2004) Death receptors in chemotherapy and cancer. *Oncogene* 23: 2950–2966.

Degos L and Wang ZY (2001) All trans retinoic acid in acute promyelocytic leukemia. *Oncogene* 20: 7140–7145.

Denecker G, Vercammen D, Declercq W and Van den abeele P (2001) Apoptotic and necrotic cell death induced by death domain receptors. *Cellular and Molecular Life Sciences* 58: 356–70.

Dimberg J, Hugander A, Sirsjo A and Soderkvist P (2001) Enhanced expression of cyclo-oxygenase 2 and nuclear beta catenin are related to mutations in the APC gene in human colorectal cancer. *Anticancer Research* 21: 911-915.

Elmore S (2007) Apoptosis: A Review of Programmed Cell Death. *Toxicologic pathology* 35 (4): 495 – 516.

Ethell DW and Buhler LA (2003) Fas ligand-mediated apoptosis in degenerative disorders of the brain. *Journal of Clinical Immunology* 23: 439–446.

Evan Gl, and Vousden KH (2001) Proliferation, cell cycle and apoptosis in cancer. *Nature* 411: 342-348.

Ewald B, Sampath D and Plunket W (2008) Nucleoside analogs: molecular mechanisms signaling cell death responses to nucleoside analogs. *Oncogene* 27: 6522-6537.

Fadeel B, Ahin A, Henter J, Orrenius S and Hampton MB (2003) Involvement of caspases in neutrophil apoptosis: regulation by reactive oxygen species *Blood* 92: 4808-4818.

Feng L, Achanta G, Pelicano H, Zhang W, Plunkett W and Huang P (2000) Role of p53 in cellular response to anticancer nucleoside analog-induced DNA damage. *International Journal of Molecular Medicine* 5: 597-604.

Ferlay J, Shin HR, Bray F, Forman D, Mathers C and Parkin DM (2010) Estimates of worldwide burden of cancer in 2008. *International Journal of Cancer* 127: 2893-2917.

Fogh J, Fogh JN and Orfeo T (1977) One hundred and twenty seven cultured human tumor cell lines producing tumors in nude mice. *Journal of the National Cancer Institute*. 59: 221-226.

Forslund A, Kressner U, Lonnroth C, Andersson M, Lindmark G. and Lundholm K (2002) P53 mutations in colorectal cancer assessed in both genomic DNA and cDNA as compared to the presence of p53 LOH. *International Journal of Oncology* 21: 409-415.

Freshney, RI (1987) Culture of Animal Cells: A Manual of Basic Technique, 2nd Edition. Alan R. Liss, Inc., New York. p117, p317.

Freshney, RI (1994) Culture of Animal Cells: A Manual of Basic Technique, 3rd Edition. John Wiley & Sons, Inc., New York. pp 90-91.

Fulda S, Galluzzi L and Kroemer G (2010) Targeting mitochondria for cancer therapy. *Nature Reviews* 9: 447-463.

Gagna CE, Kuo HR, Florea E, Shami W, Taormin R, Vaswani N, Gupta M, Vijh R and Lambert WC (2001). Comparison of apoptosis and terminal differentiation: the mammalian aging process. *Journal of Histochemistry and Cytochemistry* 49 (7): 929–930.

Galmarini CM, Mackey JR and Dumontet C (2001) Nucleoside analogues: mechanisms of drug resistance and reversal strategies. *Leukemia* 15: 875-890.

Geske FJ, Nelson AC, Lieberman R, Strange R, Sun T and Gerschenson, LE (2000) DNA repair is activated in early stages of p53-induced apoptosis. *Cell Death and Differentiation* 7: 292-401.

Gottlieb S (1999) COX 2 inhibitors might be useful in cancer prevention. *British Medical Journal* 319 (7218): 1155.

Goyns MH and Hancock BW (1991) Importance of oncogenes research to the cancer clinician. *Clinical oncology* 3: 168-176.

Graz CJ and Cowley HM (1997) Energy state in HT-29 cells is linked to differentiation. *In Vitro Cellular and Developmental Biology - Animal* 33: 277–281.

Griffith TS, Brunner T, Fletcher SM, Green DR and Ferguson TA (1995) Fas ligand induced apoptosis as a mechanism of immune privilege. *Science* 270: 1189–1192.

Gudmundsson KS and Johns BA (2003) Synthesis of Novel Imidazo[1,2-a]pyridines with Potent Activity Against Herpesviruses. *Organic Letters* 5: 1369–1372.

Guillen-Ahlers H, Suckow MA, Castellino FJ and Ploplis VA (2010) *Fas/CD95* Deficiency in *Apc*$^{Min/+}$ Mice Increases Intestinal Tumor Burden. *PLoS ONE* 5 (2): e9070. http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0009070 [Accessed on 21 January 2012].

Guo L, Pietkiewicz D, Pavlov EV, Grigoriev SM, Kasianowicz JJ, Dejean LM, Korsmeyer SJ, Antonsson B, and Kinnally KW (2004) Effects of cytochrome c on the mitochondrial apoptosis-induced channel MAC. *American Journal of Physiology* 286: 1109-1117.

Gupta RA, Tan J, Krause WF, Geraci MW, Wilson TM, Dey SK and Dubois RN (2000) Prostacyclin-mediated activation of peroxisome proliferator-activated receptor δ in colorectal cancer. *Proceedings of the National Academy of Sciences* 97: 13275-13280.

Hamilton W and Sharp D (2004) Diagnosis of colorectal cancer in primary care: the evidence base for guidelines. *Family Practice* 21**:** 99–106.

Hanahan D and Weinberg RA (2000) The hallmarks of cancer. *Cell*. 100: 57-70.

He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B and Kinzler KW (1998) Identification of c-myc as a target of the APC pathway. *Science* 281: 1509-1512.

Hengartner MO (2000) The biochemistry of apoptosis. *Nature* 407: 770-776.

Heidelberger C, Danenberg PV and Moran RG (1983) Fluorinated pyrimidines and their nucleosides. *Advances in Enzymology and Related Areas of Molecular Biology* 54: 58–119.

Hidalgo IJ, Raub TJ and Borchardt RT (1989) Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability *Gastroenterology* 96: 3 736-749.

Hoffman WH, Biade S, Zilfo JT, Chen J and Murphy M (2002) Transcriptional repression of the anti-apoptotic surviving gene by wild type p53. *Journal of Biological Chemistry* 277: 3247-3257.

Hu and Kavanagh (2003) Anticancer therapy targeting the apoptotic pathway. *The Lancet Oncology* 4 (12): 721-729.

Huang ME, Ye YC, Chen SR, Chai JR, Lu JX, Zhoa L, Gu LJ and Wang ZY (1988) Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia. *Blood* 72: 567–572.

Iwamoto M, Ahnen DJ, Franklin WA and Waltzman TH (2000) Expression of β-catenin and full-length APC protein in normal and neoplastic colonic tissues. *Carcinogenesis* 21: 1935-1940.

Jamie H, Dyason K, Milne PJ, Grant G and Graz CJ (2001) The influence of acetoacetate and butyrate on calcium influx and ATP concentrations in HT-29 cells. *Pharmazie* 56 (4): 332-336.

Jaramillo C, de Diego JE, Hamdouchi C, Collins E, Keyser H, Sánchez-Martínez C, del Prado M, Norman B, Brooks HB, Watkins SA, Spencer CD, Dempsey JA, Anderson BD, Campbell RM, Leggett T, Patel B, Schultz RM, Espinosa J, Vieth M, Zhang F and Timm DE (2004) Aminoimidazo[1,2-a]pyridines as a new structural class of cyclin-dependent kinase inhibitors. Part 1: design, synthesis, and biological evaluation. *Bioorganic and Medicinal Chemistry Letters* 14: 6095–6099.

Jeffrey AM and Mayer RJ (2005) Systemic Therapy for Colorectal Cancer. *The New England Journal of Medicine* 352: 476-487.

Jemal A, Bray F, Center MM, Ferlay J, Ward E. and Forman D (2011) Global cancer statistics. *CA: A Cancer Journal for Clinicians* 61: 69-90.

Jiang X and Wang X (2004) Cytochrome C-mediated apoptosis. *Annual Review of Biochemistry* 73: 87–106.

Jin Z and El-Deiry WS (2005) Overview of cell death signaling pathways. *Cancer Biology and Therapy* 4:139-163.

Johnstone RW, Ruefli AA and Lowe SW (2002) Apoptosis. *Cell* 108 (2): 153 – 164.

Katoaka T, Budd RC, Holler N Thome M, Martinon F, Irmler M, Burns K, Hahne M, Kennedy N, Kovacsovics M and Tschopp J (2000) The caspase-8 inhibitor FLIP promotes activation of NF-kappaB and Erk signalling pathways. *Current Biology* 10: 640-648.

Koornstra JJ, Kleibeuker JH, Van Geelen CM, Rijcken FE, Hollema H, de Vries EG and de Jong S (2003) Expression of TRAIL (TNF-related apoptosis-inducing ligand) and its receptors in normal colonic mucosa, adenomas and carcinomas. *Journal of Pathology* 200: 327-335.

Kerr JF, Wyllie AH and Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer* 26: 239-257.

