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The effect of novel compounds, imidazo[1,2-a]pyridines and azaindoles on colon cancer cell lines.



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A dissertation submitted to the Faculty of Health Sciences, University
of the Witwatersrand, Johannesburg, in fulfilment of the
requirements for the degree of Masters of Science in Medicine
(Pharmacology)

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:.....*SRagie*.....

On this.....01.....day
of.....June.....2016

Dedication

I dedicate this thesis to the following people:

- ❖ My supervisor, Dr. Leonie Harmse, who has provided me with support and encouragement. I want to thank her for her patience, understanding, motivation and commitment. Thank you for encouraging me to take the leap into research.
- ❖ My parents who have given me the courage and the support to fulfill all my dreams and aspirations.
 - My mom, who has been a true inspiration, she has showered my life with undying love, support and encouragement. My mom has taught me so many lessons. I want to thank my mom for teaching me that through self-belief, dedication and hard-work you can reach your dreams.
 - My dad, who has made so many sacrifices in order to give his children the best education. I would like to thank him for emphasizing the importance of education, for inspiring me to set high goals and for giving me the confidence to achieve them.
- ❖ My siblings, for being there throughout this entire experience. Thank you for your support and patience.
- ❖ My fellow students, we shared this experience together. Each and every one of you became a lifelong friend.

Abstract

Colorectal cancer (CRC) is the third most common cancer worldwide, and annually accounts for more than 600 000 deaths. In South Africa colorectal cancer is the fifth most common cancer and each year one in 99 people are diagnosed with the disease. Despite the advances in early detection, screening and treatment, the mortality figures due to colorectal cancer in South Africa remains high. More than 40% of patients with colorectal cancer develop metastatic disease which has a poor prognosis. Chemotherapy is used alone or in combination with radiotherapy for the management of metastatic colorectal cancer and palliation therapy. Despite an increase in the understanding of the molecular basis of colorectal cancer and the introduction of targeted drug therapy, the metastatic phase of the disease remains resistant to treatment. Current chemotherapeutic management of metastatic disease is plagued by serious adverse drug reactions, drug resistance and therapeutic failure. Therefore, there is a need to discover new and improved treatment options.

Based on previous studies with imidazo[1,2-*a*]pyridine derivatives this study aimed to evaluate a group of novel azaindole and imidazo[1,2-*a*]pyridine derivatives against two colorectal cancer cell lines, HT-29 and Caco-2 cells. In addition the mechanism of cell death was investigated to determine whether or not apoptosis was induced. The effect of active test compounds on normal human peripheral leucocytes was evaluated as a preliminary investigation into their non-specific toxicity. The compounds were synthesized by the School of Chemistry of the University of the Witwatersrand and tested for purity using NMR and X-ray crystallography.

Cell viability was measured and IC_{50} values determined by making use of the 3-[4, 5-dimethyl thiazol-2, 5-diphenyl tetrazolium bromide] (MTT) cell viability assay with camptothecin as a positive control. Phase-contrast and fluorescence microscopy using ethidium bromide, acridine orange and Hoechst-33342 as fluorochromes was used to evaluate the morphological changes in cells. The potential of the most active compounds to induce apoptosis was evaluated by determining caspase-3 activity, annexin-V binding and measuring the subcellular location of cytochrome c.

The imidazo[1,2-*a*]pyridine compounds showed cytotoxicity towards both HT-29 and Caco-2 cell lines with some compounds being comparable to and superior to camptothecin. The HT-29 cell line proved more sensitive to the imidazo[1-2*a*]pyridine compounds with seven test compounds showing IC_{50} values lower than 10 μ M against the HT-29 cell line. Two test

compounds had IC₅₀ values less than 10 µM against the Caco-2 cell line. Compared to camptothecin, four of the test compounds displayed lower toxicity towards normal unstimulated leucocytes. Fluorescence microscopy using the vital stains showed that the four most active compounds were able to induce apoptosis. The cells showed apoptotic features including pre-apoptotic nuclei, apoptotic nuclei, blebbing, chromatin condensation and lysosomal aggregation.

Moreover, the four compounds were able to induce apoptosis as measured by an increase in caspase-3 activity above that of the control in both cell lines. This was supported by the annexin-V/propidium iodide binding assay which demonstrated that phosphatidyl serine was translocated to the outer leaflet of the plasma membrane after exposure to the compounds. The leakage of cytochrome c from the mitochondria to the cytoplasm was observed for one test compound which indicated the possible activation of the intrinsic apoptotic pathway.

In conclusion, six imidazo[1,2-*a*]pyridine compounds, JD-88, JD-96M, JD-137, JD-253, JD-256 and JD-258 showed cytotoxic effects comparable to camptothecin against the HT-29 and Caco-2 cells. Four of these imidazo[1,2-*a*]pyridines proved to be inducers of apoptosis and are therefore worthy of further investigation. Future studies with the active test compounds should include testing the compounds in an animal model of colorectal cancer.

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Research outputs

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Abbreviations, Acronyms and Symbols

AIF	-	Apoptosis-inducing factor
Apaf-1	-	Apoptosis protease activating factor
APC	-	Adenomatous polyposis coli
AO	-	Acridine Orange
ATP	-	Adenosine triphosphate
Bcl-2	-	B-cell lymphoma-2
Bcl-xL	-	B-cell lymphoma-extra large
BSA	-	Bovine Serum Albumin
CO ₂	-	Carbon dioxide
CRC	-	Colorectal cancer
DCC	-	Deleted in Colorectal Carcinoma gene
DMEM	-	Dulbecco's Modified Eagle's Medium
DMSO	-	Dimethyl sulphoxide
DNA	-	Deoxyribonucleic Acid
EB	-	Ethidium Bromide
EDTA	-	Ethylene diamine tetra-acetic acid
EGFR	-	Epidermal growth factor receptor
FAP	-	Familial Adenomatous Polyposis
FADD	-	Fas associated death domain
FBS	-	Foetal Bovine Serum
5-FU	-	5-flourouracil
Hr	-	Hour(s)
HO	-	Hoechst
HNPCC	-	Hereditary non-polyposis colorectal cancer
IAP	-	Inhibitor-of-apoptosis
IC ₅₀	-	50 % inhibitory concentration

IL	-	1 β - Interleukin-1 beta
LOH	-	Loss of Heterozygosity
LV	-	Leucovorin
M	-	Molar
mA	-	Milliampere
MAC	-	Mitochondrial Apoptosis-induced Channel
MCC	-	mutated in colon cancer
mL	-	Milliliter
mM	-	Millimolar
MMR	-	Mismatch repair
MTT	-	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
O ₂	-	Oxygen
PARP	-	Poly (ADP-ribose) polymerase
PBS	-	Phosphate Buffered Saline
PI	-	Propidium iodide
pNa	-	p-nitroaniline
PS	-	Phosphatidyl serine
RBC	-	Red blood cell
ROS	-	Reactive oxygen species
rpm	-	Rotations per minute
SEM	-	Standard error of the mean
TNF- α	-	Tumour necrosis factor-alpha
TNFR1	-	Tumor necrosis factor receptor
TRADD	-	TNFR1 associated death domain
TRAIL	-	Tumor necrosis factor-related apoptosis-inducing ligand
μ g/mL	-	Micrograms per millilitre
μ L	-	Microliter
μ M	-	Micromolar

mU/ml	-	milliunits per milliliter
v/v	-	Volume per volume
w/v	-	Weight per volume

CHAPTER 1: Introduction

1.1 Cancer

Overall, there were an estimated 14.1 million cancer cases around the world and 8.2 million cancer related deaths in 2012. By 2030 the prevalence of cancer is estimated to increase to around 21.4 million cases globally (DeSantis *et al*, 2014). Worldwide cancer causes more deaths than HIV, tuberculosis and malaria, when they are grouped together. In 2012 lung cancer was the most commonly diagnosed cancer with 1.82 million cases, breast cancer was second with 1.67 million cases diagnosed followed by colorectal cancer with 1.36 million new cases diagnosed (Ferlay *et al*, 2015).

Cancer is a disease that is caused by various molecular events that changes the normal functioning and characteristics of cells (Bernstein *et al*, 2015). Factors that can increase the risk of developing cancer include changes to the genes that control the way cells function, divide and grow. In cancer cells the regulatory systems that prevent proliferation and the invasion of other tissues are absent. Cancer cells act as individual cells that divide without control to form tumours. The development of a tumour occurs in a series of stages (Figure 1.1).

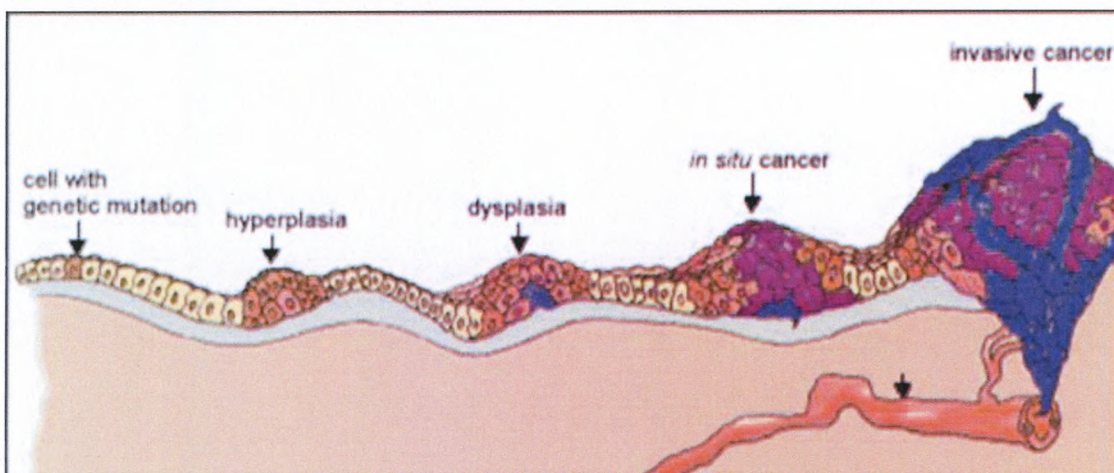


Figure 1.1. The development of a tumour. The growth of a tumour from a single genetically changed cell occurs in a step-wise progression. The first step is hyperplasia which is followed by dysplasia. Thereafter the tumour undergoes a process that is known as carcinoma in situ. Lastly the tumour gains the ability to invade surrounding tissues. (<http://static-content.springer.com>)

The first stage is hyperplasia which is characterized by an increase in cellular growth as result of uncontrolled proliferation. These cells appear normal, but changes have occurred

that result in some loss of control of cellular growth (Alberts *et al*, 2014). The second stage that occurs is dysplasia; this is caused by a further increase in cell growth and abnormal alterations to the cells are observed. The next step that the cell undergoes requires further alterations, which results in cells that are more abnormal and are now able to spread over a wider area of tissue (Carlo and Clark, 2008). During this step the cells begin to lose their original function and are now known as anaplastic. At this stage of the tumour growth the tumour is situated in its original location and is not invasive. The last stage occurs when the cells in the tumour metastasize, this means that the tumour can now invade surrounding tissue, including the bloodstream and can spread to other areas (Underwood and Cross, 2013).

The cancerous cells divide and proliferate in the presence of signals that usually prevent cell growth (Hanahan and Weinberg, 2011). Proto-oncogenes are present in all cells and are essential regulators of normal cellular functions, including the cell cycle (Croce, 2008). In cells, the proto-oncogenes are proteins that are responsible for sending signals to the nucleus to stimulate cell division (Carlo and Clark, 2008; Bernstein *et al*, 2015).

Cancers are classified into four major groups namely carcinomas, sarcomas, leukaemia and lymphomas. The type of cancer that develops depends on the type of cell that was originally changed (Merlo *et al*, 2006). Carcinomas result from changes in the epithelial cells, which cover the surface of our skin and internal organs, most cancers are carcinomas. Sarcomas arise due to changes in muscle, bone, fat, or connective tissue (Bleyer *et al*, 2008). Leukaemia results from malignant white blood cells. Lymphoma is a type of cancer that affects the lymphatic system. Myelomas are cancers that occur in specialized white blood cells which are responsible for the production of antibodies (Alberts *et al*, 2014).

1.2 Colorectal cancer (CRC)

The colon and rectum make up the large intestine which makes up part of the human digestive system. The colon is a large muscular tube that is responsible for the collection and storage of waste which passes into the rectum. CRC originates from epithelial cells that line the colon or rectum (Underwood and Cross, 2013). In 2012, CRC accounted for over 9% of total cancer incidence and it was the third most common cancer in the world (Domati *et al*, 2013). According to the World Cancer Research Fund International (WCRFI) in 2012 nearly 1.4 million new colorectal cancer cases was diagnosed. In 1989, colorectal cancer was estimated as the 10th most common cancer diagnosed in males and females in South Africa but figures released in 2014 have shown that it is now the fifth most common cancer in South Africa (World Cancer Report, 2014).

1.2.1 Types of colorectal cancer

Colorectal cancer can be divided into three broad categories, namely adenocarcinoma, mucinous adenocarcinoma and medullary carcinoma (Watson and Collins, 2011).

1.2.1.1 Adenocarcinoma

This subtype is the most commonly occurring colorectal tumour. Most of these tumours are moderately differentiated and do not have the distinct histological features that are generally associated with tumours (Hakama *et al*, 2008). Although this type of colorectal tumour does present with cribriform patterns associated with central necrosis. Dysplasia in adjacent mucosa is often observed (Xie and Itzkowitz, 2008).

1.2.1.2 Mucinous adenocarcinoma

This is a subtype of an adenocarcinoma where the tumour secretes extracellular mucin. This type of tumour is often associated with microsatellite instability. Mucinous changes can also be seen in adenocarcinomas treated with neoadjuvant chemo-radiotherapy (Watson and Collins, 2011).

1.2.1.3 Medullary carcinoma

Medullary carcinoma is the most important subtype of colorectal cancer. It has a characteristic phenotype where tumours are located on the right side and have sheets of cells where various tumour-infiltrating lymphocytes are observed on microscopy. This phenotype is associated with the hereditary non-polyposis colorectal cancer. Patients with this syndrome often develop endometrial, ovarian and skin tumours (Treanor and Quirke, 2007).

1.2.2 Causes of colorectal cancer

Epidemiologic studies have indicated that a number of factors are responsible for the development of colorectal cancer. These include age, inflammatory bowel disease, smoking, alcohol consumption, obesity, dietary factors and genetic factors (Cronje *et al*, 2009; Latchford and Philips, 2011). The HT-29 colorectal cells are positive for genetic mutations of the KRAS, BRAF, PIK3CA and the TP53 (p53 tumour suppressor) gene. The Caco-2 colorectal cells are positive for a genetic mutation of the TP53 gene (Ahmed *et al*, 2013).

1.2.2.1 Lifestyle causes of colon cancer

A number of different dietary factors have been associated with an increased risk of developing colorectal cancer. These include increased levels of red meat consumption, decreased consumption of fruit and vegetable and a diet that is low in fibre. High fat consumption increases the amount of acid in the digestive tract which could promote tumour growth (Haggard and Boushey, 2009).

Tobacco use does not only increase the risk for the development of lung, mouth, and oesophageal cancers but it also increases the risk for the development of colorectal cancer. Cigarette smoking has been linked with a higher risk of developing large colorectal adenomas, which are generally accepted as being precursor lesions for colorectal cancer (Huang and Chen, 2010). Therefore chronic exposure to tobacco could lead to the development of colorectal carcinogenesis (Cronje *et al*, 2009).

Among men, a higher Body Mass Index (BMI) is strongly linked to a higher risk of developing colorectal cancer. Epidemiological data shows that obesity in men is associated with a 30-70% higher risk of developing colorectal cancer (Bardou *et al*, 2013). As with smoking, excessive alcohol consumption is associated with a higher risk of developing colorectal cancer. Reactive metabolites such as acetaldehyde can be carcinogenic. The effects of alcohol may be linked to an increase in the release of prostaglandins, lipid peroxidation and the production of free radical oxygen species (Federiko *et al*, 2011).

1.2.2.2 Genetic predisposition

Germline mutations in six DNA Mismatch Repair genes are associated with hereditary non-polyposis colorectal cancer (HNPCC) syndrome. The DNA mismatch repair genes are a group of genes that detect and repair insertions, deletions and mismatches in newly transcribed DNA (Lindor, 2009). Mutations in these genes inhibit the repair activity of the proteins and can result in genome-wide mutations (Labianca *et al*, 2010). One gene that is regularly involved in mutations that occur due to microsatellite instability is the transforming growth factor beta receptor. This is a tumour suppressor gene and its inactivation leads to an increase in cellular growth. Approximately 4% of colorectal cancer cases are linked to hereditary non-polyposis colorectal cancer (Bosman *et al*, 2010). Lynch syndrome is an autosomal dominant disorder that is characterized by early onset of colorectal cancer and extracolonic cancers in multiple members of a family (Lindor, 2009; Bosman *et al*, 2010).