Kim PK, Zamora R, Petrosko P and Billiar TR (2001) The regulatory role of nitric oxide in apoptosis. *International Immunopharmacology* 1: 1421-1441.

Kim HS, Lee JW, Soung YH, Park WS, Kim SY, Lee JH, Park JY, Cho YG, Kim CJ, Jeong SW, Nam SW, Kim SH, Lee JY, Yoo NJ and Lee SH (2003) Inactivating mutations of caspase-8 gene in colorectal carcinomas. *Gastroenterology* 125 (3): 708-715.

Kim YH, Park JW, Lee JY and Kwon TK (2004) Sodium butyrate sensitizes TRAIL-mediated apoptosis by induction of transcription from the DR5 gene promoter through Sp1 sites in colon cancer cells. *Carcinogenesis* 25: 1813-1820.

Kovvali G, Shiff S, Telang N, Das K, Kohgo Y, Narayan S and Li H (2003) Carcinogenesis: the more we seek to know the more we need to know-challenges in the post Genomic Era. *Journal of Carcinogenesis* 2 (1): 1-5.

Kroemer G and Reed JC (2000) Mitochondrial control of cell death. *Natural Medicine*. 5: 513-519.

Kumar S (2007) Caspase function in programmed cell death. *Cell Death and Differentiation* 14: 32– 43.

Lacerda RB, de Lima CKF, da Silva LL, Romeiro NC, Miranda ALP, Barreiro EJ and Fraga CAM (2009) Discovery of novel analgesic and anti-inflammatory 3-arylamine-imidazo[1, 2-a]pyridine symbiotic prototypes. *Bioorganic and Medicinal Chemistry* 17: 74-84.

Lamkanfi M, Festjens N, Declercq T, Van den Berghe T, and Van den abeele P (2007) Caspases in cell survival, proliferation and differentiation. *Cell Death and Differentiation*. 14: 44-55.

Lavie Y, Cao H, Bursten SL, Giuliano AE and Cabot MC (1996) Accumulation of glucosylceramides in multidrug-resistant cancer cells. *Journal of Biological Chemistry* 271: 1930-1936.

Leslie A and Steele RJC (2002) Management of colorectal cancer. *Postgraduate Medical Journal* 78: 473-478.

Levine AJ and Oren M (2009) The first 30 years of p53: growing ever more complex. *Nature Reviews Cancer* 9: 749-758.

Li M, Wu X and Xu XC (2001) Induction of Apoptosis in Colon Cancer Cells by Cyclooxygenase-2 Inhibitor NS398 through a Cytochrome *c*-dependent Pathway. *Clinical Cancer Research* 7: 1010-1016.

Li Q and Verma IM (2002) NF-kB regulation in the immune system. *Nature Reviews Immunology* 2: 725-734.

Liu X, Kim CN, Yang J, Jemmerson R and Wang X (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 86: 147–57.

Liu LF, Desai SD, Li TK, Mao Y, Sun M and Sim SP (2000) Mechanism of Action of Camptothecin. *Annals of the New York Academy of Sciences* 922: 1–10.

Liu Q, Chan STF and Mahendran R (2003) Nitric oxide induces cyclooxygenase expression and inhibits cell growth in colon cancer cell lines. *Carcinogenesis* 24 (4): 637-642.

Longley DB, Wilson TR, McEwan M, Allen WL, McDermott U, Galligan L and Johnston PG (2006) c-FLIP inhibits chemotherapy-induced colorectal cancer cell death. *Oncogene* 25 (6): 838-848.

Lopaczynski W and Zeisel SH (2001) Antioxidants, programmed cell death, and cancer. *Nutrition Research* 21: 295-307.

Luzzio MJ, Besterman JM, Emerson DL, Evans MG, Lackey K, Leitner PL, McIntyre G, Morton B, Myers PL, Peel M, Sisco JM, Sternbach DD, Tong WQ, Truesdale A, Uehling DE, Vuong A and Yates J (1995) Synthesis and antitumor activity of novel water solublederivatives of camptothecin as specific inhibitors of topoisomerase I. *Journal of Medicinal Chemistry* 38: 395-401.

Ly JD, Grubb DR and Lawen A (2003) The mitochondrial membrane potential (Δψm) in apoptosis; an update. *Apoptosis* 8 (2): 115-128.

Majno G and Joris I (1995) Apoptosis, oncosis and necrosis. An overview of cell death. *The American Journal of Pathology* 146: 3-15.

Maldonado O, Demasi R, Maldonado Y, Taylor M, Troncale F and Vender R (1998) Extremely high levels of alkaline phosphatase in hospitalized patients. *Journal of Clinical Gastroenterology* 27 (4): 342-345.

Martinez ME, O'Brien TG, Fultz KE, Babbar NE, Yerushalmi H, Qu N, Guo Y, Boorman D, Einspahr J, Alberts DS and Gerner EW (2003) Pronounced reduction in adenoma recurrence associated with aspirin use and a polymorphism in the ornithine decarboxylase gene. *The Proceedings of the National Academy of Sciences of the United States of America* 100: 7859-7864.

Meyerhardt JA and Mayer RJ (2005) Systemic Therapy for Colorectal Cancer. *New England Journal Medicine* 352 (5): 476-487.

Mikhailov V, Mikhailova M, Degenhardt K, Venkatachalam MA, White E, and Saikumar P (2003) Association of Bax and Bak homo-oligomers in mitochondria. Bax requirement for Bak reorganization and cytochrome c release. *Journal of Biological Chemistry* 278: 5367-5376.

Ming L, Wang P, Bank A, Yu J and Zhang L (2006) PUMA dissociates Bax and Bcl-XL to induce apoptosis in colon cancer cells. *Journal of Biological Chemistry* 281: 16034–16042.

Monks A, Scudiero D, Skehan P, Shoemaker R, Paull K, Vistica D, Hose C, Langley J, Cronise P, Vaigro-Wolff A, Gray-Goodrich M, Campbell H, Mayo J, and Boyd M (1991) Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines in culture. *Journal of the National Cancer Institute* 83**:** 757-766.

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of immunological methods* 65(1-2): 55-63.

Musch T, Öz Y, Lyko F and Breiling A (2010) Nucleoside Drugs Induce Cellular Differentiation by Caspase-Dependent Degradation of Stem Cell Factors. *PLoS ONE* 5 (5): e10726. http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0010726. [Accessed on 13 February 2012]

Nadege B, Patrick L and Rodrigue R (2009) Mitochondria: from bioenergetics to the metabolic regulation of carcinogenesis. *Frontiers in Bioscience* 14: 4015-4034.

Norat T and Riboli E (2003) Dairy products and colorectal cancer. A review of possible mechanisms and epidemiological evidence. *European Journal of Clinical Nutrition* 57: 1-17.

O'Connell J, Bennett MW, O'Sullivan GC, Collins JK and Shanahan F (1997) The Fas counterattack: a molecular mechanism of tumor immune privilege. *Molecular Medicine* 3 (5): 294-300.

O'Neil BH and Goldberg RM (2008) Innovations in Chemotherapy for Metastatic Colorectal Cancer: An Update of Recent Clinical Trials. *The Oncologist* 13 (10): 1074-1083.

Pan G, O'Rourke K, and Dixit VM (1998) Caspase-9, Bcl-XL, and Apaf-1 Form a Ternary Complex. *Journal of Biological Chemistry* 273: 5841-5845.

Park BH and Vogelstein B (2003) Tumor-suppressor genes in Holland-Frei Cancer Medicine. 6th edition. Hamilton, Ontario: BC Decker : 87–106.

Parkin DM, Bray F, Ferlay J and Pisani P (2005) Global Cancer Statistics, 2002. *Cancer Journal for clinicians* 55: 74-108.

Peters U, Chatterjee N, McGlynn KA, Schoen RE, Church TR, Bresalier RS, Gaudet MM, Flood A, Schatzkin A and Hayes RB (2004) Calcium intake and colorectal adenoma in a US colorectal cancer early detection program. *The American Journal of Clinical Nutrition* 80: 1358-1365.

Petit PX, Zamzami N, Marchetti P, Castedo M, Decaudin D, Macho A, Hirsch T, Susin SA, Mignotte B, and Kroemer G (1995) Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. *The Journal of Experimental Medicine* 182: 367–377.

Pettus BJ, Chalfant CE and Hannun YA (2002) Ceramide in apoptosis: an overview and current perspectives. *Biochimica et Biophysica Acta* 1585: 114– 125.

Pietinen P, Rohan TE, Sellers TA, Speizer FE, Willett WC, Wolk A and Hunter DJ (2004) Alcohol Intake and Colorectal Cancer: A Pooled Analysis of 8 Cohort Studies. *Annals of Internal Medicine* 140: 1-55.

Pinto M, Robine-Leon S, Appay M, Kedinger M, Triadou N, Dussaulx E, Lacroix B, Simon-Assman P, Haffen K, Fogh J and Zweibaum A (1983) Enterocyte-like Differentation and Polarisation of the Human Colon Carcinoma Cell Line Caco-2 in Culture, *Biology of the Cell* 47: 323-330.

Pitari GM, Zingman LV, Hodgson DM, Alekseev AE, Kazerounian S, Bienengraber M, Hajnoczky G, Terzic A and Waldman SA (2003) Bacterial enterotoxins are associated with resistance to colon cancer. *Proceedings of the National Academy of Sciences* 100: 2695-2699.

Poincloux L, Durando X, Seitz JF, Thivat E, Bardou VJ, Giovannini MH, Parriaux D, Barriere N, Giovannini M, Delpero JR and Monges G (2009) Loss of Bcl-2 expression in colon cancer: a prognostic factor for recurrence in stage II colon cancer 18 (4): 357-365.

Porter AG and Janicke RU (1999) Emerging roles of caspase 3 in apoptosis. *Cell Death and Differentiation* 6: 99-104.

Ravi R and Bedi A (2002) Requirement of BAX for TRAIL/Apo2L induced apoptosis of colorectal cancer: synergism with sulindac-mediated inhibition of Bcl-x(L). *Cancer Research* 62: 1583-1587.

Reers M, Smiley ST, Mottola-Hartshorn C, Chen A, Lin M and Chen LB (1995) Mitochondrial membrane potential monitored by JC-1 dye. *Methods in Enzymology*. 260: 406-417.

Rogers AW (1983) Cells and Tissues. Academic Press, London, England. Pg 190.

Ruemmelle FM, Schwartz S, Seidman EG, Dionne S, Levy E and Lentze MJ (2003) Butyrate induced Caco-2 cell apoptosis is mediated via the mitochondrial pathway. *Gut* 52: 94-100.

Saelens X, Festjens N, Vande Walle L, van Gurp M, van Loo G and Vandenabeele P (2004) Toxic proteins released from mitochondria in cell death. *Oncogene* 23: 2861–74.

Saif MW, Alexander D and Wicox CM (2005) Serum Alkaline Phosphatase Level as a Prognostic Tool in Colorectal Cancer: A Study of 105 patients. *The Journal of Applied Research* 5 (1): 88-95.

Sakamaki K and Satou Y (2009) Caspases: evolutionary aspects of their functions in vertebrates. *Journal of Fish Biology* 74: 727-753.

Saleh HA, Jackson H and Banerjee M (2000) Immunohistochemical expression of Bcl-2 and p53 oncoproteins: correlation with Ki67 proliferation index and prognostic histopathologic parameters in colorectal neoplasia. *Applied Immunohistochemistry and Molecular Morphology* 8: 175-182.

Salvioli S, Ardizzoni A, Franceschi C and Cossarizza A (1997) JC-1, but not $DiOC_6(3)$ or rhodamine 123, is a reliable fluorescent probe to assess $\Delta\Psi$ changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. *Federation of European Biochemical Societies* 411 (1): 77-82.

Sen R and Baltimore D (1986) Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism. *Cell* 47: 921-928.

Sethi G, Sung B and Aggarwal BB (2008) Nuclear factor-kappaB activation: from bench to bedside. *Experimental Biology and Medicine* 233: 21–31.

Shacter E, Williams J, Hinson R, Sentürker S and Lee Y (2000) Oxidative stress interferes with cancer chemotherapy: inhibition of lymphoma cell apoptosis and phagocytosis. *Blood* 96 (1): 307-313.

Shaw P, Bovey R, Tardy S, Sahli R, Sordat B and Costa J (1992) Induction of apoptosis by wildtype p53 in a human colon tumor-derived cell line. *Proceedings of the National Academy of Sciences of the United States of America* 89: 4495-4499.

Sherr CJ (2004) Principles of tumor suppression. *Cell* 116: 235–246.

Schroeder CP and Maurer HR (2002) Tributyrin-induced differentiation promotes apoptosis of LS 174T colon cancer cells in vitro. *International Journal of Oncology* 20:195-200.

Shimada T, Kojima K, Yoshiura K, Hirashi H and Terano A (2002) Characteristics of the peroxisome proliferators activated receptor γ (PPARγ) ligand induced apoptosis in colon cancer cells. *Gut* 5: 658-664.

Shimizu S, Ide T, Yanagida T and Tsujimoto Y (2000) Electrophysiological study of a novel large pore formed by bax and the voltage dependent anion channel that is permeable to cytochrome c. *Journal of Biological Chemistry* 275: 12321–12325.

Shureiqi I, Jiang W, Zuo X, Wu Y, Stimmel JB, Leesnitzer LM, Morris JS, Fan HZ, Fischer SM and Lippman SM (2003) The 15-lipoxygenase-1 product 13-S-hydroxyoctadecadienoic acid down-regulates PPAR- delta to induce apoptosis in colorectal cancer cells. *Proceedings of the National Academy of Sciences* 100: 9968-9973.

Soung YH, Lee JW, Kim SY, Park WS, Nam SW, Lee JY, Yoo NJ and Lee SH (2004) Somatic mutations of CASP3 gene in human cancers. *Human Genetics* 115: 112–115.

Strater J, Walczak H, Pukrop T, Von Muller L, Hasel C, Kornmann M, Mertens T and Moller P (2002) TRAIL and its receptors in the colonic epithelium: a putative role in the defense of viral infections. *Gastroenterology* 122: 659-666.

Sykorova E and Fajkus J (2009) Structure-function relationships in telomerase genes. *Biology of the cell* 101: 375-392.

Talanian RV, Quinlan C, Trautz S, Hackett MC, Mankovich JA, Banach D, Ghayur T, Brady KD and Wong WW (1997) Substrate specificities of caspase family proteases. *Journal of Biological Chemistry* 272: 9677-9682.

Tanizawa A, Fujimori A, Fujimori Y and Pommier Y (1994) Comparison of topoisomerase I inhibition, DNA damage, and cytotoxicity of camptothecin derivatives presently in clinical trials. *Journal of the National Cancer Institute* 86: 836–842.

Tapon N, Harvey KF, Bell DW, Wahrer DCR, Schiripo TA, Haber DA and Hariharan IK (2002) Salvador promotes both cell cycle exit and apoptosis in *Drosophila* and is mutated in human cancer cell lines. *Cell* 110: 403-406.

Urbano A, Lakshmanan U, Choo PH, Kwan JC, Ng PY, Guo K, Dhakshinamoorthy S and Porter A (2005) AIF suppresses chemical stress-induced apoptosis and maintains the transformed state of tumor cells. *The EMBO Journal* 24: 2815-2826.

Van Cruchten S and Van Den Broeck W (2002) Morphological and biochemical aspects of apoptosis, oncosis and necrosis. *Anatomy, Histology and Embryology* 31: 214-223.

Van Loo G, van Gurp M, Depuydt B, Srinivasula SM, Rodriguez I, Alnemri ES, Gevaert K, Vandekerckhove J, Declercq W and Vandenabeele P (2002) The serine protease Omi/HtrA2 is released from mitochondria during apoptosis. Omi interacts with caspase-inhibitor XIAP and induces enhanced caspase activity. *Cell Death and Differentiation* 9: 20 – 26.

Vermes I, Haanen C and Reutelingsperger CPM (1995) A novel assay for apoptosis: flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescence labelled annexin V. *Journal of Immunological Methods* 180: 39–52.

Vousden KH (2002) Activation of the p53 tumor suppressor protein. *Biochemica et Biophysica Acta* 1602: 47-59.

Voux DL (2011) Apoptogenic factors released from mitochondria. *Biochimica et Biophysica Acta* 1813: 546-550.

Wang LM, Li QY, Zu YG, Fu YJ, Chen LY, Lv HY, Yao LP and Jiang SG (2008) Anti-proliferative and pro-apoptotic effect of CPT13, a novel camptothecin analog, on human colon cancer HCT8 cell line. *Chemico-biological interactions* 176(2-3): 165-72.

Wilkinson DG, Peters G, Dickson C, and McMahon AP (1988) Expression of the FGF-related proto-oncogene int-2 during gastrulation and neurulation in the mouse. *The European Molecular Biology Organization Journal* 7 (3): 691–695.

Willett WC (2001) Diet and cancer: one view at the start of the millennium. *Cancer Epidimiology, Biomarkers and Prevention* 10 (1): 3-8.

Wils J (2007) Adjuvant Treatment of Colon Cancer: Past, Present and Future *Journal of Chemotherapy* 19: 115 – 122.

Winslow T (2005) Stages of colon cancer. http://www.collaborativecurriculum.ca/en/modules/oncologyintro/oncology-fundamentals-cancertreatment-02.jsp [Accessed 6 November 2011]

Wolpin BM and Mayer RJ (2008) Systemic Treatment of Colorectal Cancer. *Gastroenterology* 134 (5)**:** 1296-1310.

Wyllie AH (1997) Apoptosis: an overview. *British Medical Bulletin* 53: 451-465.

Yu J, Wang Z, Kinzler KW, Vogelstein B and Zhang L (2003). *PUMA* mediates the apoptotic response to p53 in colorectal cancer cells. *Proceedings of the National Academy of Sciences* 100: 1931-1936.

Zangemeister-Wittke U and Simon HU (2001) Apoptosis - Regulation and clinical implications. *Cell Death and Differentiation* 8: 537-544.

Zhang L, Yu J, Park BH, Kinzler KW and Vogelstein B (2000a) Role of BAX in the apoptotic response to anticancer agents. *Science* 290: 982-983.