Genetic vulnerability to colon cancer has been attributed to either polyposis or non-polyposis syndromes. The major polyposis syndrome that is linked to the development of colorectal cancer is the familial adenomatous polyposis (FAP), which is associated with mutations or a loss of the adenomatous polyposis coli (APC) gene (Markowitz and Bertagnolli, 2009; Wark, 2009). Familial adenomatous polyposis coli accounts for about 0.5% of all cases of colorectal cancer. The pro-apoptotic APC gene inhibits the growth of potential cancer cells and when mutated the gene disrupts the balance between cell growth and cell death. The formation of a colorectal polyp is often a result of an inherited mutation in the APC gene (Pino and Chung, 2010)

Mutations in the APC gene can result in a loss of beta-catenin regulation, altered cell migration and chromosome instability. Beta-catenin is a transcription factor that is an integral part of the Wingless-Wnt pathway (Roberts *et al*, 2011; Cosimelli *et al*, 2014). The Wingless-Wnt signalling pathways are a group of signal transduction pathways that relay signals into a cell via cell surface receptors. The APC/Wnt/B-catenin pathway is important in colorectal cancer (Minde *et al*, 2013). The APC tumour suppressor gene prevents the transition from the G1 phase of the cell cycle to the S phase of the cell cycle. The Wnt signalling pathway mediates the maintenance of stem cells in their undifferentiated state at the bottom of the colonic crypts. In normal colonic mucosa, cells migrate from the colonic crypts and are sloughed off after apoptosis and this process is controlled by the B-catenin protein (Markowitz and Bertagnolli, 2009; Klaus and Birchmeier, 2008).

The APC gene induces the breakdown of the b-catenin protein and therefore plays a role as a negative regulator of the Wnt signalling pathway (Roberts *et al*, 2011). Accumulation of the b-catenin protein can result in the retention of a stem cell phenotype and this prevents the cells from migrating to the surface to be sloughed off. This accumulation of undifferentiated cells in the colonic crypts leads to the formation of a polyp (Minde *et al*, 2011).

The tumour suppressor p53 gene is important in the control of the cell cycle and apoptosis. The anti-cancer properties of the p53 gene include the activation of the DNA repair proteins and the prevention of cell growth by arresting the cell cycle at the G1/S phase (Levine and Oren, 2009). Various genetic mutations and deletion of the p53 gene are observed in approximately 85% of colorectal tumours and generally develop during the transition from adenoma to adenocarcinoma (Naccarati *et al*, 2012). If DNA repair is unsuccessful the p53 genes indirectly induces the apoptotic process by decreasing the levels of the survivin protein. Survivin belongs to the inhibitors of apoptosis (IAP) family and evidence suggests that survivin is overexpressed in 64% of colorectal cancer patients (Armaghany *et al*, 2012).

Loss of Heterozygosity (LOH) of the chromosome 18q is commonly seen in metastatic colorectal cancer. The Deleted in Colorectal Carcinoma (DCC) gene is usually found on the long arm of chromosome 18 and it is responsible for encoding the transmembrane protein that is also called DCC. The DCC gene is a conditional tumour suppressor gene that plays an integral role in the prevention of cell proliferation when in the absence of its ligand netrin-1 (Ogino *et al*, 2009). Netrin-1 is produced deep in the crypts of the mucosae and as epithelial cells differentiate and move towards the surface of the mucosae, the level of netrin-1 decreases (Ren *et al*, 2008). The decrease in netrin-1 contributes to the initiation of the apoptotic process and the sloughing of the epithelial cells. Mutations in the DCC gene,

prevents netrin-1 from binding to the DCC transmembrane protein which results in an overexpression of netrin-1 and an abnormal cell survival (Grady and Carethers, 2008).

The DCC gene, in the absence of its ligand, interacts with caspase-9 and promotes the activation of the caspase-activating complex. This stimulates the release of caspase-3 and the initiation of apoptotic process. This occurs without the formation of an apoptosome or the release of cytochrome c from mitochondria (Ogino *et al*, 2009).

Physiological and oncogenic activation of the RAS kinase stimulates various downstream signalling pathways (Rajalingam *et al*, 2007). The first RAS effector pathway is the RAS-MEK-ERK pathway. The RAF-MEK-ERK pathway is a key cellular proliferation signal transduction pathway from the cells surface to the nucleus (Castellano and Downward, 2010). This pathway controls inflammation, cell growth, cell differentiation, senescence and apoptosis. The activation of the MAPK pathway requires various proteins including RAS, RAF and MEK. RAS activates the protein kinase activity of the RAF kinase. The RAF kinase then phosphorylates and activates the MEK. Activated MEK phosphorylates and activates extracellular signal-regulated kinases driving cell division (Lewis *et al*, 1998).

Mutation to the RAS and the RAF genes can lead to significant changes and can initiate cell growth and cell survival that is independent of the EGFR receptor. Mutations of the RAS genes are thought to occur after the APC mutations in the adenoma-carcinoma sequence. Activating mutations in RAF has been identified in about 10% colorectal carcinomas and patients with a RAF mutation have a poorer prognosis (Fernandez-Medarde and Santos, 2011).

The second RAS effector pathway that is important in cell proliferation is the Akt-PI3K pathway (Kim *et al*, 2014). The Akt pathway is activated through the phosphorylation of phosphoinositide 3-kinase (PI3K) and the pathway is regulated by RAS (Reviewed in Agarwal *et al*, 2013). PI3K is mutated in 25% of colorectal cancers and has an important role in the mediation of RAS-mediated cell survival and proliferation (Manning and Cantley; 2007).

The activated Akt pathway regulates the functioning of various substrates that are responsible for the regulation of cell growth, cell cycle progression and cell survival. The survival of cancer cells and its resistance to apoptosis is induced by several receptors that are controlled by the Akt pathway (Cantrell, 2001; Castellano and Downward, 2010).

1.2.3 Staging and Presentation of colorectal cancer

Epithelial tumours of the colon and rectum are staged according to the guidelines of the TNM classification which corresponds to the Dukes' tumour classification system (Figure 1.2).

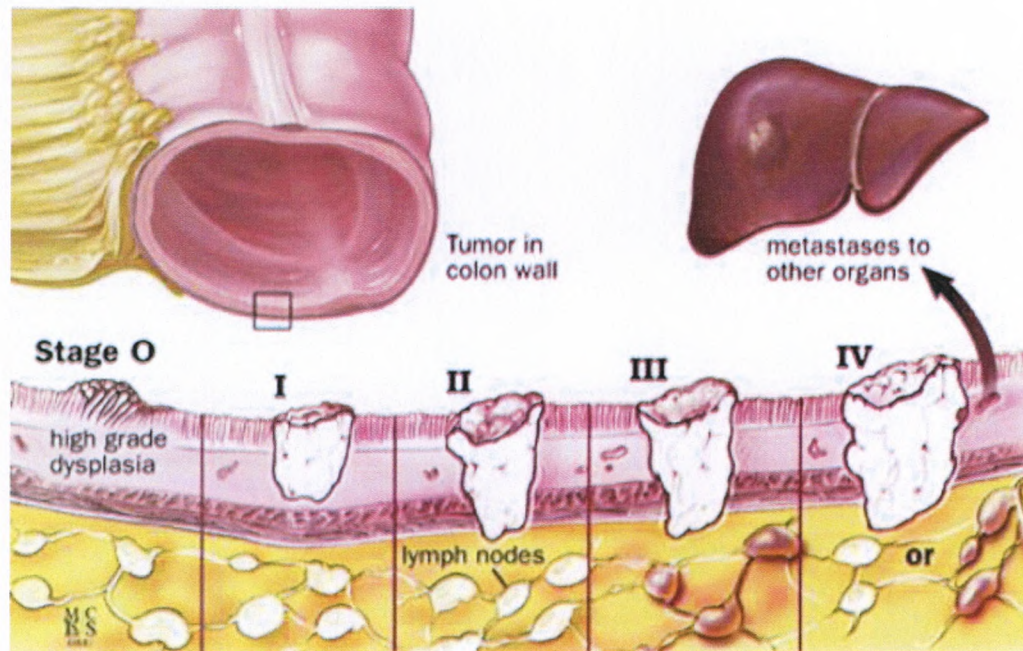


Figure 1.2. The different stages of colon cancer illustrating the relative size and penetration of the tumour (<http://cancer.tipstimes.com>)

Tumours are categorized into four categories. In stage I (Dukes' A), lesions spread through the muscularis mucosa into the submucosa (T1) or they have reached the muscular wall (T2). In stage II (Dukes' B) the neoplasms have spread beyond the muscular wall and into the pericolic tissues (T3) and could have already reached the serosal surface (T4) or infiltrated the surrounding organs (Labianca *et al*, 2010). In stage III (Dukes 'C) lymph nodes are affected, although the degree of infiltration of the primary tumours is not taken into account (C-1, 1–3 metastatic lymph nodes; C-2, 4 or more metastatic lymph nodes). Lastly, stage IV (Dukes' D'), at this point there is evidence of distant haematogenous metastasis that commonly occurs in the liver or the lung (Sobin, 2003; Strorli *et al*, 2011).

The signs and symptoms of CRC vary and depend on the site of the tumour and whether it has metastasized. The most common symptoms of colorectal cancer are constipation, bloody stool, decreased appetite, weight loss and nausea and vomiting (Watson and Collins, 2011).

Right-sided carcinomas are often diagnosed after an obstruction or fatigue and weakness is observed due to iron deficiency anaemia, whereas left-sided malignancies tend to cause

changes in bowel habit, abdominal discomfort or bloody mucus in the stool (Ganapathi *et al*, 2011). Changes in bowel habits may include changes 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 occurs in the rectum, fresh red blood on the stool is observed whereas haemorrhagic lesions result in dark stools (Astin *et al*, 2011).

1.2.4 Management of colorectal cancer

When diagnosis occurs early in the development of CRC, patients undergo curative surgical resection. In Stage I, patients are treated surgically and approximately 95% of patients are cured. Most stage II tumours have grown through the wall of the colon but have not yet metastasized to the lymph nodes therefore surgery to remove the section of the colon may be the only treatment that is necessary (Katzung *et al*, 2013; van Custem *et al*, 2011). For patients with high risk stage II colorectal cancer adjuvant chemotherapy is recommended and main options includes 5-fluorouracil (5-FU), leucovorin or capecitabine. For the management of stage III colon cancer, surgery to remove the section of the colon along with nearby lymph nodes as well as adjuvant chemotherapy, are used. Chemotherapy treatment includes the use of either FOLFOX (5-FU, leucovorin and oxaliplatin) or CapeOX (capecitabine and oxaliplatin) regimens. In some cases patients are treated with 5-FU with leucovorin or capecitabine alone (Katzung *et al*, 2013).

1.2.5 Management of metastatic CRC

Data released by the *American Cancer Society* recommends that patients with stage IV metastatic disease should be treated with chemotherapy and/or targeted therapies (Chibaudel *et al*, 2012). Targeted therapy is the newer method of cancer treatment and it uses drugs that specifically identify and attack cancer cells, usually while doing little damage to normal cell. Some of the most common regimens used include:

- FOLFOX regimen (leucovorin, 5-FU and oxaliplatin)
- FOLFIRI regimen (leucovorin, 5-FU and irinotecan)
- CapeOx regimen (capecitabine and oxaliplatin)
- One of the above combinations plus the addition of either a drug that specifically targets the vascular endothelial growth factor (VEGF) (Bevacizumab, ziv-aflibercept or Ramucitumab) or a drug that targets epidermal growth factor receptors (EGFR) (Cetuximab or Panitumumab)
- 5-FU and Leucovorin with or without a targeted drug
- Capecitabine with or without a targeted drug
- Irinotecan with or without a targeted drug

- Cetuximab
- Panitumumab

The choice of regimens depends on several factors including previous treatments and the aggressiveness of the tumour (Kasper *et al*, 2015).

1.2.6 Chemotherapeutic agents used in colorectal cancer

1.2.6.1 Fluorouracil (5-FU) and Leucovorin

Fluorouracil is used in cancer chemotherapy but resistance to the drug remains a clinical problem. 5-FU is inactive in its parent form and therefore activation of the drug is required. This occurs via a complex series of enzymatic reactions that is necessary to yield ribosyl and deoxyribosyl nucleotide metabolites (Kasper *et al*, 2015). One of these metabolites, 5-fluoro-2-deoxyuridine-5-monophosphate (FdUMP), forms a complex with thymidylate synthase and the reduced folate 5,10-methylenetetrahydrofolate which is a critical reaction for the synthesis of thymidylate (Medina and Shord, 2011). Thymidylate synthase converts deoxyuridine monophosphate (dUMP) to thymidine monophosphate (dTMP), which is phosphorylated to thymidine triphosphate for use in DNA synthesis and repair. 5-FU is converted to 5-fluorouridine-5-triphosphate (FUTP), which is integrated into RNA, where it interferes with RNA processing and mRNA translation. 5-FU is also converted to 5-fluorodeoxyuridine-5-triphosphate (FdUTP), which, when integrated into cellular DNA, inhibits DNA synthesis and function (Katzung *et al*, 2013).

Administration of 5-FU causes a decrease in dTMP therefore rapidly dividing cancerous cells die as a result of a thymidine deficiency. Fluorouracil derivatives cause a pharmacogenetic syndrome that involves a partial or a complete deficiency of the dihydropyrimidine dehydrogenase (DPD) enzyme. DPD is an enzyme that is responsible for the processing of thymine and uracil. Thymine and uracil make up part of the structure of genes. This deficiency results in severe toxicity that includes symptoms such as diarrhoea, myelosuppression, nausea and vomiting and neurotoxicity (Chabner *et al*, 2011).

Leucovorin, a 5-formyl derivative of tetrahydrofolic acid, is given in combination with 5-FU. 5-FU can only bind to thymidylate synthase when leucovorin has locked the enzyme in an active conformation. Leucovorin stabilises the binding of fluorouracil to thymidylate synthase and causes a cytotoxic deficiency in dTMP. Leucovorin on its own has no cytotoxic activity (Medina and Shord, 2011). Evidence suggests that 5-FU combined with leucovorin improves overall survival rates of patients with metastatic disease by an average of 3-7 months (Hassan *et al*, 2014).

1.2.6.2 Capecitabine

Capecitabine is an orally active pyrimidine analog of uracil and is a prodrug of 5-FU. The mechanism of capecitabine is the same as 5-FU. The most common toxicities of fluoropyrimidines include neutropenia, thrombocytopenia, and anaemia when administered as an IV bolus administration and hand–foot syndrome and diarrhoea when administered as a continuous IV infusion (Chabner *et al*, 2011).

1.2.6.3 Oxaliplatin

Oxaliplatin is not effective when used as a single-agent therefore the drug is used with 5-FU. Oxaliplatin is a third-generation diaminocyclohexane platinum analog (Kasper *et al*, 2015). Platinum analogs kill tumour cells in any stage of the cell cycle by binding DNA through the formation of intrastrand and interstrand cross-links. The formation of these cross-links leads to the prevention of DNA synthesis and an inhibition of DNA functioning (Chabner *et al*, 2011).

The adverse effects of oxaliplatin include effects such as nausea and vomiting, laryngopharyngeal spasms, myelosuppression, diarrhoea and peripheral sensory neuropathy. Other common toxicities include damage by sloughing of the gastrointestinal epithelium, pneumonitis, cardiac, and endothelial damage, which become evident during high-dose therapy (Hassan *et al*, 2014).

1.2.6.4 Irinotecan

Irinotecan has been established as an effective treatment either as a single agent or in combination with 5-FU. Irinotecan is a pro-drug that is converted in the liver by the carboxylesterase enzyme to the active SN-38 metabolite which inhibits the activity of topoisomerase I (Douillard *et al*, 2010). The DNA topoisomerase enzyme controls the number of topological links between two DNA strands by catalysing single or double strand breaks, crossing the strands through one another, then resealing the breaks. The topoisomerase enzymes have numerous functions which include the removal of DNA supercoils during transcription and DNA replication; it is responsible for strand breakage during recombination; it plays a role in chromosome condensation; it is responsible for the disentangling of intertwined DNA during mitosis (Katzung *et al*, 2013).

Myelosuppression and severe diarrhoea are two of the most common adverse effects that occur in patients that are being treated with irinotecan (Kasper *et al*, 2015). The diarrhoea that occurs can be very severe and can lead to major electrolyte imbalances and dehydration. Another side-effect that occurs in patients that are using irinotecan is the development of hand and foot syndrome (Chabner *et al*, 2011).