Zhang ZW, Patchett SE and Farthing M (2000b) Topoisomerase I inhibitor (camptothecin)-induced apoptosis in human gastric cancer cells and the role of wild-type p53 in the enhancement of its cytotoxicity. *Anti-Cancer Drugs* 11 (9): 757-764.

Zhivotovsky B and Orenius S (2003) Defects in the apoptotic machinery of cancer cells: role in drug resistance. *Seminars in Cancer Biology* 13: 125-134.

Zhu C, Wang X, Deinum J, Huang Z, Gao J, Modjtahedi N, Neagu MR, Nilsson M, Eriksson PS, Hagberg H, Luban J, Kroemwe G and Blomgren K (2007) Cyclophilin A participates in the nuclear translocation of apoptosis-inducing factor in neurons after cerebral hypoxia-ischemia. *Journal of Experimental Medicine* 204 (8): 1741-1748.

Zumkeller N, Brenner H, Chang-Claude J, Hoffmeister M, Nieters A and Rothenbacher D (2007) *Helicobacter pylori* infection, interleukin-1 gene polymorphisms and the risk of colorectal cancer: evidence from a case-control study in Germany. *European Journal of Cancer* 43: 1283–1289.

http://www.abcam.com. 2011. Microplate Elisa Format. Available at http://www.abcam.com/index.html?pageconfig=resource&rid=13855. [Accessed 1 June 2011].

VII. Appendices

Appendix A: MTT results from "non-active" compounds

Table A1 The effect of the "non active"CDK group of imidazo[1,2-a]pyridines on the survival of the HT-29 and Caco-2 cells.

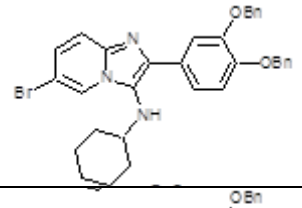
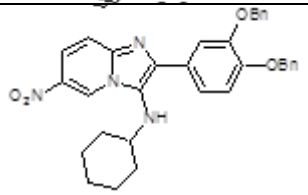
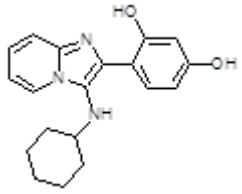
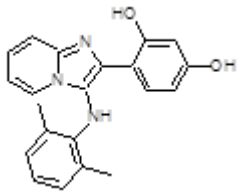
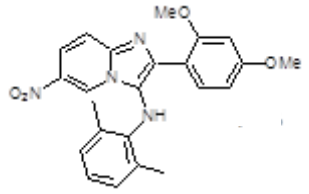
		Cell Viability %	
		Cell Lines	
Name of compound	**Structure of compound**	**Caco2**	**HT29**
CDK 1		86.46 ± 1.09	90.32 ± 0.74
CDK 2		93.71 ± 1.12	89.27 ± 0.45
CDK 4		86.92 ± 1.33	82.95 ± 0.84
CDK 5		79.48 ± 0.93	82.53 ± 2.16
CDK 7		82.46 □ 0.74	88.62 □ 1.03

Table A2 The effect of the "non active"CL group of imidazo[1,2-a]pyridines on the survival of the HT-29 and Caco-2 cells.

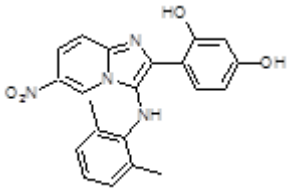
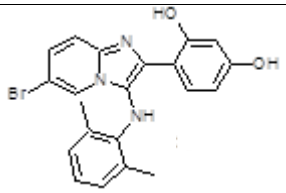
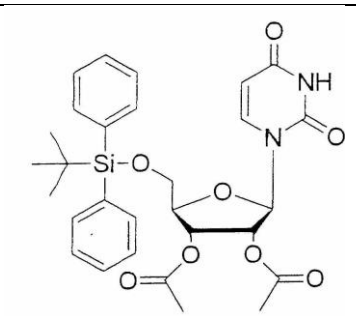
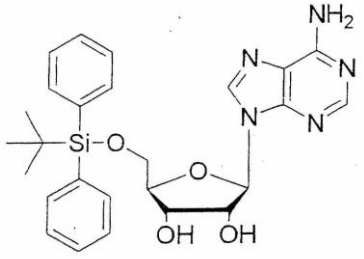
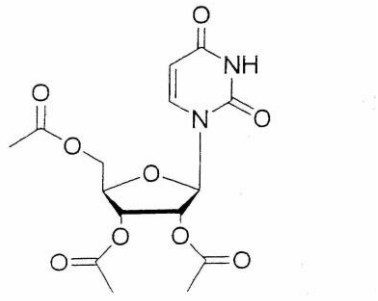
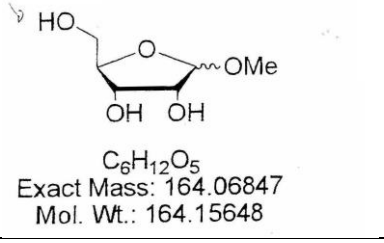
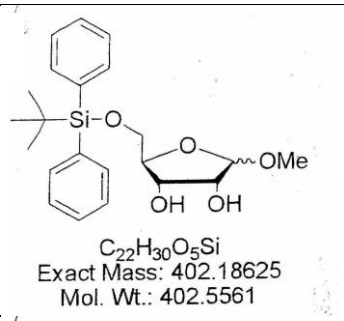
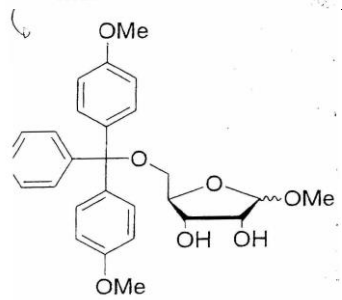
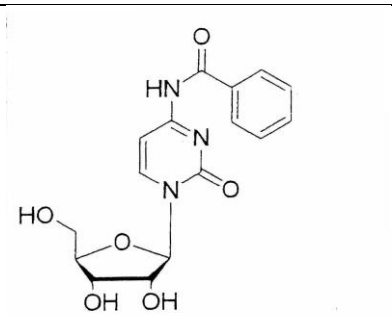
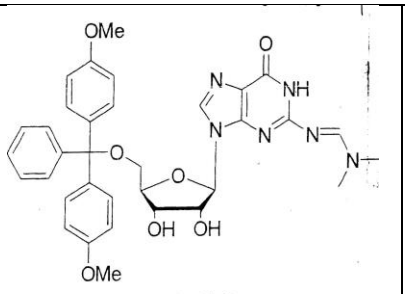
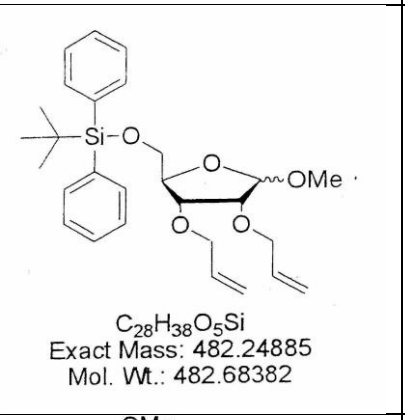
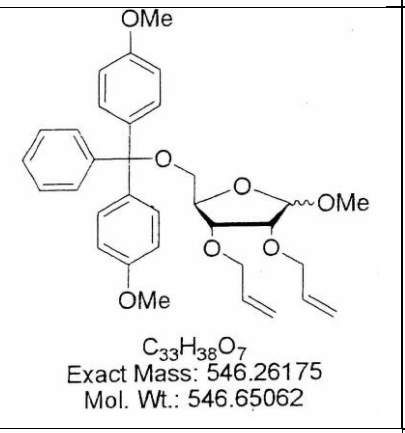
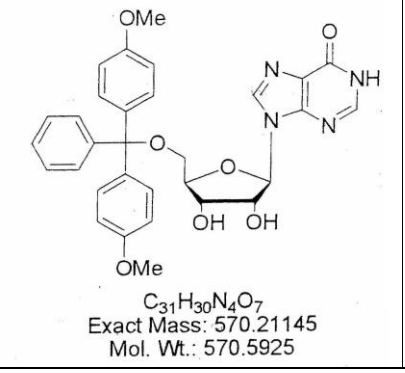
		Cell Viability %	
		Cell Lines	
Name of compound	**Structure of compound**	**Caco2**	**HT29**
CL 1		118.79 ± 1.13	113.43 ± 1.01
CL 2		108.30 ± 0.69	109.77 ± 1.42

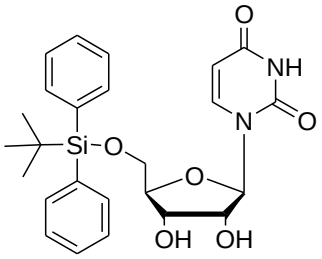
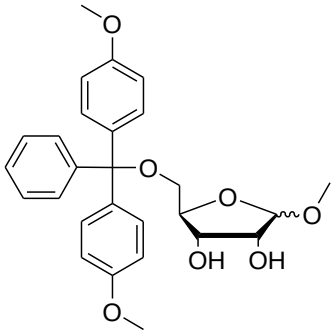
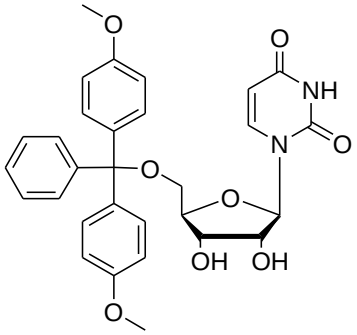
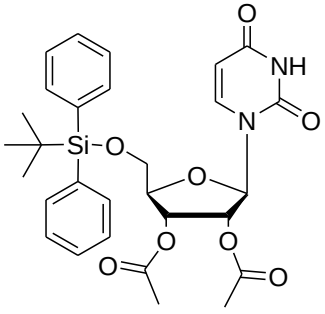
Table A3 The effect of the "non active" nucleoside analogues on the survival of the HT-29 and Caco-2 cells.