1.2.6.5 Monoclonal antibodies

The monoclonal antibody bevacizumab exerts its mechanism of action by inhibiting angiogenesis. The prevention of angiogenesis occurs via the binding of the antibody to the vascular endothelial growth factor-A (VEGF-A). A major clinical problem with the use of bevacizumab is the fact that the drug inhibits the proliferation of blood vessels, which is part of the body's normal healing and maintenance (Katzung *et al*, 2013).

The main adverse reactions induced by bevacizumab include hypertension and an increased risk of bleeding. Bowel perforation has also been reported in some cases. Other adverse reactions that have been reported are nasal septum perforation and renal thrombotic micro-angiopathy (Chabner *et al*, 2011).

Cetuximab is a monoclonal antibody that binds to the epidermal growth factor receptor (EGFR). EGFR is a transmembrane glycoprotein that is used in many signalling pathways that affect and stimulate cell growth, proliferation, differentiation and apoptosis. The receptor is located on the surface of the normal epithelium and is 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 (Bokemeyer *et al*, 2012).

Cetuximab is only effective as an anticancer agent in some patients. Cetuximab is only effective in patients with wild type KRAS and NRAS therefore making it ineffective in patients with a mutated RAS gene. Studies have observed that the presence of RAS mutations is significantly correlated with resistance to EGFR-targeted therapies (Reviewed in Saletti *et al*, 2015). Adverse effects observed in patients treated with cetuximab include the development of rashes, hypertension, dizziness, bronchospasms, wheezing, fevers, puritis and angioedema (Chabner *et al*, 2011).

1.2.7 Prognosis of metastatic disease

Approximately 35% of patients diagnosed with CRC will develop stage IV metastatic disease at the time of diagnosis and 20%-25% of patients with stage II and stage III CRC will progress to stage IV during the course of the disease. Various factors can influence the prognosis and treatment outcomes include the following: age, gender and the presence of tumour-related symptoms (Pastrello *et al*, 2011).

The 5-year survival rate for stage IV CRC is in range of 5%-8% and the average survival time of patients with stage IV colorectal cancer that are given optimal supportive care without chemotherapy is approximately five months. With the integration of chemotherapy and

surgical resection, overall 5-year survival rates are improved to 30% to 40% (Bartlett and Chu, 2015).

It is clear that metastatic colorectal cancer has a poor prognosis and the new monoclonal antibodies have a limited efficacy against some genetic mutations (Pastrello *et al*, 2011). Therefore the need to discover novel therapeutic agents is a necessity.

1.3 Apoptosis

Apoptosis is a complex, controlled and active cellular process where individual cells are triggered to undergo self-destruction in a physiological manner. Biochemical events lead to characteristic changes in cell morphology and cell death (Alberts *et al*, 2008). The changes observed in the apoptotic cell include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation and changes in cell membrane permeability. There are three phases that occur during the apoptotic process namely, the initiation phase, effector phase and degradation phase (Wong, 2011). In the initiation phase, the cells receive a stimulus that induces the apoptotic process. In the effector phase, the apoptotic machinery is activated, although the process is still reversible. In the degradation phase, the cell begins to disintegrate and the process is now irreversible. In this phase morphological characteristics and DNA degradation becomes evident. The duration of the apoptotic cell death process depends on the stimulus and the cell type and is usually estimated to take from 12 to 24 hours (Wong, 2011).

1.3.1 Morphological features of apoptosis

Morphological features of apoptosis in the nucleus includes chromatin condensation, nuclear fragmentation and the exposure of phosphatidyl serine. These key characteristics are accompanied by rounding up of the cell and reduction in cellular volume (pyknosis). Chromatin condensation starts at the periphery of the nuclear membrane and forms a crescent or ring-like structure. The chromatin further condenses until it breaks up inside a cell with an intact membrane. This is known as karyorrhexis. The plasma membrane remains intact throughout the process (Wong, 2011; Elmore, 2007).

At the later stages of apoptosis some of the morphological changes that are observed include cell membrane blebbing, lysosomal aggregation, structural changes of cytoplasmic organelles and a loss of cell membrane integrity. *In vivo*, phagocytic cells engulf apoptotic cells before the occurrence of apoptotic bodies. If the apoptotic cells are not phagocytosed such as in the case of a cell culture environment, they will undergo degradation that

resembles necrosis (Wong, 2011). Figure 1.3 is a representation of the changes in cell morphology observed during the apoptotic process.

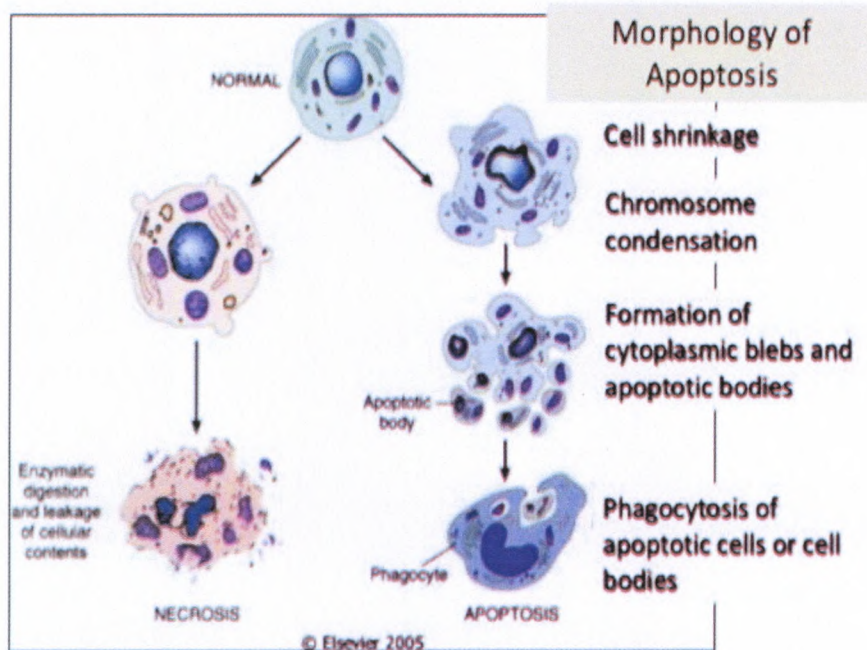


Figure 1.3. The morphological features observed during the apoptotic process. Changes that are observed during apoptosis include cell shrinkage, chromatin condensation, cell membrane blebs and apoptotic bodies (<http://www.slideshare.net/Nimralqbal/apoptosis-15088884>).

1.3.2 Apoptotic Pathways

The apoptotic process is initiated by various signal pathways and it is regulated by extrinsic and intrinsic ligands. There are two major apoptosis pathways namely the extrinsic (death) receptor and the intrinsic (mitochondrial) pathway. The extrinsic pathway is initiated by various extracellular ligands that bind to cell-surface death receptors. The binding of ligands to receptors lead to the formation of the death-inducing signalling complex (DISC). The intrinsic pathway is activated by intracellular signals that are stimulated when cells are stressed. This pathway is highly dependent on the release of proteins from the intermembrane space of the mitochondria (Pennarun *et al*, 2010).

1.3.2.1 The death receptor pathway

The death receptor pathway is initiated by extracellular stimuli and is controlled either by the cell surface receptors which includes the Fas receptor and the tumour necrosis factor (TNF) receptor system or by perforin and granzyme-B. Perforin and granzyme-B are released from activated, cytotoxic lymphocytes. The extrinsic death receptor pathway is activated when death ligands bind to death receptors (Jin and El-Deiry, 2005). The extrinsic pathway is able to activate the caspase cascade within seconds of binding to a ligand; therefore apoptosis is

induced very rapidly. The most important death receptors that are involved in the extrinsic pathway are the type-1 TNF receptor (TNFR1), a related protein called Fas (CD95), TNF-related apoptosis inducing ligand receptor 1 (TRAIL-R1, also known as APO-2), and the TNF-related apoptosis-inducing ligand receptor 2 (TRAIL-R2) (Schneider and Tschopp, 2000; Nadege *et al*, 2009).

The binding of the TNF- α cytokine to the TNFR1 receptor induces the apoptotic process by activating caspases via the intermediate membrane proteins, the TNF receptor-associated death domain protein (TRADD) and Fas-associated death domain (FADD). The binding of the Fas ligand to the Fas death receptors results in the formation of a complex that is known as the death-inducing signalling complex (DISC). DISC then induces the activation of pro-caspase-8 (Kischkel *et al*, 1995). Caspase-8 is an initiator caspase and initiates apoptosis by cleaving other downstream or executioner caspases such as caspase-3 and caspase-7. Caspase-3, in turn, cleaves and activates procaspase-6. The activation of procaspase-6 results in the cleavage of cellular components such as lamin, poly (ADP-ribose) polymerase (PARP) and DNA degradation. In other instances the formation of the DISC complex increases the release of proapoptotic factors from the mitochondria including the second mitochondria-derived activator of caspase/direct inhibitor-of-apoptosis (IAP) protein binding protein with a low PI (Smac/DIABLO) and apoptosis-inducing factor (AIF) (Wang and Youle, 2009; Huphreys and Halppern, 2008).

Studies have shown that cancer cells can either downregulate the Fas expression or can have a disabled intracellular Fas apoptotic signal transduction and therefore renders the cell resistant to Fas-mediated apoptosis. The resistance to Fas mediated apoptosis is important in providing colorectal cancer cells an opportunity to evade the immune system. FasL expression in colorectal cancer induces the apoptosis of tumour infiltrating lymphocytes but not the cancer cells. This provides colon cancer cells an opportunity to evade the immune system (Hoogwater *et al*, 2012; Zhang *et al*, 2005).

1.3.2.2 The intrinsic (mitochondrial) apoptotic pathway

The intrinsic apoptotic pathway is initiated inside the cell. Various stimuli such as genetic damage, hypoxia, high concentrations of cytosolic calcium and oxidative stress are a few of the triggers that are responsible for the initiation of the intrinsic mitochondrial pathway. Regardless of the stimuli, this pathway is the result of an increase in the mitochondrial permeability which allows the release of pro-apoptotic molecules such as cytochrome-c into the cytoplasm (Voux, 2011; Kroemer *et al*, 2009).

A decrease in the membrane potential ($\Delta\psi_m$) is an early feature of apoptosis and is a result of the asymmetrical distribution of protons on either side of the inner mitochondrial

membrane. The asymmetrical distribution of protons increases the chemical (pH) and electrical gradient, which is important for mitochondrial functioning. A decrease in the mitochondrial membrane potential results in the release of mitochondrial pro-apoptotic factors such as cytochrome c (Hockenbery, 2010).

The mitochondrial proteins, second mitochondria-derived activator of caspase (SMACs) are released into the cytosol of the cell following an increase in the mitochondrial permeability. SMACs bind to the proteins that inhibit apoptosis (IAPs) which results in a deactivation of these proteins (Voux, 2011). Following the formation of a channel that is known as the mitochondrial apoptosis-induced channel (MAC) in the outer membrane, cytochrome c is released into the cytosol of the cell. Once cytochrome c is released into the cytosol it binds to the apoptotic protease activating factor (Apaf-1) and ATP which then binds to procaspase-9. The binding to procaspase-9 creates a complex that is known as the apoptosome. This apoptosome complex cleaves the pro-caspase-9 to its active form which activates caspase-3 (Rastogi *et al*, 2009).

The formation of the mitochondrial apoptosis-induced channel is tightly controlled by a group of proteins that belong to the Bcl-2 family. There are two important groups of the Bcl-2 proteins, which are known as the pro-apoptotic proteins Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim and Hrk and the anti-apoptotic proteins including Bcl-2, Bcl-XL, Bcl-W, Bfl-1 and Mcl-1 (Brunelle and Letai, 2009). Bcl-2 proteins are able to promote or inhibit apoptosis by directly influencing the formation of MAC. The pro-apoptotic proteins, Bax and Bak form the pore while the anti-apoptotic proteins inhibit its formation (Gibson and Davids, 2015). The Bax/Bak protein forms a high conductance channel, which leads to a decrease in the cell membrane potential, therefore allows the passage of cytochrome c to the cytoplasm. The balance between the pro- and anti-apoptotic proteins determines whether the apoptotic process will be induced (Brunelle and Letai, 2009; Gibson and Davids, 2015). Further detail on the intrinsic and extrinsic pathways is shown in Figure 1.4.

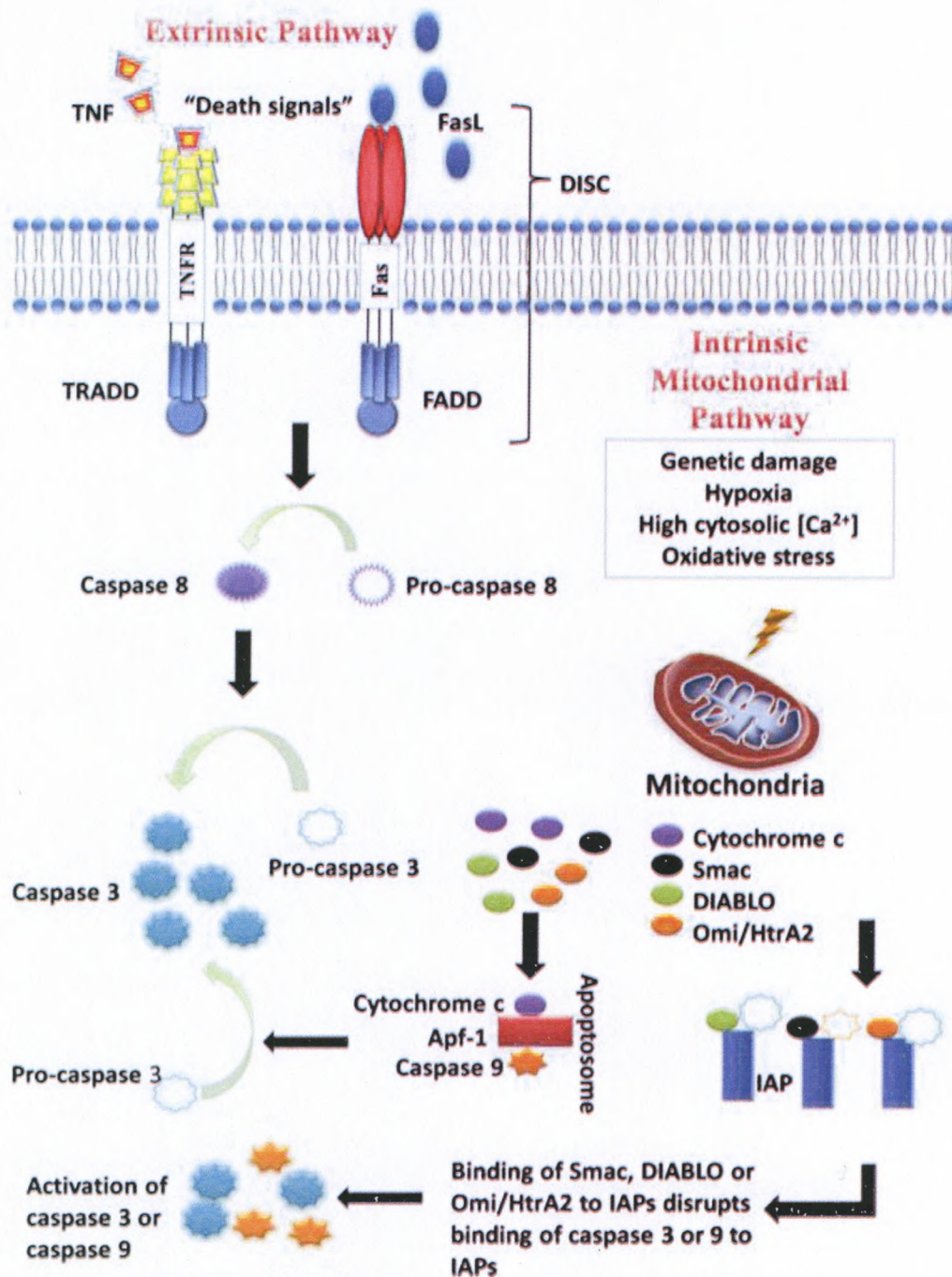


Figure 1.4. A schematic representation of apoptosis and the two major pathways

(<http://jeccr.biomedcentral.com>). The extrinsic pathway induces apoptosis by binding Fas ligand to its corresponding Fas death receptor. This results in the recruitment of the adaptor protein; Fas-associated death domain (FADD) and procaspase-8. A death-inducing signalling complex (DISC) is formed, which activates procaspase-8. Caspase-8 induces the activation of effector caspases 3, 6 and 7. The intrinsic pathway is induced in response to stress. Cytochrome c is released into the cytosol and associates with Apaf-1 to form the apoptosome complex and the activation of caspase-9. Caspase-9 cleaves and activates procaspase-3 (Wong, 2011) (Permission for use of figure was granted by author, Appendix F).

1.4 Apoptosis in cancer

Cancer is caused by various genetic alterations during which normal cells are transformed into malignant cells by evading cell death. Reduced apoptosis or resistance to apoptosis has an important role in the development of cancer (Indran *et al*, 2011). In malignant cells the apoptotic process is reduced or cells become resistant to apoptosis through various mechanisms. The mechanisms by which evasion of apoptosis occurs can be divided into three categories namely, the disruption of the balance between pro-apoptotic and anti-apoptotic proteins, reduction in caspase functioning and an impairment in the death receptor signalling (Letai, 2008).

Defects in the apoptotic pathway make it possible for epithelial cells to survive in a suspended state, without attachment to the extracellular matrix. This promotes the ability of cancer cells to metastasize. Cancer-associated defects in the apoptotic process are associated with treatment failure to chemotherapy and radiation (Indran *et al*, 2011).

1.4.1 Apoptosis in the colon

Normal homeostasis of the colonic mucosae relies on a balance between proliferation of stem cells at the base of the crypt and apoptosis at the surface of the epithelium. In the intestine the epithelium cells proliferate and turn over rapidly. The growth and differentiation of these epithelial cells occurs via distinct stages that depend on the location of a specific cell in the crypt (Barker *et al*, 2009). Stem cells that are found at the base of the crypts, replicate to form daughter cells, which grow rapidly and move up the crypts. After migration the stem cells differentiate into columnar epithelium, goblet cells and enteroendocrine cells. Usually in the colonic mucosa the proliferative areas of the colon is located in the lower half to two-third of the crypt. As cells migrate up the segments, they differentiate, lose their ability to proliferate, and die within a couple of days via apoptosis (West *et al*, 2009).

Apoptotic rates are determined from the number of apoptotic cells in each colonic crypt, and the rate of cell renewal. This supports the concept that the apoptotic process is integral in the control of the epithelial cell population (Heller *et al*, 2008). At the luminal surface a segment of the apoptotic cells are extruded from the epithelial layer into the lumen, while the rest of the cells are engulfed either by the surrounding epithelial cells or lymphocytes. This engulfment of the apoptotic cells is mainly seen at the bottom of the crypt. The occasional apoptotic events that are found in the lower, proliferative zones of the crypts occur in response to the genetic damage (Barker *et al*, 2009).

Various genes are responsible for the regulation of apoptosis including the Bcl-2 family of proteins. The Bcl-2 proteins are integral for the control of the downstream effector pathway of apoptosis. In the colon Bak is the member of the Bcl-2 family that is primarily involved in the apoptotic process (West *et al*, 2009).

Apoptosis along the surface epithelium is a result of a physiological turnover of senescent cells. The cells divide and proliferate in the basal or crypt areas and move up the surface of the villi where they are shed into the intestinal lumen. The molecular signals that create the niche at the bottom of the colonic crypt are responsible for the regulation of apoptosis (Reviewed in Watson, 2004). The niche refers to an anatomic position that control how stem cells participate in tissue regeneration, maintenance and repair. The niche saves stem cells from depletion while preventing increased cellular proliferation in the colon. The regulation of B-catenin/T cell factor activity by the Wnt signalling pathway is central to the functioning of the stem cell niche (Tan and Barker, 2014).

1.4.2 Apoptosis in the development of CRC

A recognized mechanism in the development of CRC is an imbalance between cell growth and cell death, with cell growth being favoured. A balance between new and old cells is important for the maintenance of organ size and the colonic crypt structure (Anant, 2013).

1.4.2.1 Colon cancer and the intrinsic apoptotic pathway

Bcl-2 is an inhibitor of apoptosis and is overexpressed in colorectal adenomas and carcinomas. Several studies have observed that the levels of the Bcl-2 protein is increased in the initial stages of colorectal cancer but it is decreased as the tumour progresses to the late stages of the adenoma-to-carcinoma sequence (Poincloux *et al*, 2009). The Bcl-2 and the Bax protein are transcriptional targets for the p53 gene. The increase in the expression of Bcl-2 causes a decline in p53 protein and enhances the development of a colorectal carcinoma. Bcl-2 alters the activity of p53 from the induction of the apoptotic process to the induction of growth arrest, and it is thus identified as a regulator of the functioning of the p53 gene (Zhang *et al*, 2011).

Bax and Bak are important in the control of the intrinsic (mitochondria) apoptotic pathway (Poincloux *et al*, 2009). Their insertion into the mitochondrial membrane induces the release of cytochrome c from the mitochondria into the cytosol. The release of cytochrome c leads to an increase in caspase activity. Bax and Bak are redundant in functioning therefore, a decrease in the activity of either one does not change the cells ability to undergo apoptosis, while a decrease in expression of both Bax and Bak leads to the prevention of apoptosis (Miquel *et al*, 2005; Nehls *et al*, 2007). Bax has been extensively studied in the development

of CRC. Studies have observed that an increase in Bax expression positivity correlates with better survival rates than Bax negative tumours in advanced metastatic CRC. 50% of colorectal tumours with microsatellite instability contain mutations in the Bax protein (Katkooi *et al*, 2010).

Genetic mutations of the p53 gene are observed in numerous tumours, including CRC (Brosh and Rotter, 2009). Mutations and deletions of the p53 gene can be detected in up to 85% of colorectal cancers. Mutations of the p53 gene commonly occur during the development of an adenoma to adenocarcinoma. When p53 is mutated, it prevents apoptosis of faulty cells and allows damaged cells to proliferate (Anant, 2013).

1.4.2.2 Colon cancer and the death receptor pathway

Apoptosis that is mediated via Fas is involved in numerous functions within the immune system. Despite the fact that colon cancers and colon cancer cell lines express Fas receptors (Fas-R), several studies have observed that a few colorectal cancer cell lines are resistant to Fas-mediated apoptosis. This therefore implies that there is a mutation in the Fas-mediated apoptotic pathway. In contrast, the normal colonic epithelium continues to be responsive to Fas mediated apoptosis (Fulda, 2010). Because of this the efficacy of the Fas ligand (FasL) or Fas ligand agonists like the TNF- α and interleukin-1 beta (IL-1 β) in chemotherapy is diminished since the cancer cells are resistant to apoptosis. Normal colonic cells easily undergo apoptosis and this favours the survival of the cancer cells. Studies have shown that tumour cells develop a resistance to FasL from anti-tumour immune effector cells. This resistance is thought to occur via mechanisms that are complex, and several possibilities have been studied. These possibilities include the upregulation of FasL and a downregulation of the Fas receptor in tumour cells. Mutations in the p53 gene in colorectal cancers can also inhibit Fas signalling directly (Kykalos *et al*, 2012).

TRAIL is an Apo-2 ligand, and belongs to the TNF family. The binding of TRAIL DR4 or DR5 receptors which are expressed in colonic mucosa and colon carcinomas, activates the TRAIL death receptor pathway of apoptosis (Fulda, 2010). A number of studies have observed that TRAIL can induce the apoptotic process in numerous cancer cells *in vitro*. An estimated 80% of human cancer cell lines, including lung, colon, kidney, breast, brain and skin tumours are sensitive to TRAIL, whereas most normal cell types are resistant to TRAIL (Huphreys and Halppern 2008). The expression of the TRAIL receptor DR4 has been proposed to have a protective role against metastatic diseases and therefore it may be important in removing tumour cells by the immune system (Hector and Prehn, 2009).

Evidence suggests that an alteration in the activity of caspases, at both the genetic and protein levels increases the risk of colorectal cancer. The impaired expression or function of caspase-8 has been shown to stimulate the formation of tumours (Fulda, 2010).

1.4.3 Clinical significance of apoptosis in cancer therapy

As mentioned before, the development of cancer arises as a result of a dysregulation of cell growth and a decrease in apoptosis. Therefore both of these abnormalities can be prevented by a clinical intervention (Gimenez-Bonafe *et al*, 2009). Many of the current chemotherapeutic agents are designed to prevent cellular proliferation which results in damage to normal cells and limitations in clinical efficacy. Therefore, the design of chemotherapeutic agents that target abnormalities in cancer cells that suppress apoptosis may potentially avoid the adverse drug reactions that are normally observed with conventional chemotherapeutic agents (Hassan *et al*, 2014).

Necrosis, as opposed to apoptosis is seen as a passive and vaguely regulated process, this process is triggered by external harmful agent instead of an internal cellular mechanism. The most important difference observed between the two is that the necrotic process can lead to a destruction of a large number of cells in a particular area, where in the apoptotic process a small number of scattered cells are involved (Eum *et al*, 2007). The most important mechanistic difference is that in necrosis, the cell membrane is damaged; which leads to swelling of the cytoplasm and the bursting of the cell occurs. The swelling results in a release of lysosomal enzymes and ultimately to inflammation which is not advantageous to the patient (Saey, 2012).

On the other hand in apoptosis, the entire process is limited to a particular cell without destroying neighboring cells. A significant number of differences in cellular morphology are observed (Reviewed in Watson, 2004). The chromatin in necrosis is unevenly distributed as clumps. These clumps are irregular in shape and are poorly defined. In necrosis nuclear fragmentation, cellular shrinking and cell membrane blebbing is absent. Although there are various differences seen between necrosis and apoptosis, there are mechanistically related (Eum *et al*, 2007).

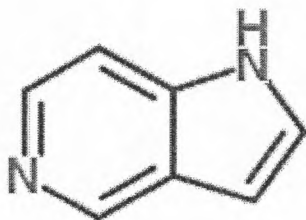
The Bcl-2 protein represents a promising target for the regulation of a tumour cell's ability to undergo apoptosis. An increase in the levels the of anti-apoptotic Bcl-2 proteins is observed in many cancer types (Czabotar *et al*, 2014). Initiating the apoptotic process via Bcl-2 or Bcl-xL would allow the restoration normal cellular homeostasis, prevent drug resistance and restore the tumour cell's sensitivity to chemotherapeutic agents. Alternative approaches

include the use of BH3 peptides or molecules that are able to mimic BH3 domains and can therefore induce apoptosis (Volger, 2014).

1.5 Biological activity of azaindoles and imidazopyridine derivatives

Despite an increase in the understanding of the molecular mechanisms underlying CRC the prognosis of metastatic disease remains poor. Targeted therapy with monoclonal antibodies targeting overactive or overexpressed receptors like EGFR is only effective in specific patients (Reviewed in Saletti *et al*, 2015). Therapy targeting specific kinases like erlotinib has failed since they were designed for mutated forms of EGFR and failed to improve the clinical outcome of colorectal cancer. Therefore there is a need in the phenotypic evaluation of novel compounds in order to identify new lead compounds. The azaindoles and imidazopyridines are two classes of compounds that have been investigated in recent times (Hassan *et al*, 2014).

1.6.1 Azaindoles



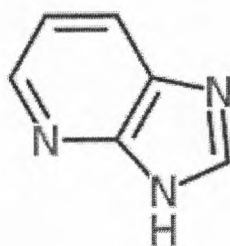
Azaindole compounds have been shown to prevent the formation of microtubules. This process that is necessary for mitosis to be completed (Prudent *et al*, 2013).

Regulation of microtubule dynamics is an important complex that is necessary for processes such as the cell cycle, shape maintenance and motility to occur. Disturbances of microtubule dynamics by drugs is one of the most important ways of suppressing tumour growth. These drugs achieve a clinical response by stopping G2 and the M phases of the cell cycle as a result of abnormal mitotic spindle assembly and chromosome segregation (Kavallaris, 2010).

Indoles are one of the most unique groups of compounds and these compounds have a wide range of biological activities. The indole structure is found in many natural products including vinca alkaloids, fungal metabolites and natural marine products. Derivatives containing the indole ring constitute an important class of therapeutic agents in medicinal chemistry. Medicinal activities of these derivatives include antihypertensive,

antiproliferative, antiviral, antitumor, analgesic, anti-inflammatory, antimicrobial, antifungal activities (Lalit *et al*, 2012).

1.6.2 Imidazopyridines



A group of compounds known as the imidazopyridines has proven to have an effect on the carcinogenic process. Imidazopyridine derivatives show activity against the cytomegalovirus and the antivaricella-zoster virus and have antibacterial activity, hypnoselective effects, and anxiolytic activities. The mechanism of action of these compounds includes their ability to act as an α -amyloid formation inhibitor. The imidazopyridine derivatives constitute a class of orally active non-peptide bradykinin B₂-receptor antagonists (Martínez-Urbina *et al*, 2010). The structure of imidazopyridine derivatives consists of a six membered benzene ring that is fused to a pyrrole ring.

A study conducted by Dahan-Farkas *et al*, (2011) investigated the *in vitro* effects of a group of novel imidazo[1,2-*a*]pyridine derivatives on the HT-29 and Caco-2 cells. These compounds showed cytotoxic activity against the HT-29 and Caco-2 cells within 24 hours whilst showing significant decrease in toxicity against white blood cells. Four of the seven compounds that were tested demonstrated cytotoxicity towards the HT-29 cells with IC₅₀ below 10 μ M. From the seven compounds that were tested three compounds had IC₅₀ values less than 10 μ M against the Caco-2 cells. The study also observed that the apoptotic process was initiated within two hours of exposing the cells to the imidazo[1,2-*a*]pyridine compounds. Results obtained from the study indicated that the imidazo[1,2-*a*]pyridine derivatives initiated cell death in the HT-29 and Caco-2 cells via a pathway that included the leakage of cytochrome c from the mitochondria to the cytosol and an increase in the activity of caspase-3 and caspase-8.

Kim *et al*, (2014) investigated the inhibitory effects of a group of imidazo [1,2-*a*]pyridine (IP)-based dipeptides on the Akt (PKB) kinase. Akt is an important regulator of the downstream response of EGFR and regulates cell growth and survival, cell-cycle progression, cell migration and angiogenesis by phosphorylating a range of intracellular molecules. Many of these substrates are associated with the induction of apoptosis and the arrest of cell

proliferation and are inactivated upon phosphorylation by Akt. Mutations of proteins in the Akt pathway have been shown to directly correlate with the development of colorectal cancer (Kim *et al*, 2014).

1.7 Motivation and Aims

The chemotherapy used in the treatment of advanced metastatic CRC has a significant adverse reaction profile and development of resistance and is plagued by therapeutic failure. Targeted therapy with monoclonal antibodies such as cetuximab and bevacizumab is more expensive and has limited efficacy.

Currently used chemotherapeutic agents have limited efficacy and are associated with a serious adverse reaction profile. It is therefore desirable that new and improved treatment options are discovered. Compounds that are able to induce apoptosis would have a therapeutic advantage.

1.7.1 Aims

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

- The cytotoxic effects of the azaindole and imidazo[1,2-a]pyridine compounds on the cell viability of the HT-29 and Caco-2 cells.
- Evaluate the cytotoxicity of the most active test compounds by using mononuclear peripheral blood cells.
- Determine whether the most active compounds induce cell death by apoptosis.

1.7.2 Objectives

- Screen a small library of compounds at a fixed concentration (100 μ M) to identify potentially active compounds.
- Determining the IC₅₀ values (inhibitory concentration showing 50 % cell death) for compounds showing more than an 80% reduction in cell growth during screening.
- Determine the effects of the most active compounds on human leucocyte viability as a model of normal cells.
- Evaluate whether the most active compounds induce the apoptotic process by evaluating cell morphology, specific apoptotic processes including caspase activity, phosphatidyl serine exposure, and the subcellular distribution of cytochrome c.

CHAPTER 2: Methods and Materials

2.1 Cell culture

2.1.1 Maintenance of cell cultures

Both the HT-29 and Caco-2 cell lines were obtained from Highveld Biologicals, South Africa. The HT-29 and Caco-2 cell lines were grown in Dulbecco's Modified Eagles Medium (DMEM) (Sigma) that was supplemented with heat-inactivated foetal bovine serum (FBS) (10% v/v). An antibiotic solution (1 mL) containing 10 kU penicillin G and 10 mg streptomycin sulphate (Sigma) was added to 100 mL of the media. The cells were maintained at 37°C, in a humidified incubator containing 5% CO₂ (Afrox, Johannesburg). The cell cultures were routinely observed for bacterial contamination, by using the Olympus inverted CX41 microscope. Permission to carry out the study on cell lines was obtained from the University of Witwatersrand Human Ethics Committee (W-CJ-130614-1), as shown in Appendix A1.

2.1.2 Sub-culturing

The confluent HT-29 and Caco-2 cells were subcultured according to the following procedure; firstly the spent media in the 75 cm² flat-bottomed flask (Sigma) was discarded. The flask was rinsed twice with Phosphate Buffered Saline (3.2 mM Na₂HPO₄, 0.5 mM KH₂PO₄, 1.3 mM KCl and 135 mM NaCl, pH 7.4). Thereafter 3 mL of a 0.25% trypsin solution containing 0.1% EDTA (Sigma) was added. The flask was then incubated for 5-10 minutes, until the cells detached from the surface.