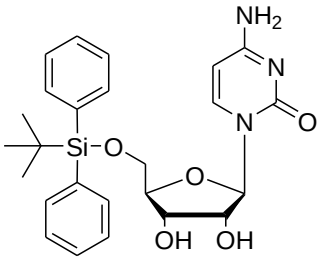
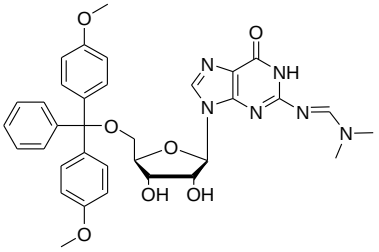
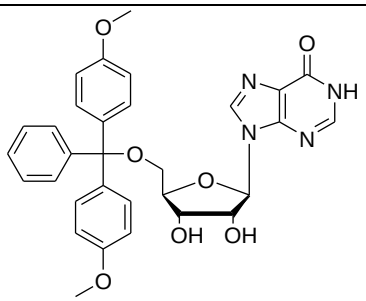
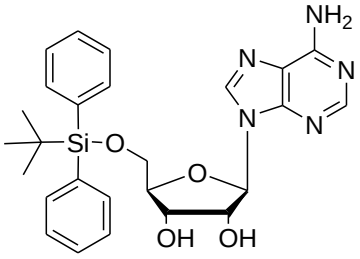
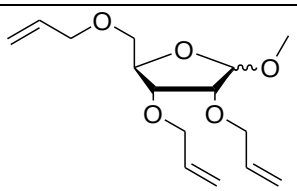
CYTOTOXICITY

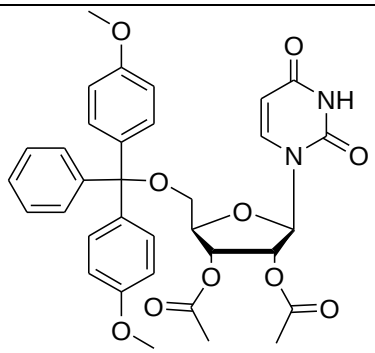
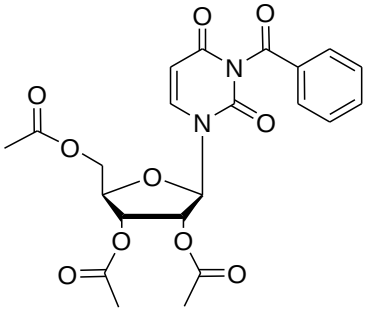
		Cell Viability %	
		Cell Lines	
Name of compound	**Structure of compound**	**Caco2**	**HT29**
JLP 029	 $C_{29}H_{34}N_2O_8Si$ Exact Mass: 566.20844 Mol. Wt.: 566.67436	78.615 + 1.702	76.448 + 0.892
JLP 035	 $C_{26}H_{31}N_5O_4Si$ Exact Mass: 505.21453 Mol. Wt.: 505.64094	96.978 + 1.665	96.869 + 0.998
JLP 045.2	 $C_{15}H_{18}N_2O_9$ Exact Mass: 370.10123 Mol. Wt.: 370.31142	87.972 + 1.309	87.751 + 2.001
JLP 046.2	 $C_6H_{12}O_5$ Exact Mass: 164.06847 Mol. Wt.: 164.15648	73.447 + 1.401	72.757+ 0.351

JLP 052	 $C_{22}H_{30}O_5Si$ Exact Mass: 402.18625 Mol. Wt.: 402.5561	90.554 + 0.784	89.720 + 0.396
JLP 053	 $C_{27}H_{30}O_7$ Exact Mass: 466.19915 Mol. Wt.: 466.5229	92.776 + 1.114	93.320 + 0.115
JLP 054	 $C_{16}H_{17}N_3O_6$ Exact Mass: 347.11174 Mol. Wt.: 347.32268	94.663 + 0.859	93.213 + 1.298
JLP 055	 $C_{13}H_{18}N_6O_5$ Exact Mass: 338.13387 Mol. Wt.: 338.31922	87.821 + 2.004	86.395 + 0.981

JLP 056	 $C_{34}H_{36}N_6O_7$ Exact Mass: 640.26455 Mol. Wt.: 640.68564	96.954 + 0.134	97.334 + 0.112
JLP 057	 $C_{28}H_{38}O_5Si$ Exact Mass: 482.24885 Mol. Wt.: 482.68382	86.056 + 1.239	84.565 + 1.762
JLP 058	 $C_{33}H_{38}O_7$ Exact Mass: 546.26175 Mol. Wt.: 546.65062	80.334 + 1.094	82.320 + 0.952
JLP 60.1	 $C_{31}H_{30}N_4O_7$ Exact Mass: 570.21145 Mol. Wt.: 570.5925	84.223 + 0.963	83.318 + 1.117

JLP 089	 $C_{25}H_{30}N_2O_6Si$ Exact Mass: 482.18731 Mol. Wt.: 482.601	86.544 ± 0.474 %	89.193 ± 0.837 %
JLP 099	 $C_{27}H_{30}O_7$ Exact Mass: 466.19915 Mol. Wt.: 466.5229	84.718 ± 0.052 %	74.824 ± 1.138 %
JLP 090.3	 $C_{30}H_{30}N_2O_8$ Exact Mass: 546.20022 Mol. Wt.: 546.5678	71.892 ± 0.452 %	67.609 ± 0.558 %
JLP 117.1	 $C_{29}H_{34}N_2O_8Si$ Exact Mass: 566.20844 Mol. Wt.: 566.67436	92.572 ± 1.096 %	87.113 ± 0.645 %

JLP 093	 $C_{25}H_{31}N_3O_5Si$ Exact Mass: 481.2033 Mol. Wt.: 481.61624	64.432 ± 3.818 %	69.982 ± 4.193 %
JLP 077.1	 $C_{34}H_{36}N_6O_7$ Exact Mass: 640.26455 Mol. Wt.: 640.68564	86.177 ± 4.411 %	80.298 ± 1.655 %
JLP 065	 $C_{31}H_{30}N_4O_7$ Exact Mass: 570.21145 Mol. Wt.: 570.5925	79.748 ± 2.245 %	73.629 ± 1.475 %
JLP 095.2	 $C_{26}H_{31}N_5O_4Si$ Exact Mass: 505.21453 Mol. Wt.: 505.64094	91.563 ± 2.411 %	89.208 ± 3.514 %
JLP 107.1	 $C_{15}H_{24}O_5$ Exact Mass: 284.16237 Mol. Wt.: 284.34806	79.728 ± 1.873 %	89.491 ± 3.224 %

JLP 118.1	 $C_{34}H_{34}N_2O_{10}$ Exact Mass: 630.22135 Mol. Wt.: 630.64116	86.053 ± 12.721 %	74.961 ± 6.230 %
JLP 087	 $C_{22}H_{22}N_2O_{10}$ Exact Mass: 474.12744 Mol. Wt.: 474.41748	81.847 ± 0.075 %	64.805 ± 2.448 %