After trypsinization, 7 mL of media was added to the flask; thereafter the cells were centrifuged in the Sorvall T6000D centrifuge at 250 x *g* for 5 minutes. Following centrifugation, the cell pellet was suspended in 5 mL fresh culture media and the supernatant was discarded. The suspended cells (1-2 mL) were transferred back to the flask and 20 mL of fresh media was added. The HT-29 and Caco-2 cells were subcultured 2-3 times a week depending on the confluency of the cells.

2.1.3 Cryopreservation

Cryopreservation of cells was carried out according to the method of the American Type Culture Collection (ATCC, Manassas, VA, USA) protocol. The freezing media consisted of 95% complete culture medium and 5% dimethyl sulfoxide (DMSO). Cell cultures were subcultured as described above and centrifuged with the Sorvall T6000D at 250 x *g* for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in the freezing media. Aliquots of 1 mL were transferred into 2 mL plastic cryotubes (Nunc, Denmark). The cultures were cooled for 10 minutes at 4°C, and then placed in a CoolCell® LX cell freezing

container (Biocision) and stored at -70°C for three days after which they were transferred to liquid nitrogen.

2.1.4 Determination of cell number and viability

Cell number and viability was determined by using the trypan blue (0.2%) exclusion method. Cells were counted in a Neubauer haemocytometer chamber with the Nikon bright field microscope. Cells were observed with the 400X objective.

Trypan blue (25 µL) was added to 25 µL of a cell suspension; the mixture was mixed and transferred to the haemocytometer. Trypan blue is a dye that does not penetrate cells with intact cell membranes; therefore dead cells are observed as a distinctive blue colour under the microscope. The number of cells in the chamber was determined by directly counting the live cells using a hand tally counter; the number of live cells in a 16 square area was counted.

2.2. Preparation of compounds

The School of Chemistry at the University of the Witwatersrand supplied 40 novel compounds that included azaindole and imidazo[1,2-*a*]pyridine derivatives. The compounds were synthesized using multi-component reactions. They were checked for purity by nuclear magnetic resonance spectroscopy and X-ray crystallography. Depending on solubility, a working solution (1mM) of each stock solution (10 mM) was prepared in dimethyl sulfoxide (DMSO), acetone or ethanol and stored at -20°C until used.

2.2.1 Positive control

The positive control, camptothecin, was used in all assays and experiments. Camptothecin, the parent drug of irinotecan which is used to treat colorectal cancer and camptothecin is also known for its ability to induce apoptosis. A working solution (1 mM) of the camptothecin stock solution (10 mM) was prepared in dimethyl sulfoxide (DMSO) and was stored at -20°C until used.

2.3. Cell viability assay

2.3.1 MTT assay

The viability of the HT-29 and Caco-2 cells after exposure to test compounds was determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT assay is a quantitative, colorimetric assay that measures the conversion of the yellow tetrazole to a purple water-insoluble formazan crystal. This is assumed to be

directly related to the dehydrogenase activity of the cells and therefore it is used as a measure of the number of metabolically active cells in the well. By designing the experiment appropriately the viability, cell number, or cytotoxicity can be determined. . The cleavage of the yellow tetrazolium salt is catalysed by the mitochondrial enzyme, succinate-dehydrogenase, reductive conditions as well as a number of cytoplasmic dehydrogenases which are only found in viable cells (Mosmann, 1983). The insoluble formazan crystals are solubilised by using a dimethyl sulfoxide (DMSO), an organic solvent. The absorbance of the solubilized formazan crystals is measured spectrophotometrically at 540 nm. This is relative to other cells under the same growth conditions and cannot be used directly to determine cell numbers.

The aim of this assay was to determine which compounds had an 80% cell growth inhibition after exposing cells to the compounds for 48 hours. Compounds showing less than 20% cell viability at 100 μ M after 48 hours were then evaluated to determine their IC_{50} values. The IC_{50} value of each compound was determined by constructing sigmoidal dose-response curves, using the Graph-Pad Prism software package. Experiments conducted in this study were repeated at least five times with each data point done in quadruplicate.

2.3.1.1 Procedure

To perform the cytotoxic assay the cultured cells were plated in 96-well microtitre plates (Nunc, Denmark) at a cell density of 25 000 cells in a volume of 180 μ L/well. Thereafter the plates were incubated for a two hour period before exposure to compounds to allow the cells to adhere and thereafter 20 μ L of the test compound was added to the wells. The culture plates were then incubated for 48 hours at 37°C, after which forty microlitres of 5 mg/mL MTT (w/v) prepared in PBS, was added to each well. After the addition of MTT, the plates were incubated at 37 °C for another two hours. Following the incubation, 200 μ L of the medium was carefully aspirated and the formazan crystals were dissolved in 150 μ L of DMSO. The absorbance was then determined at 540 nm with a reference wavelength of 690 nm using the Labsystems iEMS Multiskan plate reader. The negative control (8 wells/plate) used in the assay consisted of cells only. The positive control used in the assay was camptothecin with concentration values of 200, 100, 50, 25, 10, 1, 0.5 and 0.125 μ M or in the case of screening assays at four wells at a concentration of 100 μ M. The percentage cell viability in reference to the growth of the control sample (cells only) of the various compounds was calculated as follows:

Equation 1:

$$\% \text{ Cell viability} = \frac{\text{A540 test sample} - \text{blank}}{\text{Mean A540 negative control} - \text{blank}} \times 100$$

ANOVA was used to determine if the differences between the average IC₅₀ values obtained for the test compounds were statistically significant. Statistically significant differences between the positive control and the test compounds as well as the differences in the IC₅₀ values between the two cell lines were evaluated via the Student t-test with the significance set at 0.05.

2.3.2 Leucocyte viability assay

The MTT assay as described in Section 2.3.1 was carried out on human leucocytes exposed to the active test compounds for 24 hours. This was done in order to determine their potential cytotoxic effects on normal unstimulated leucocytes as opposed to the cancer cells. Drug-free, human peripheral blood was obtained from healthy volunteers. Permission to study the effects of the test compounds on leucocytes was obtained from the University of the Witwatersrand Human ethics committee (M140669), shown in Appendix A2.

Using the HISTOPAQUE-1077 (5.7 g/dL polysucrose and 9.0 g/dL sodium diatrizoate) kit (Sigma) the isolation of human peripheral white blood cells was carried out as follows: HISTOPAQUE-1077 (3 mL) was added to a 15 mL conical tube and brought to room temperature. Whole blood (3 mL) that was collected in an anti-clotting factor- K3-EDTA containing vacutainer tube was carefully layered on top of the histopaque. The tubes were centrifuged for 400 x g for 30 minutes at room temperature. Thereafter the upper layer in the tube was carefully aspirated and discarded. The opaque interface (Figure 2.1) was carefully transferred to a clean conical centrifuge tube. To this tube 10 mL of PBS was added and gently mixed thereafter the tube was centrifuged for 10 minutes at 250 x g. After the centrifugation the supernatant was aspirated and discarded. The cell pellet was re-suspended in 5 mL of PBS and centrifuged for a further 10 minutes at 250 x g. This step was repeated three times to remove the platelets. The cells were then suspended in 5 mL of RPMI-1640 which contained 2 mM L-glutamine, and was supplemented with 10% heat-inactivated FBS (v/v).

The number of mixed lymphocytes/monocytes was counted in a haemocytometer using the trypan blue exclusion method as described in Section 2.1.3. The cells were then plated in 96-well microtitre plates (Nunc, Denmark) at a cell density of 50 000 cells in a volume of 180 µL/well. The MTT assay was carried out as described in Section 2.3.1. A background

control, containing 200 μL of the cells, was included in each experiment. The cells were exposed to each active compound as well as camptothecin at various concentrations. The plates were then incubated for 24 hours at 37°C , after incubation the MTT assay was carried out as described in Section 2.3.1.1

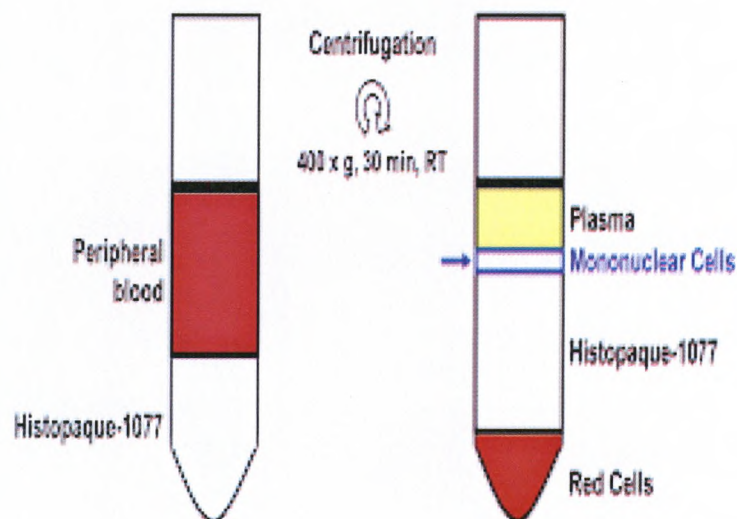


Figure 2.1. Differential sedimentation following centrifugation results in the formation of several cell layers. Lymphocytes, monocytes and platelets are found in the banded plasma-LSM interphase due to their density. The pellet that forms contains the erythrocytes and granulocytes, which have migrated through the gradient to the bottom of the tube. Lymphocytes/monocytes are recovered by aspirating the plasma layer and then removing the cells (www.sigmaaldrich.com).

2.4. Effects of the test compounds on cell morphology

2.4.1. Time studies of the test compounds against the HT-29 and Caco-2 cells

The Olympus inverted phase-contrast microscope was used to observe morphological changes in the HT-29 and Caco-2 cells after exposure to test compounds. Cells were seeded at a cell density of 50 000 cells per well in 6-well plates (Sigma) and incubated for 24 hours at 37°C . Thereafter, cells were treated with the active test compounds. Cells were exposed for 8, 18, 24 and 48 hours. After the 8, 18, 24 and 48 hour exposure period cells were observed under the Olympus inverted CX41 microscope. Photomicrographs were captured with the Olympus DP21 digital camera using a 200X magnification.

2.4.2 Fluorescence microscopy

Morphological and apoptotic changes were determined after staining treated cells with a dye combination of acridine orange (AO), ethidium bromide (EB) and the Hoechst-33342 stain

(HO) (Life Technologies) by fluorescence microscopy. AO is permeable to the cell membrane and stains the nuclei green (Ribble *et al*, 2005). Ethidium bromide only penetrates cells which have lost membrane integrity and stains their nuclei red (Ho *et al*, 2009). Hoechst-33342 (2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole trihydrochloride trihydrate) is a cell-permeable DNA stain that is excited by ultraviolet light and emits blue fluorescence at 460 to 490 nm. Hoechst-33342 is used specifically for the staining of nuclei of living or fixed cells and tissues (Mpoke and Wolfe, 1997). The untreated control cells and the treated cells were seeded on sterile coverslips in 6-well plates (Sigma) at a cell density of 50 000 cells per coverslip. The plates were incubated for at 37°C for a 24 hour period before exposure to the IC₅₀ concentration of the four most active compounds and camptothecin. After addition of the test compounds, plates were incubated for a further 24 hours at 37°C.

This was followed by three gentle washing steps with PBS and the removal of the cover slips from the wells. Thereafter 30 µL of a 1:1:1 combination of AO (100 µg/mL), EB (100 µg/mL) and HO (3 µL/30 µL) was added to a slide and the cover slips were mounted cells facing downwards onto a glass microscope slide. Thereafter the slides were incubated at room temperature for 20 minutes before observation with the BX21 Olympus fluorescence microscope (Ribble *et al*, 2005). Slides were observed using the 10X and 40X objectives under a blue filter with an emission wavelength of 450-490 nm and green filter with an emission wavelength of 500-570 nm.

Images were recorded with the Olympus DP72 camera and the images were analysed using the Olympus CellSens Software package. Images were observed under a 100X and 400X magnification. HT-29 and Caco-2 cells were observed for changes in nuclear morphology.

The HT-29 and Caco-2 cells (50 cells) were observed and analysed to determine the changes in the nuclear morphology after exposing cells to active compounds. Cells were seen as normal or apoptotic after observing the staining pattern of the Hoechst stain. Differences in the staining pattern illustrate the various changes in nuclear morphology. Necrotic cells stained a reddish colour. Figure 2.2 gives an indication of the various staining patterns observed after staining treated cells with the Hoechst stain.

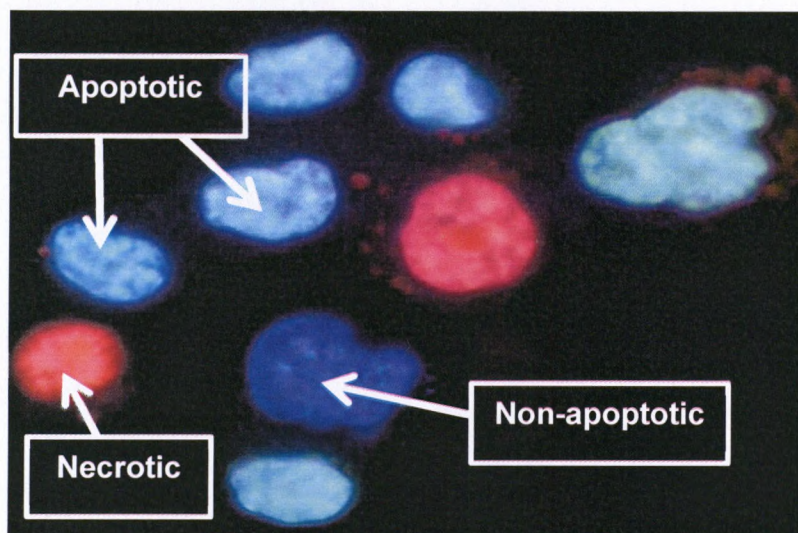


Figure 2.2. Staining patterns of non-apoptotic, apoptotic and necrotic cells after staining with the Hoechst stain

2.5. Colorimetric assay for caspase-3/7 detection

Caspases (cysteiny aspartate proteinases) are part of a family of proteolytic enzymes that cleave their substrates following an aspartate amino acid residue. During apoptosis, caspases are responsible for the cleavage of specific substrates, which are involved in the propagation of typical apoptosis-related biochemical and morphological changes (Vaculova and Zhivotovsky, 2008). Therefore, the detection of activated caspases is considered to be a significant marker for this mode of cell death. Caspase-3 is activated in the apoptotic cell by both the extrinsic and intrinsic pathways. Caspase-3 cleaves most of the cellular substrates in apoptotic cells that contains the amino acid sequence Asp-Glu-Val-Asp (DEVD) therefore it is the most important executioner caspase. The caspase-3/7 colorimetric assay is based on cleavage by hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp-p-nitroaniline (AcpNa) moiety. AcpNa is cleaved by the caspase from the substrate and its concentration is determined spectrophotometrically at a wavelength of 405 nm. A calibration curve was prepared with the pNa solution as shown in Appendix B3. Caspase-3 and Caspase-7 share similar substrate specificity therefore this assay did not distinguish between the two types of caspases (Droin *et al*, 2008).

The HT-29 and Caco-2 cells (2 000 000 cells/well) were exposed to the test compounds and tested for protease activity by the addition of a caspase-specific peptide that is conjugated to the chromophore p-nitroaniline (pNA). HT-29 and Caco-2 cells were harvested and lysed according to the method described below. 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. A pNA calibration curve was constructed by preparing a series of solutions with concentrations ranging from 10 to 100 μ M that was diluted in 1X Assay buffer. To each well 100 μ L of the dilution was added and the absorbance read at 405 nm using the Thermo Scientific™ Multiskan GO plate reader.

To determine caspase-3/7 activity, the following procedure using the kit from Sigma was performed. The HT-29 and Caco-2 cells were seeded at a density of 2 000 000 cells/mL in a 24-well plate (Sigma) and incubated overnight at 37°C. Cells were treated with the active compounds; and a positive (camptothecin) and a negative control (cells only) were included in all experiments. After exposure the plates were incubated for various time periods (2, 4, 12, 24 hours).

Cells were harvested according to the following procedure; the treated and untreated cells were trypsinised, washed with PBS and transferred into sterile 1.5 mL Eppendorf tubes. The subcultured cells were then centrifuged at 1000 x *g* for 5 mins at room temperature.