Appendix B Calibration Curves

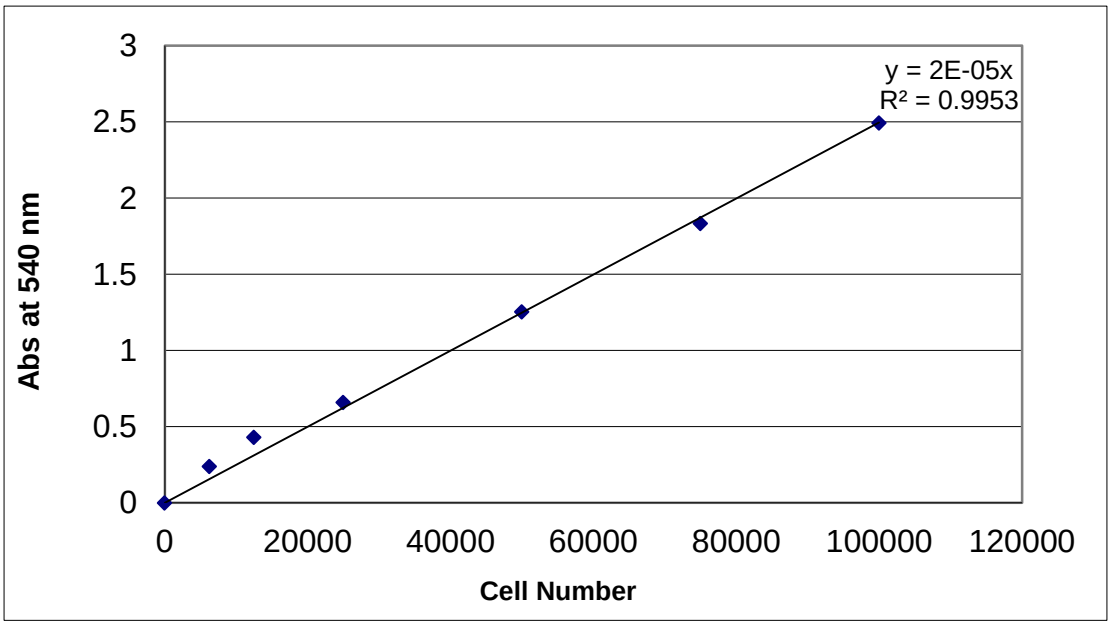


Figure B1 MTT HT-29 Calibration Curve

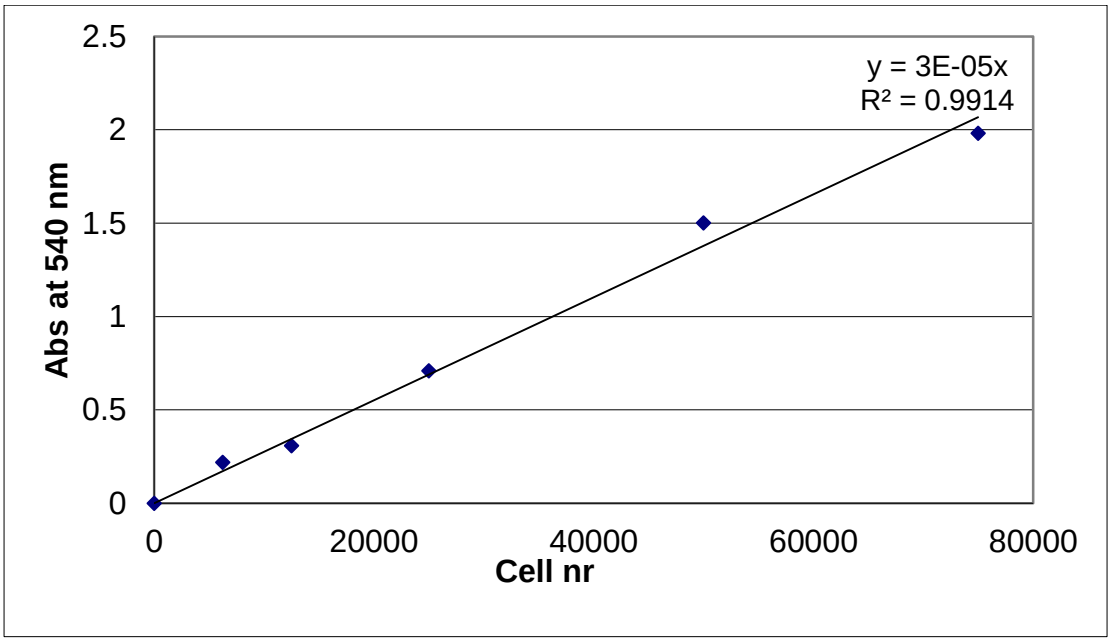


Figure B2 MTT Caco-2 Calibration Curve

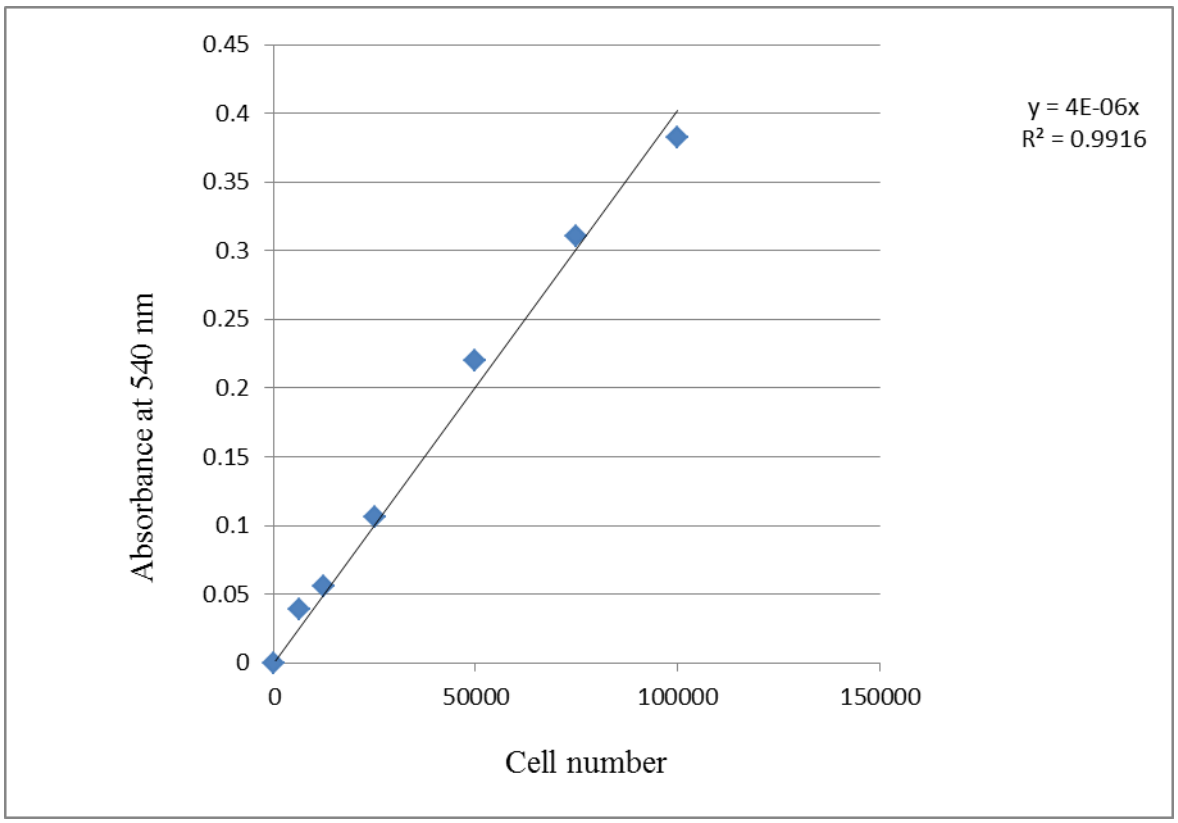


Figure B3 MTT Leucocyte Calibration Curve

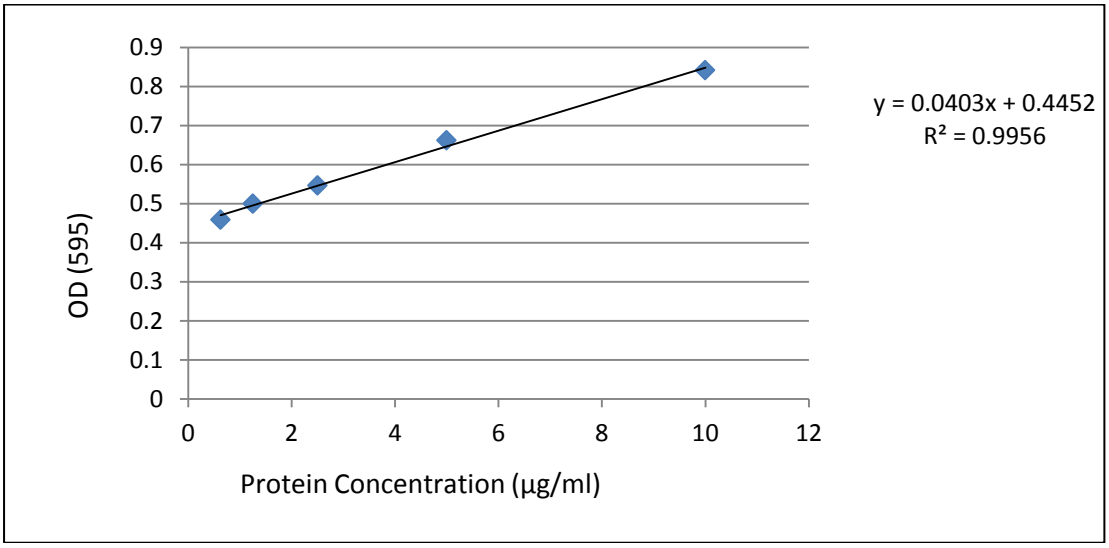


Figure B4 Bio-Rad Protein Assay Calibration Curve

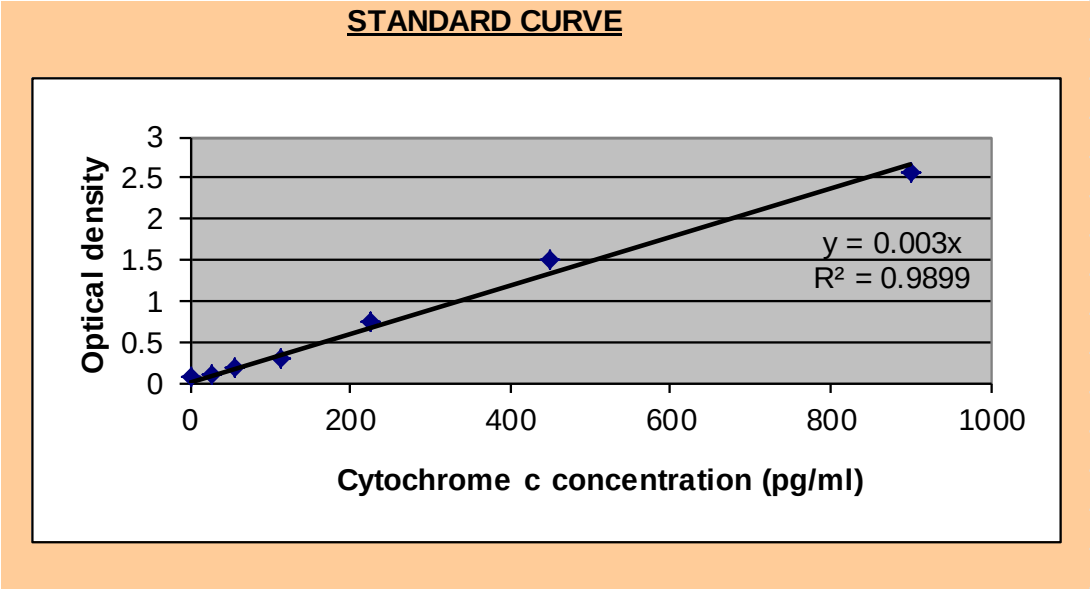