Thereafter the supernatant was removed and cells were washed with 1mL PBS. After harvesting, the treated and untreated cells were resuspended in 50 μ L of chilled 1x cell lysis buffer (50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 1 mM DTT, 100 mM EDTA, pH 7.4) and incubated on ice for 15 minutes. The lysed cells were centrifuged for 15 minutes at 16000 x *g* at 4°C and the supernatant (cytosolic extract) was transferred to a fresh tube. The protein concentration of the cytosolic extract was assayed using the Bradford assay as described in section 2.5.1.

To determine caspase-3/7 activity, 5 μ L of the lysate was placed in the appropriate wells as shown in Table 2.1. Thereafter 85 μ L of 1X Assay Buffer (20 mM HEPES, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, pH 7.4) was added to each well. The reaction was started by the addition of 5 μ L of the 2 mM caspase-3 substrate (Ac-DEVD-pNA) to each well. The samples were incubated at 37°C for 24 hours and the absorbance was read at 405 nm using the Thermo Scientific™ Multiskan GO plate reader. Time dependent studies were performed to determine caspase-3/7 activity by harvesting cells after 2, 4, 8, 12, and 24 hours post-exposure.

Table 2.1. Reaction scheme for the 96-well Plate Microassay Method

	Cell lysate	Caspase-3 (1:10)	1x Assay buffer	Caspase-3 inhibitor (2 mM)	Caspase-3 substrate (20 mM)
reagent blank			90 μ L		10 μ L
non-induced cells	5 μ L		85 μ L		10 μ L
non-induced cells + inhibitor	5 μ L		75 μ L	10 μ L	10 μ L
induced cells	5 μ L		85 μ L		10 μ L
induced cells + inhibitor	5 μ L		75 μ L	10 μ L	10 μ L
caspase-3 positive control		5 μ L	85 μ L		10 μ L
caspase-3 positive control + inhibitor		5 μ L	75 μ L	10 μ L	10 μ L

2.5.1 Measurement of the protein concentration

Measurement of the protein concentration was performed according to the Bradford Assay (BIO-RAD). This is a dye-binding assay that is based on the colour change of the dye in response to different concentrations of protein (Bradford, 1976). The Bradford protein assay is a simple and accurate procedure for determining the concentration of protein in solution. This assay is based on the fact that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when protein binding occurs.

Standardized bovine serum albumin (1.4 mg/mL) (BIO-RAD) was used to construct a standard curve with concentrations of 0, 0.14, 0.28, 0.7, 0.84, 1.12, and 1.4 (mg/mL). The macro assay (typically 3 mL) was adapted to be performed in a volume of 200 μ L in a 96 well microtitre plate. The dye reagent was prepared by diluting 1 part dye reagent concentrate with 4 parts double distilled water. Thereafter 10 μ L of each standard was pipetted into separate microtiter plate wells (96-well plates, Sigma). Dye reagent (190 μ L) was added to each sample followed by mixing using a microplate shaker (10 mins, 250 rpms). Thereafter the plate was incubated at room temperature for 5 minutes. After incubation the absorbance was measured at a wavelength of 595 nm using the Thermo Scientific™ Multiskan- GO plate reader. A standard curve was constructed for each protein determination (Example in Appendix B; Figure B2). The protein concentration of the lysates of the HT-29 and Caco-2 cells after treatment with the compounds was determined from the standard curve.

2.6 Annexin-V assay

The Annexin-V assay is based on the observation that early after the initiation of the apoptotic process, mammalian cell types translocate phosphatidyl serine (PS) from the inner face of the plasma membrane to the outer cell surface. Cells are separated from the extracellular environment by the plasma membrane which prevents the diffusion of proteins, ions and other essential molecules in the extracellular space. Early in the apoptotic process which occurs at the cell surface, cells undergoing apoptosis break up the phospholipid symmetry of their plasma cell membrane. The alteration in the plasma membrane allows the translocation of phosphatidyl serine (PS) from the inner layer of the plasma membrane to the outer layer and PS is exposed at the external surface of the cell (Fadok *et al*, 1992). Once on the cell surface, phosphatidyl serine can be specifically detected by staining with fluorescein isothiocyanate (FITC)-labelled annexin-V (annexin-V-FITC), which is a protein with a strong natural affinity for PS. In order to distinguish between apoptotic and necrotic cells annexin-V was used combined with propidium iodide (PI). Propidium iodide is a DNA binding fluorescent dye that does not cross the cell membrane of viable cells and is therefore an indication of late apoptosis and/or necrosis (Vermes *et al*, 1995).

The untreated control cells and the treated cells were seeded in 6-well plates on sterile coverslips (Sigma) at a cell density of 50 000 cells per coverslip cells/mL. Cells were incubated for 24 hours at 37°C before exposure to the four most active compounds and camptothecin. After exposing cells to the test compounds, plates were incubated for various time periods at 37°C.

After the incubation period three gentle washing steps with PBS followed. Thereafter the cover slips were removed from the wells. Surface exposure of phosphatidyl serine was measured at the cell surface by making use of an annexin-V binding buffer (Invitrogen), alexa-fluor 488 (Invitrogen) and a propidium iodide working solution (Invitrogen). The 1X annexin-V binding buffer was prepared by diluting 1 mL of the stock solution in 4 mL of deionized water. To 100 µL of the 1X annexin-V binding buffer, 5 µL of conjugated annexin-V and 1 µL of Propidium iodide were added. The propidium iodide working solution (100 µg/mL) was prepared by diluting 5 µL of the 1mg/mL PI stock solution in 45 µL of 1X annexin-V binding buffer.

Thereafter 30 µL the annexin V: PI mixture was added to a slide and the cover slips were mounted cells facing downwards onto the slide. Thereafter the slides were incubated at room temperature for 20 minutes and were then observed using the Olympus BX41 fluorescence microscope. Slides were observed under the UV filter at a magnification of

400X. Images were recorded with the Olympus DP72 camera and analysed with the Olympus CellSens Software package.

2.7 Cytochrome c determination

The leakage of cytochrome c from the mitochondria to the cytosol is a hallmark of the intrinsic pathway of apoptosis. The determination of cytochrome c is used to characterize the mitochondria-dependent pathway of apoptosis. Cytochrome c is a soluble protein which is found in the mitochondrial inter-membrane space. The function of cytochrome c is to transfer electrons from the cytochrome bc₁ complex to cytochrome oxidase on the surface of the inner mitochondrial membrane (Campos *et al*, 2006). The Cytochrome c, ELISA kit is a complete assay system for the quantitative determination of cytochrome c in cell lysates and in mitochondria. It uses a monoclonal antibody to cytochrome c that is immobilized on a microliter plate to bind the Cytochrome c in the standards or sample.

Determination of cytochrome c was done on the HT-29 cells, as these cells were more sensitive to the test compounds. The cells were seeded at a density of 1 000 000 cells/mL in 6-well plates (Sigma). Cells were exposed to the IC₇₅ concentration of the active compounds and incubated for 24 hours. Thereafter HT-29 cells were harvested according to the procedure mentioned in Section 2.5. The cells were then centrifuged at 800 x *g* for 5 minutes at room temperature and the supernatant discarded. The remaining cell pellet was resuspended in 1 mL of a PBS solution and was washed four times with PBS. After the last PBS wash, the cells were centrifuged at 1,000 x *g* for 5 minutes and the supernatant was discarded. The cell pellet was resuspended with the Digitonin Cell Permeabilization Buffer and incubated on ice for 5 minutes. Cells were then centrifuged at 10 000 x *g* for 10 minutes at 4°C. This buffer causes permeabilisation of the cell membrane only, leaving the mitochondrial membrane intact.

After centrifugation the supernatants were transferred to new tubes, as they contained the cytosolic fraction of Cytochrome c. The remaining pellet was then resuspended with RIPA Cell Lysis Buffer 2 (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1% Sodium Deoxycholate and 0.1% SDS) vortex mixed and incubated on ice for 5 minutes. Following this the lysate were mixed by vortexing and were then centrifuged for at 4°C at 10 000 x *g* for 10 minutes (See Figure 2.3 for a flow diagram of the methodology).

The cytochrome c standard was solubilized in the provided Assay Buffer (proprietary composition) and this was then used to create a range of cytochrome c standard dilutions. A

calibration curve was constructed with final concentrations of 28.13, 56.25, 112.5, 225, 450 and 900 pg/ml. The assay buffer (100 μ L), standards (100 μ L) and samples (100 μ L) were pipetted into the appropriate wells on the provided microtitre plate. The 96-well plate was sealed and incubated at room temperature on the Labsystems iEMS Multiskan plate shaker for an hour at 500 x rpms. This allowed for the 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 μ L 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 μ L of a solution containing a biotinylated monoclonal antibody to cytochrome c was pipetted into each well except the blank. The plate was sealed and it was incubated at room temperature on a plate shaker for an hour at 500 x rpms to allow the binding of the cytochrome c to the antibody that was captured in the plate. The contents of the wells were removed and the wash step was repeated. Thereafter a solution of streptavidin (100 μ L) that is 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 x rpms. This step allowed for the binding of the quantifying enzyme to the secondary antibody bound to the cytochrome c that is captured in the plate.

The content of the wells were removed and the wash step was repeated. The substrate solution (100 μ L) containing p-nitrophenyl phosphate (pNpp) was added to each well and incubated at room temperature on a plate shaker for 45 minutes at 500 x rpms. A stop solution (25 μ L) which contained trisodium phosphate (proprietary concentration) in sterile water was added to each well. After the addition of the sterile water the optical density was read at 405 nm. A schematic diagram of the procedure can be viewed in Figure 2.4.

The absorbance readings of the standard dilutions at a wavelength of 405 nm minus the blank was used to construct a standard curve of Absorbance (405 nm) against cytochrome c concentration (pg/mL). Cytosolic and mitochondrial cytochrome c concentrations of the treated cells were determined from the standard curve.

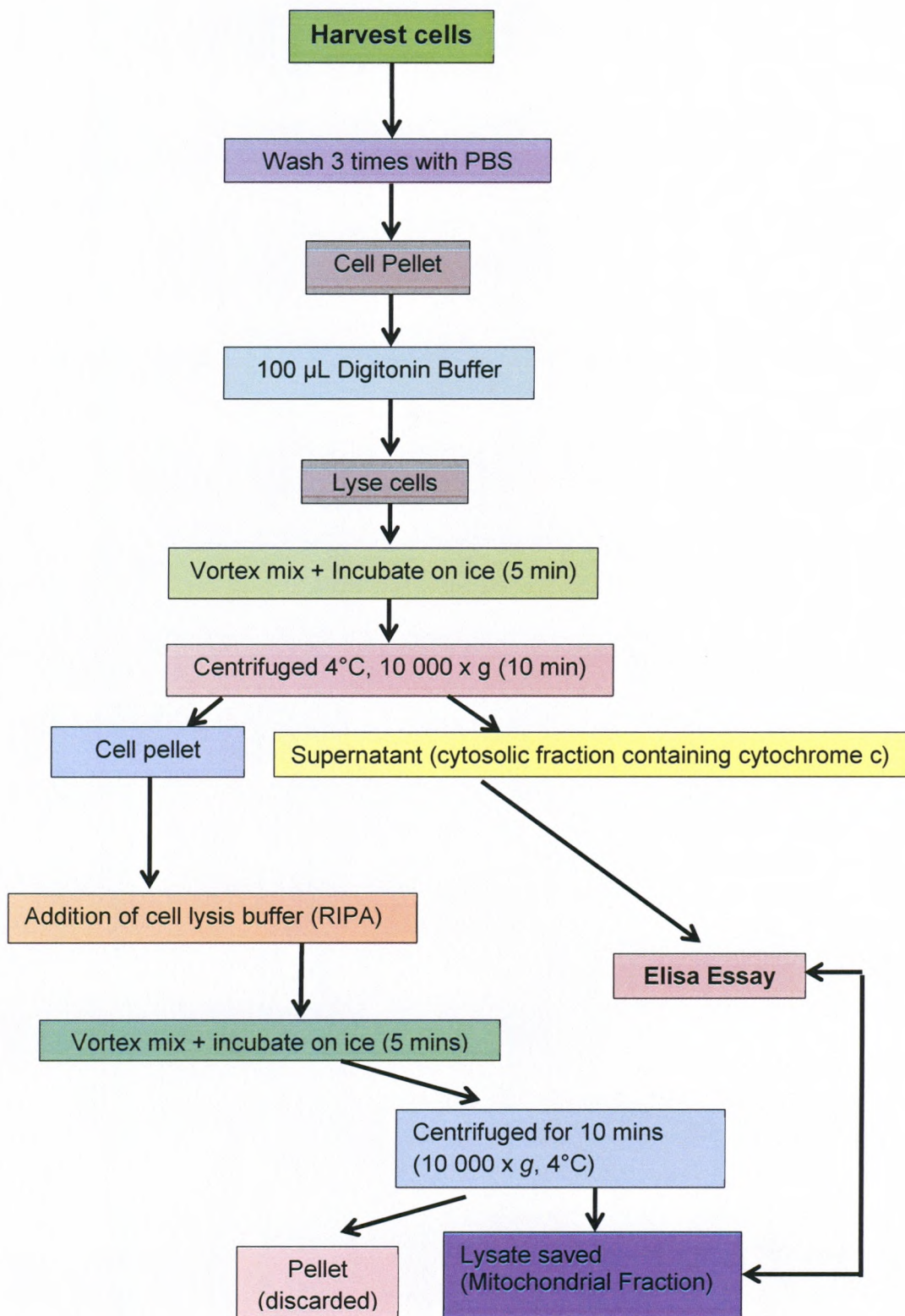


Figure 2.3. A flow diagram showing the preparation of the cytosolic and mitochondrial fractions for cytochrome c determination.

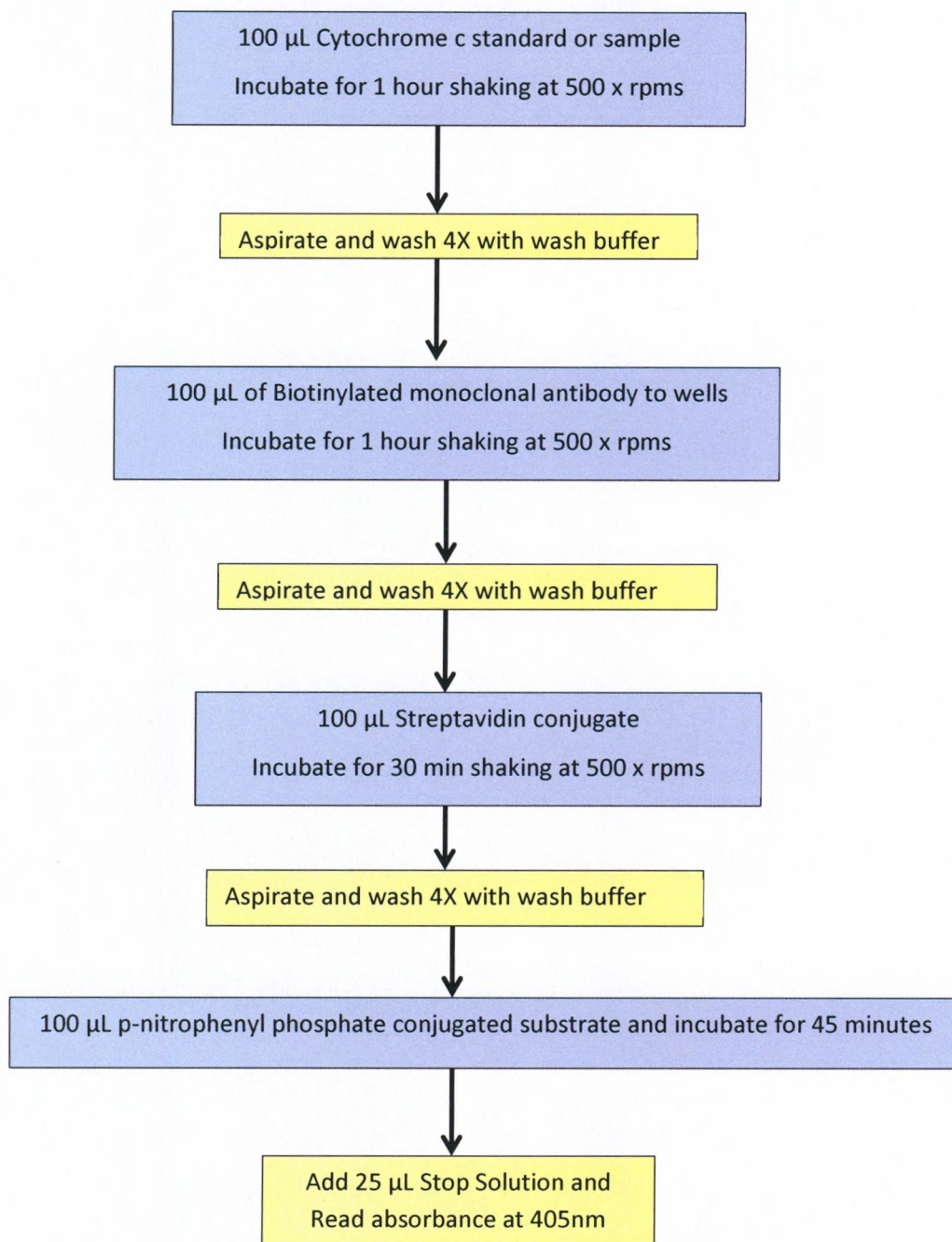


Figure 2.4. Schematic diagram of the procedural steps of the ELISA assay

CHAPTER 3: Results

3.1 Cell viability

Fourteen novel azaindole and twenty-seven imidazo[1,2-a]pyridine derivatives were subjected to a preliminary evaluation to identify compounds that decreased the cell viability of HT-29 and Caco-2 cells. Compounds were arbitrary named with the initials of the individual who synthesized them. Cell viability was determined by the MTT assay after the HT-29 and Caco-2 cells were exposed to the test compounds at a concentration of 100 μ M for 48 hours. Camptothecin was used as the positive control. The results of the initial screening assays are summarised in Table 3.1 and 3.2. IC₅₀ values (calculated from sigmoidal log dose response curves) were obtained for the compounds showing less than 20% cell viability at 100 μ M after 48 hours.

Table 3.1. The effect of imidazo[1,2-a]pyridine derivatives (100 μ M) on the percentage cell viability of the HT-29 and Caco-2 cells. The reported percentage cell viability values represent the mean + SEM of at least three values obtained from three separate MTT assays. Test compounds showing viability lower than 21% are highlighted in red (HT-29 cells) and blue (Caco-2 cells).

COMPOUNDS	% CELL VIABILITY (Mean + SEM)		COMPOUNDS	% CELL VIABILITY (Mean + SEM)	
	HT-29 cells	Caco-2 cells		HT-29 cells	Caco-2 cells
Imidazo [1,2-a] pyridines			Imidazo[1,2-a] pyridines		
JD-PT-C1	29.67 $\pm$ 3.5	43.00 $\pm$ 3.1	JD-218	65.67 $\pm$ 4.1	43.33 $\pm$ 1.7
JD-52	63.33 $\pm$ 6.4	186.03 $\pm$ 3.2	JD-224	53.00 $\pm$ 1.5	35.33 $\pm$ 2.1
JD-60	95.21 $\pm$ 0.4	211.30 $\pm$ 6.7	JD-236	49.33 $\pm$ 5.2	33.33 $\pm$ 4.3
JD-64	29.00 $\pm$ 1.0	28.02 $\pm$ 3.21	JD-245	36.01 $\pm$ 2.3	48.67 $\pm$ 0.6
JD-80	36.33 $\pm$ 3.8	12.00 $\pm$ 1.2	JD-248	62.12 $\pm$ 5.5	41.00 $\pm$ 0.6
JD-81	18.67 $\pm$ 1.9	50.30 $\pm$ 3.7	JD-249	32.67 $\pm$ 1.5	41.33 $\pm$ 0.7
JD-88	15.33 $\pm$ 0.7	18.23 $\pm$ 0.6	JD-253	18.67 $\pm$ 1.8	20.67 $\pm$ 2.2
JD-88M	22.00 $\pm$ 1.7	40.33 $\pm$ 0.3	JD-254	20.67 $\pm$ 4.2	22.67 $\pm$ 1.3
JD-96	26.32 $\pm$ 2.7	42.33 $\pm$ 1.4	JD-255	30.21 $\pm$ 1.6	46.33 $\pm$ 1.3
JD-96M	20.67 $\pm$ 2.9	17.33 $\pm$ 3.9	JD-256	16.67 $\pm$ 0.9	12.23 $\pm$ 1.2
JD-119	18.12 $\pm$ 3.5	43.00 $\pm$ 6.1	JD-257	43.10 $\pm$ 1.3	48.00 $\pm$ 0.5
JD-122	17.33 $\pm$ 5.9	12.67 $\pm$ 4.8	JD-258	11.00 $\pm$ 0.6	24.70 $\pm$ 1.8
JD-124	76.00 $\pm$ 0.3	186.21 $\pm$ 3.2	JD-259	31.00 $\pm$ 1.0	36.01 $\pm$ 3.1
JD-137	16.33 $\pm$ 5.8	11.00 $\pm$ 1.2			

Table 3.2. The effect of the azaindole derivatives (100 μ M) on the percentage cell viability of the HT-29 and Caco-2 cells. The reported percentage cell viability values represent the mean + SEM of at least three values obtained from three separate MTT assays. One test compound showing viability lower than 20% in the Caco-2 cells is highlighted in blue.

COMPOUNDS	% CELL VIABILITY (Mean + SEM)		COMPOUNDS	% CELL VIABILITY (Mean + SEM)	
Azaindoles	HT-29 cells	Caco-2 cells	Azaindoles	HT-29 cells	Caco-2 cells
TLC-26(1)	76.67 $\pm$ 0.7	82.33 $\pm$ 1.5	V-22	40.54 $\pm$ 0,4	60.67 $\pm$ 0.7
TLC-27(3)	76.00 $\pm$ 1.5	22.67 $\pm$ 1.3	V-33	85.00 $\pm$ 0.6	65.11 $\pm$ 2.4
TLC-26(3)	91.67 $\pm$ 0.9	51.67 $\pm$ 1.2	V-41	90.01 $\pm$ 0.6	91.33 $\pm$ 0,8
TLC-74	87.12 $\pm$ 1.4	211.00 $\pm$ 2.0	V-50	87.21 $\pm$ 0.8	101.70 $\pm$ 1.2
TLC-75	27.33 $\pm$ 0,9	11.04 $\pm$ 1.4	V-53	74.67 $\pm$ 0.7	85.00 $\pm$ 0.6
TLC-76	97.33 $\pm$ 0.6	109.43 $\pm$ 2.3	V-92	86.67 $\pm$ 0,8	96.33 $\pm$ 0.9
TLC-77	24.43 $\pm$ 2.3	25.03 $\pm$ 2.4	V-93	64.42 $\pm$ 0.3	95.70 $\pm$ 0.6

3.1.1 IC₅₀ values of active compounds

3.1.1.1 HT-29 cells

Studies to determine the potency of test compounds showed that nine of the imidazo[1,2-a]pyridine compounds had IC₅₀ values lower than 20 μ M (Figure 3.1) when tested against the HT-29 cell line. JD-253 had the lowest IC₅₀ value of 3.89 $\pm$ 0.6 μ M against the HT-29 cells.

Six compounds displayed promising IC₅₀ values around a concentration of 10 μ M: JD-88 (8.03 $\pm$ 0.4 μ M), JD-119 (8.13 $\pm$ 1.5 μ M), JD-137 (4.13 $\pm$ 0.4 μ M), JD-253 (3.89 $\pm$ 0.6 μ M), JD-256 (9.14 $\pm$ 0.5 μ M), JD-258 (5.87 $\pm$ 0.8 μ M).

Three compounds, JD-253, JD-258 and JD-137 proved to be the most active with IC₅₀ values of 3.89 $\pm$ 0.6 μ M, 5.87 $\pm$ 0.8 μ M and 4.13 $\pm$ 0.4 μ M, respectively. The IC₅₀ value of camptothecin was 5.70 $\pm$ 0.1 μ M.

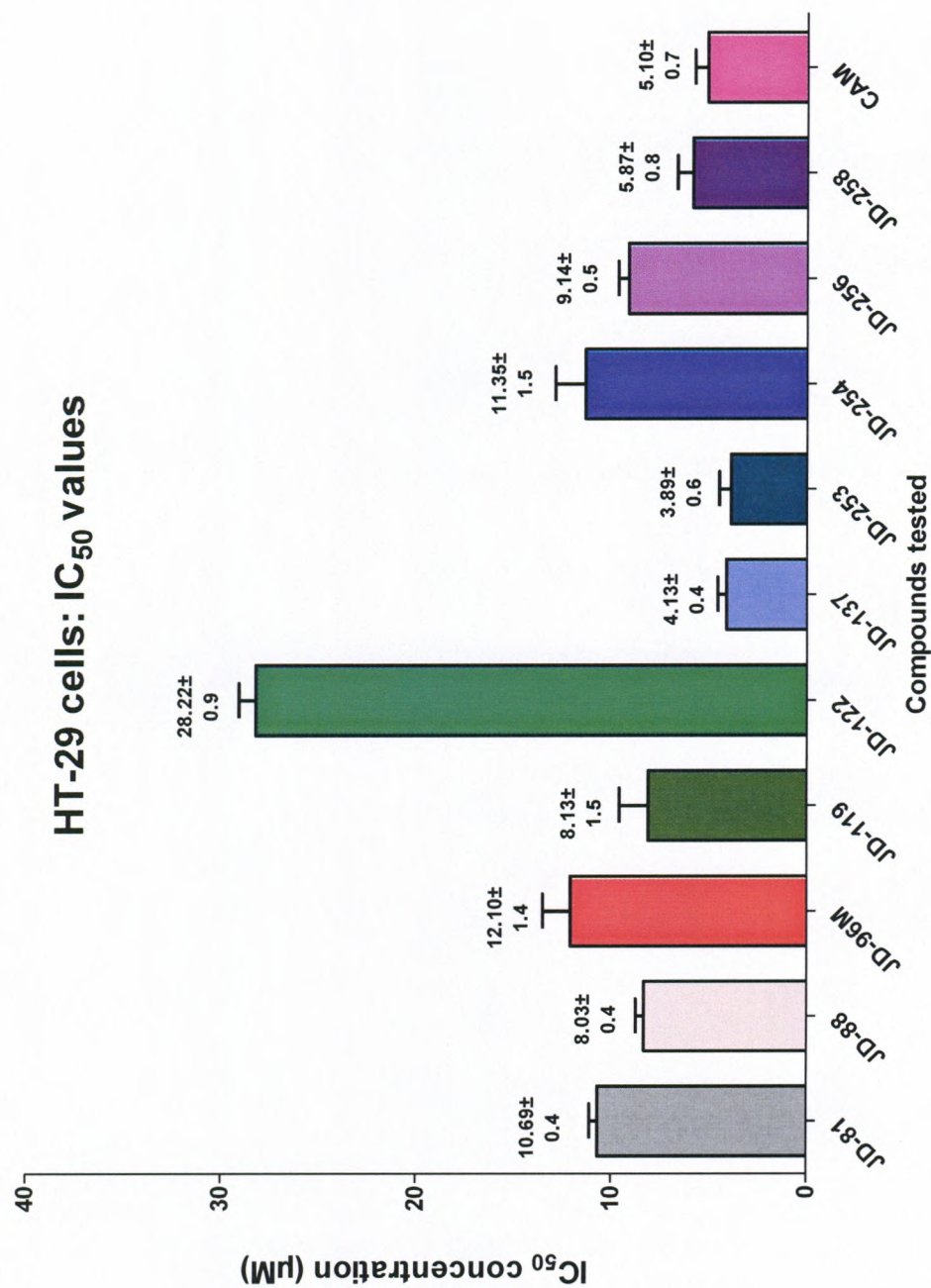


Figure 3.1. The IC₅₀ values for the test compounds and camptothecin on the HT-29 cells. The HT-29 cells were exposed to each compound for 48 hours. The IC₅₀ values of JD-81, JD-88, JD-119 and JD-122 were determined after exposing cells to concentration values of 0.5; 1; 10; 25; 50; 75; 100; 200 μM. The IC₅₀ values of JD-88, JD-119, JD-137, JD-253, JD-254, JD-256 and JD-258 were determined after exposing cells to concentration values of 0.125; 0.25; 0.5; 1; 10; 25; 50; 100 μM. The bars represent the mean + SEM of the at least three IC₅₀ values obtained from three separate MTT assays.

3.1.1.2 Caco-2 cells

The Caco-2 cells were less sensitive to inhibitory action of the test compounds than the HT-29 cells. Seven imidazo[1,2-*a*]pyridine compounds had IC₅₀ values lower than 20 µM (Figure 3.2).

Two compounds JD-80 and JD-137 showed IC₅₀ values of around 14 µM which was higher than that of camptothecin. Camptothecin had an IC₅₀ value of 10.60±1.5 µM. From the all the compounds that were tested, JD-256 had the lowest IC₅₀ value (6.22±1.5 µM).

Four imidazo[1,2-*a*]pyridine compounds (JD-88, JD-96M, JD-253 and JD-254) were similar to camptothecin with IC₅₀ values, of 12.22±0.6 µM, 10.79±0.5 µM, 9.62±0.4 µM and 11.69±2.6 µM respectively.

One azaindole derivative had an IC₅₀ value higher than 40 µM (JD-75) against the Caco-2 cells. This high IC₅₀ value indicated that the azaindoles were not viable candidates for future studies and no further investigations were carried out on this compound.

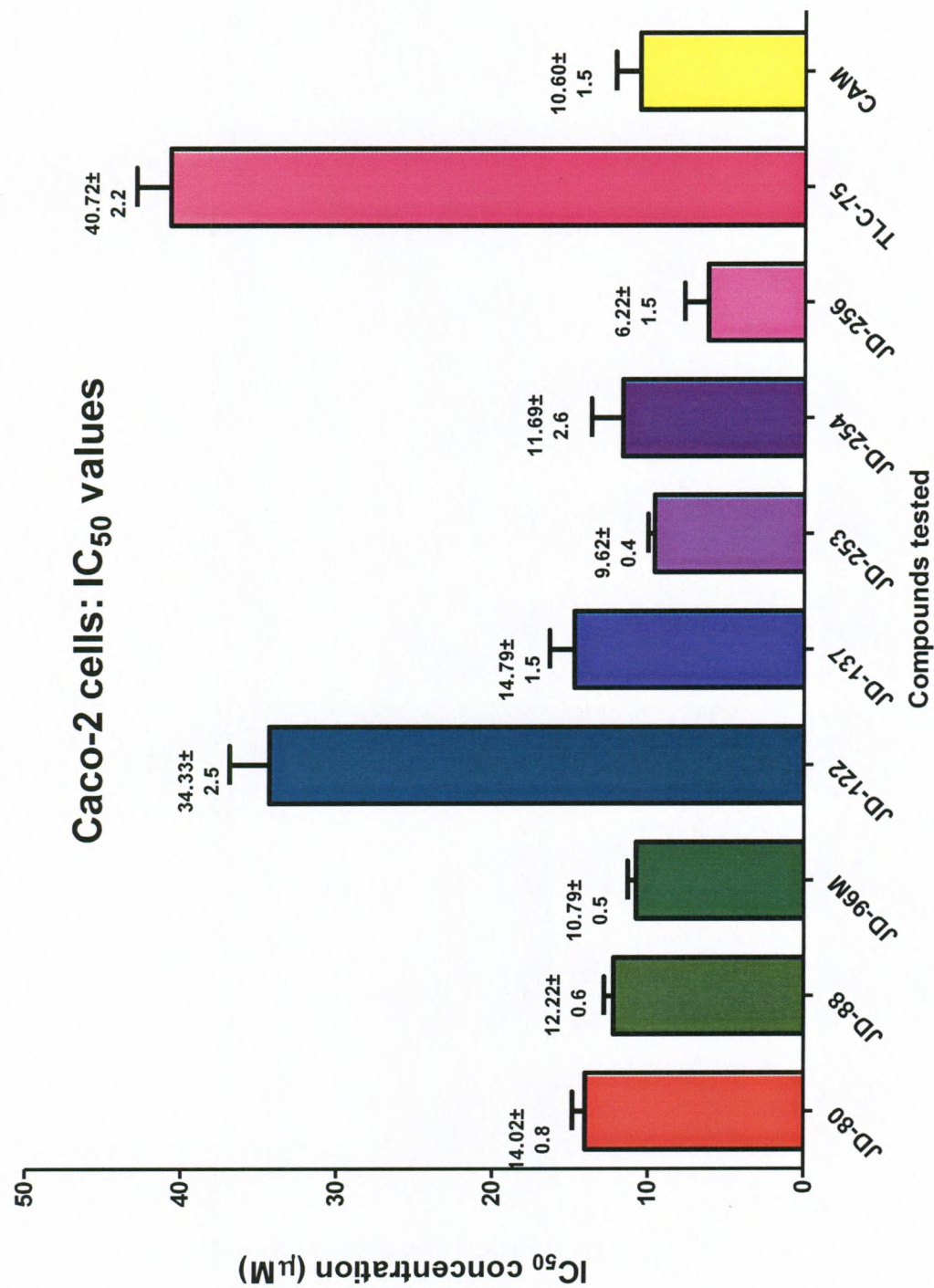


Figure 3.2. The IC₅₀ values for the test compounds and camptothecin on the Caco-2 cells. The Caco-2 cells were exposed to each compound for 48 hours. The IC₅₀ values of JD-80, JD-88 JD-122, JD-137 and TLC-75 were determined after exposing cells to concentration values of 0.5; 1; 10; 25, 50; 75; 100; 200 μM. The IC₅₀ values of JD-96M, JD-119, JD-253; JD-254 and JD-256 were determined after exposing cells to concentration values of 0.125; 0.25; 0.5; 1; 10; 25; 50; 100 μM. The bars represent the mean + SEM of the at least three IC₅₀ values obtained from three separate MTT assays.