Figure B5 Cytochrome c Assay Calibration Curve

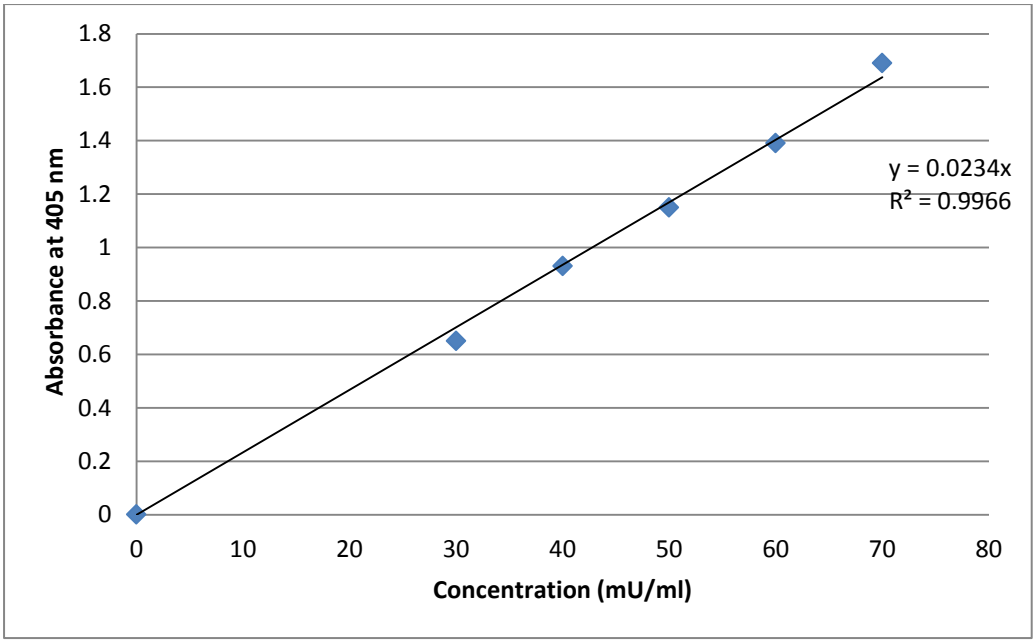
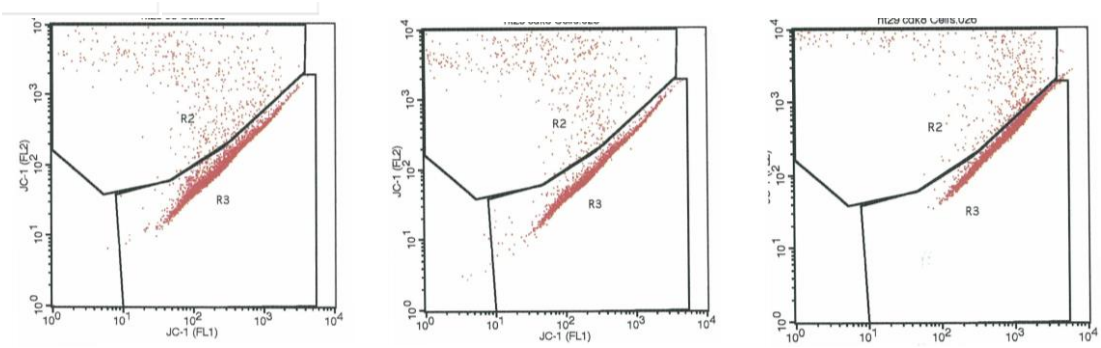


Figure B6 Alkaline Phosphatase Callibration Curve

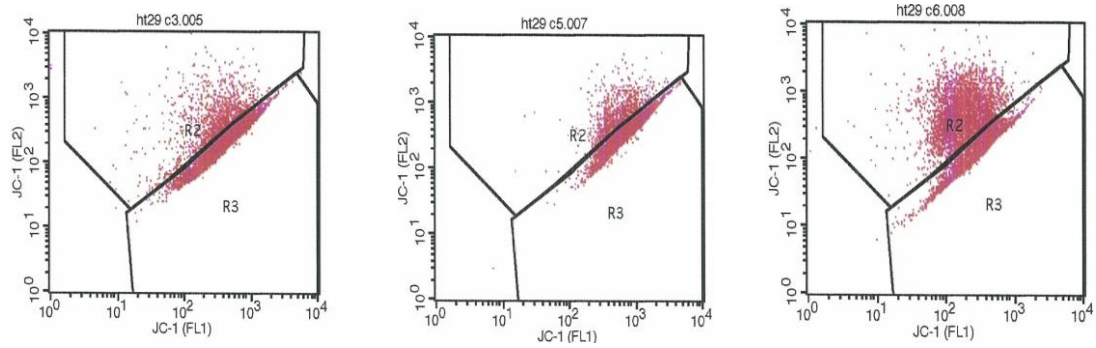
Appendix C JC-1 Flow Cytometry Scattergraphs

HT-29 cells

CDK 3 **CDK 6** **CDK 8**



CL 3 **CL 5** **CL 6**



JLP 26.5 **JLP 27.2**

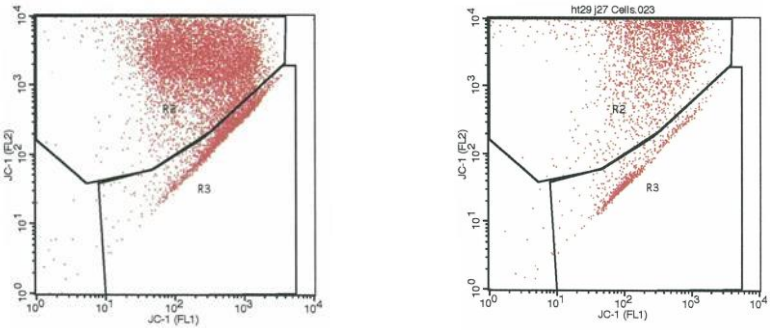
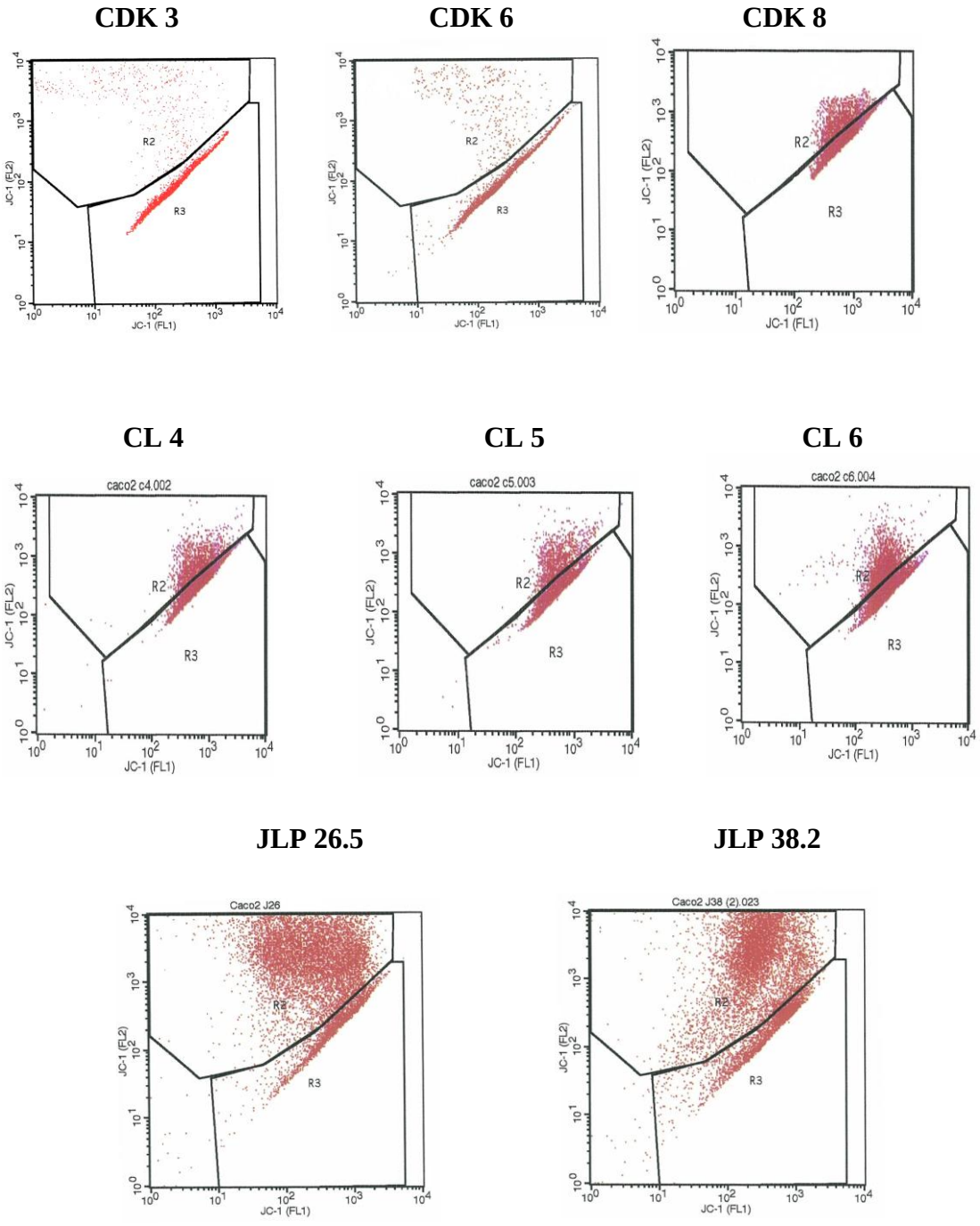