3.1.2 Dose-response curves of active test compounds

IC₅₀ values of the active compounds were calculated from sigmoidal dose response curves constructed by using the data obtained from the MTT assay.

The dose-response curves indicated that some of the compounds showed a preferential selectivity to either HT-29 or the Caco-2 cell line.

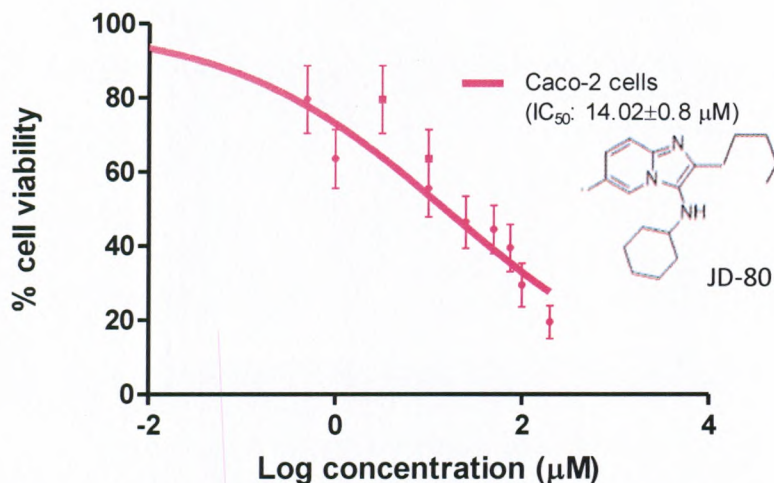


Figure 3.3A. Representative dose-response curves and the structure of test compound JD-80 on the Caco-2 cells. Cells were treated with concentration range of 0.5-200 μM for 48 hours. All experiments were repeated three times and each data point was obtained in quadruplicate. Each data point on the curve represents the mean and the standard error of the mean.

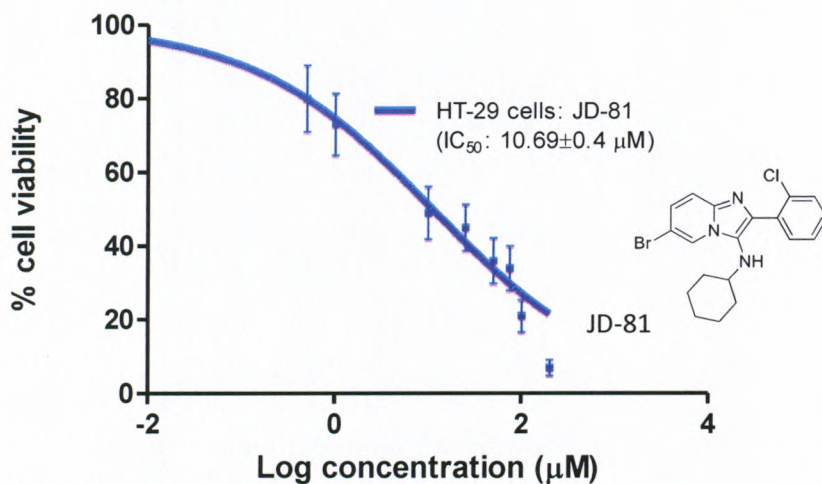


Figure 3.3B. Representative dose-response curves and the structure of test compound JD-81 on the HT-29. Cells were treated with concentration range of 0.5-200 μM for 48 hours. All experiments were repeated three times and each data point was obtained in quadruplicate. Each data point on the curve represents the mean and the standard error of the mean.

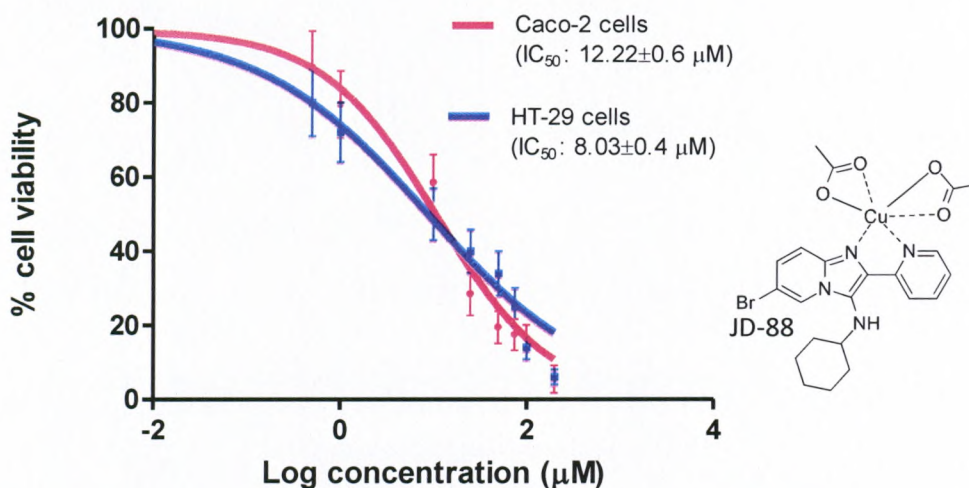


Figure 3.4. Representative dose-response curves and the structure of test compound JD-88 on HT-29 and Caco-2 cells. Cells were treated with a concentration range of 0.5-200 μM for 48 hours. All experiments were repeated three times and each data point was obtained in quadruplicate. Each data point on the curve represents the mean and the standard error of the mean.

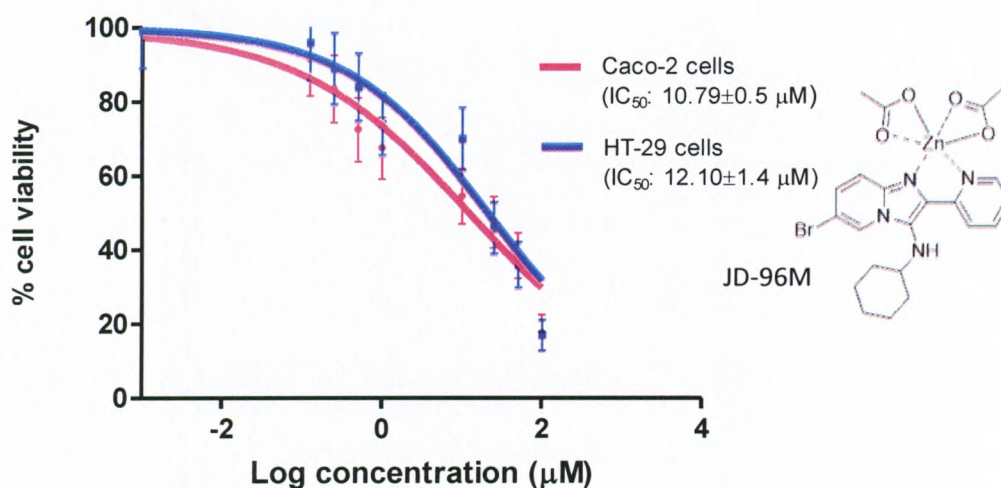


Figure 3.5. Representative dose-response curves and the structure of test compound JD-96M on HT-29 and Caco-2 cells. Cells were treated with a concentration range of 0.125-100 μM for 48 hours. All experiments were repeated three times and each data point was obtained in quadruplicate. Each data point on the curve represents the mean and the standard error of the mean.

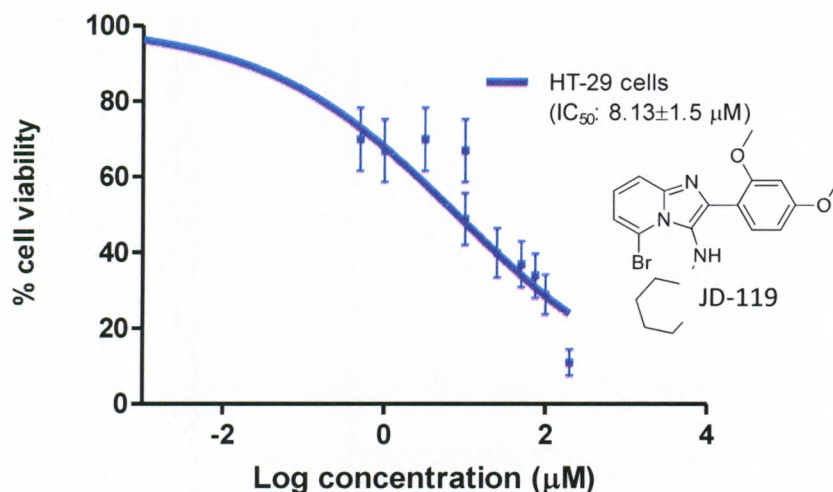


Figure 3.6. Representative dose-response curves and the structure of test compound JD-119 on HT-29. Cells were treated with a concentration range of 0.5- 200 μM for 48 hours. All experiments were repeated three times and each data point was obtained in quadruplicate. Each data point on the curve represents the mean and the standard error of the mean.

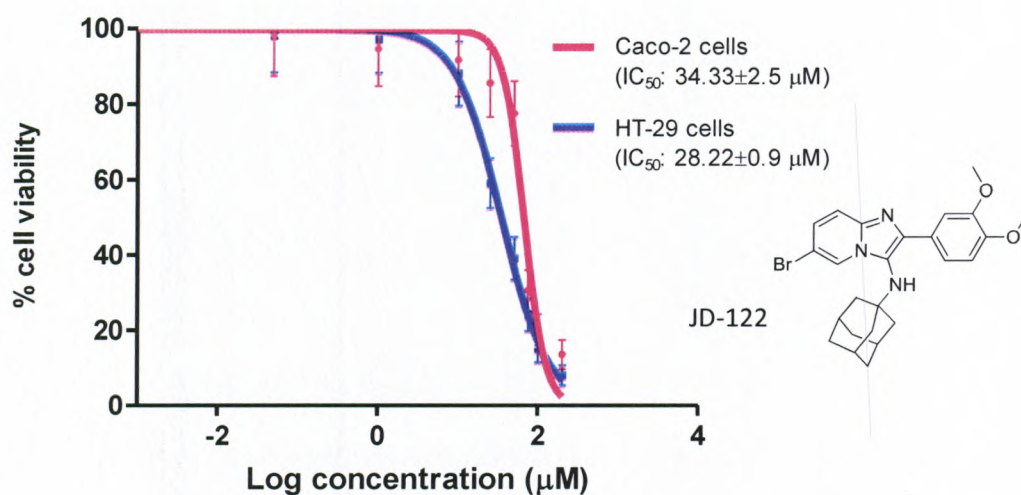


Figure 3.7. Representative dose-response curves and the structure of test compound JD-122 on HT-29 and Caco-2 cells. Cells were treated with a concentration range of 0.5- 200 μM for 48 hours. All experiments were repeated three times and each data point was obtained in quadruplicate. Each data point on the curve represents the mean and the standard error of the mean.

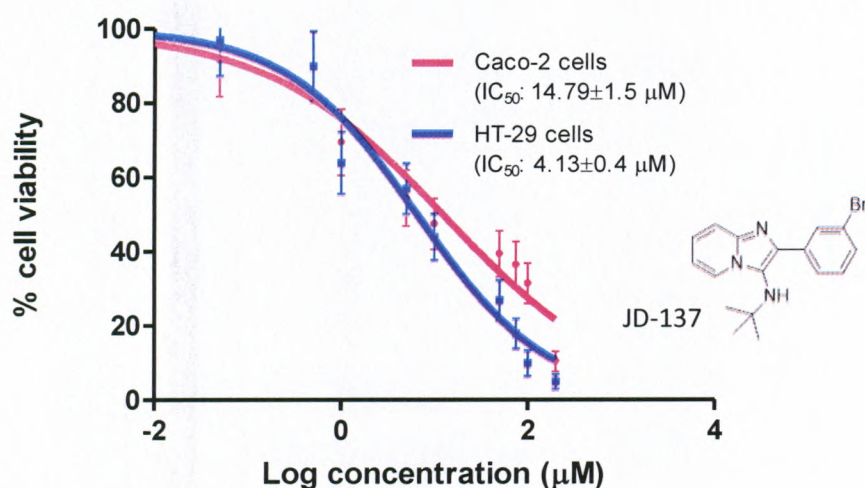


Figure 3.8. Representative dose-response curves and the structure of test compound JD-137 on HT-29 and Caco-2 cells. Cells were treated with a concentration range of 0.5- 200 μM for 48 hours. All experiments were repeated three times and each data point was obtained in quadruplicate. Each data point on the curve represents the mean and the standard error of the mean.

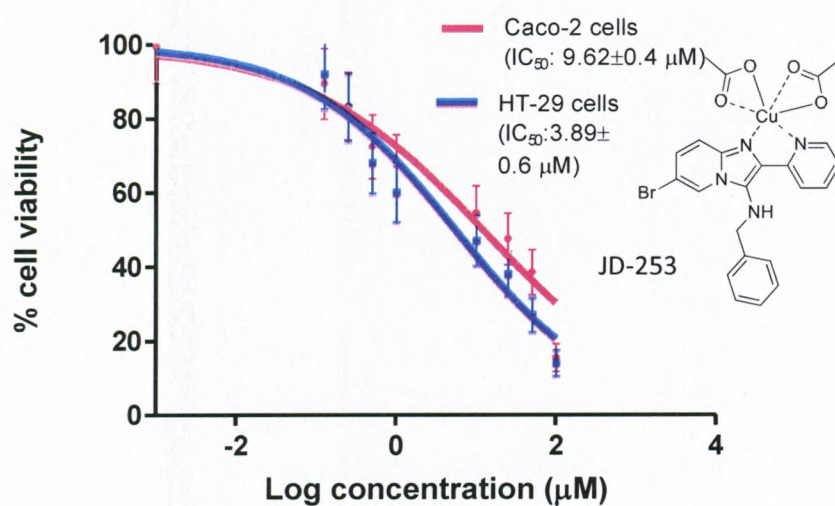


Figure 3.9. Representative dose-response curves and the structure of test compound JD-253 on HT-29 and Caco-2 cells. Cells were treated with a concentration range of 0.125- 200 μM for 48 hours. All experiments were repeated three times and each data point was obtained in quadruplicate. Each data point on the curve represents the mean and the standard error of the mean.

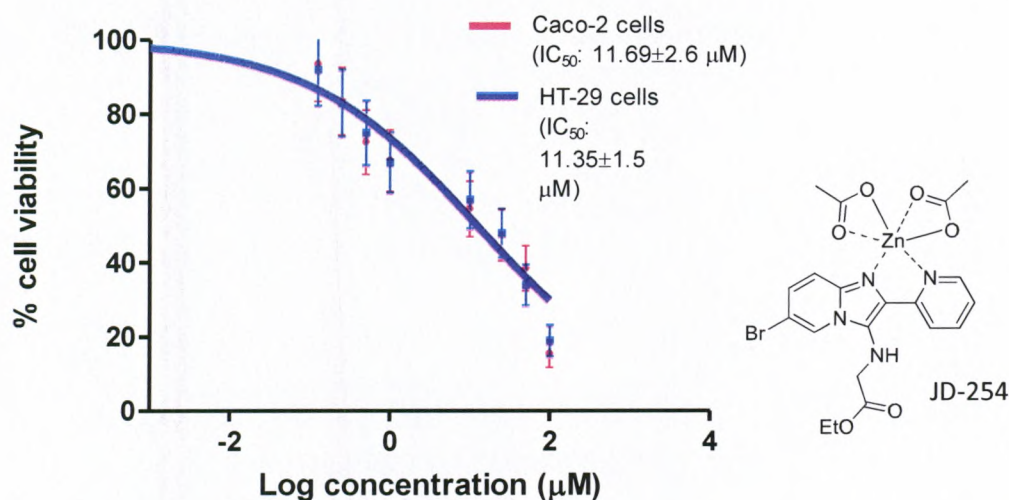


Figure 3.10. Representative dose-response curves and the structure of test compound JD-254 on HT-29 and Caco-2 cells. Cells were treated with a concentration range of 0.125-100 μM for 48 hours. All experiments were repeated three times and each data point was obtained in quadruplicate. Each data point on the curve represents the mean and the standard error of the mean