Figure C1 Flow cytometric analyses of JC-1 staining in HT-29 cells after a 24 hour treatment with the novel compounds.

Caco-2 cells



<u>Figure C2</u> Flow cytometric analyses of JC-1 staining in Caco-2 cells after a 24 hour treatment with the novel compounds.

Appendix D Cytochrome c concentrations (pg/ml)

Table D1 Cytochrome c concentrations (pg/ml) after treatment with the novel compounds in the HT-29 colon cancer cell line

Compound	Cytochrome c (pg/ml) Cytosolic fraction	Cytochrome c (pg/ml) Mitochondrial fraction	Total Cytochrome c (pg/ml)
Untreated HT-29 cells	26.98	264.04	290.15
JLP 26.5	274.48	26.24	301.63
JLP 27.2	242.29	68.34	310.63
JLP 38.2	272.55	51.92	324.47
CDK 3	296.49	40.43	336.92
CDK 6	298.44	17.14	317.49
CDK 8	264.44	32.39	297.12
CL 3	279.19	41.08	320.91
CL 4	198.22	93.28	291.50
CL 5	217.92	98.79	290.56
CL 6	206.96	85.25	293.97
Camptothecin	211.00	34.75	248.23

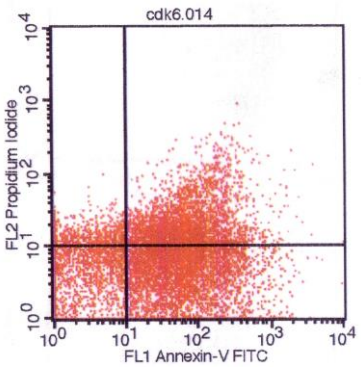
Table D2 Cytochrome c concentrations (pg/ml) after treatment with the novel compounds in the Caco-2 colon cancer cell line

Compound	Cytochrome c (pg/ml) Cytosolic fraction	Cytochrome c (pg/ml) Mitochondrial fraction	Total Cytochrome c (pg/ml)
Untreated Caco-2 cells	22.46	269.87	289.44
JLP 26.5	166.43	94.14	260.57
JLP 27.2	165.79	77.76	242.99
JLP 38.2	164.69	69.13	233.87
CDK 3	204.79	36.75	241.30
CDK 6	194.70	45.45	239.70
CDK 8	225.35	27.99	252.83
CL 3	200.45	30.23	229.53
CL 4	204.58	20.01	224.59
CL 5	177.91	34.52	212.43
CL 6	217.01	30.50	247.76
Camptothecin	22.46	269.87	289.44

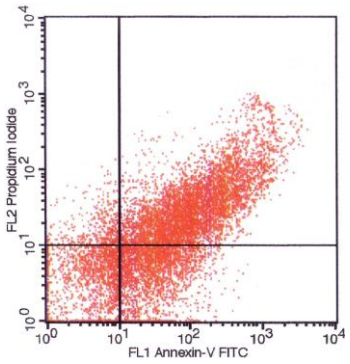
Appendix E Annexin V-FITC Flow Cytometry Scattergraphs

HT-29

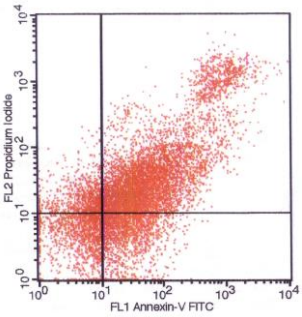
CDK 6



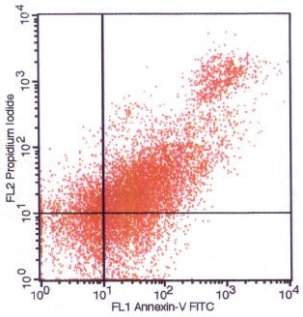
CDK 8



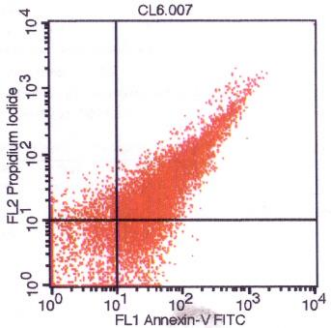
CL 4



CL 5

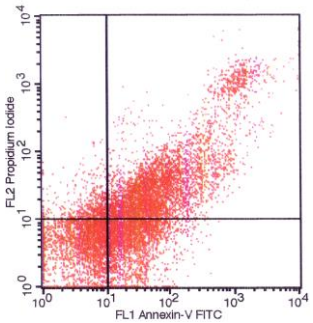


CL 6

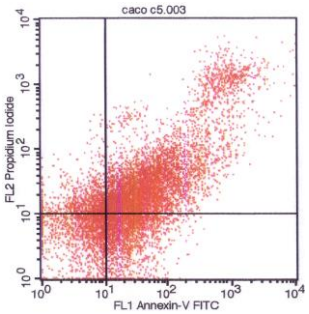


Caco-2

CL 4



CL 5



CL 6

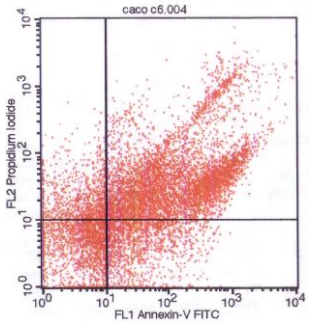


Figure E1 Flow cytometric analyses of Annexin V-FITC staining in HT-29 and Caco-2 cells after a 24 hour treatment with the novel compounds

Appendix F

Postgraduate Office, Faculty of Health Sciences
Wits Medical School, 7 York Road, PARKTOWN, 2193, Johannesburg • Tel: (011) 717 2000 • Fax: (011) 717 2119

APPLICATION FOR CHANGE OF TITLE OF APPROVED RESEARCH REPORT, DISSERTATION OR THESIS

Motivation / Reason for title change:
The old title was not a good representation of the work

Recommendation of Department / School:
Recommended.

Student Surname and initials: Dabaa-Farkas NE Student Number: 0303576H
Degree: MSc
Department: Pharmacy & Pharmacology Telephone: 082 902 3272 E-mail: nurit.dabaa@gmail.com

Previous Title: Novel Synthetic compounds in the induction of colonic cell differentiation and apoptosis.

New Title: The effect of Novel compounds on cell survival and apoptosis in colon cancer cell lines.

Supervisor/s: Dr Leonie Harmse
Departments: Pharmacy & Pharmacology
Supervisor's Telephone:
Supervisor's E-mail: leonie.harmse@wits.ac.za

Signatures of Student: N Farkas Supervisor 1: L Harmse Supervisor 2:

*HEAD OF DEPARTMENT / HEAD OF SCHOOL: *(Where the HOD is Supervisor, the HOS must sign)*

Van Zyl RL (Surname and Initials) RLvanZyl (Signature) 31 Oct 2011 (Date)

DECISION OF CHAIR OF THE PG COMMITTEE: Signature:

Date:

Appendix G

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Davids

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M070519**
PROJECT	Novel compounds in the induction of Apoptosis in various cancer cell lines
INVESTIGATORS	Dr H Davids
DEPARTMENT	Pharmacy & Pharmacology
DATE CONSIDERED	07.05.25
DECISION OF THE COMMITTEE*	APPROVED UNCONDITIONALLY

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 07.06.14 **CHAIRPERSON** .....
(Professors PE Cleaton-Jones, A Dhai, M Vorster, C Feldman, A Woodiwiss)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Dr H Davids

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10005, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES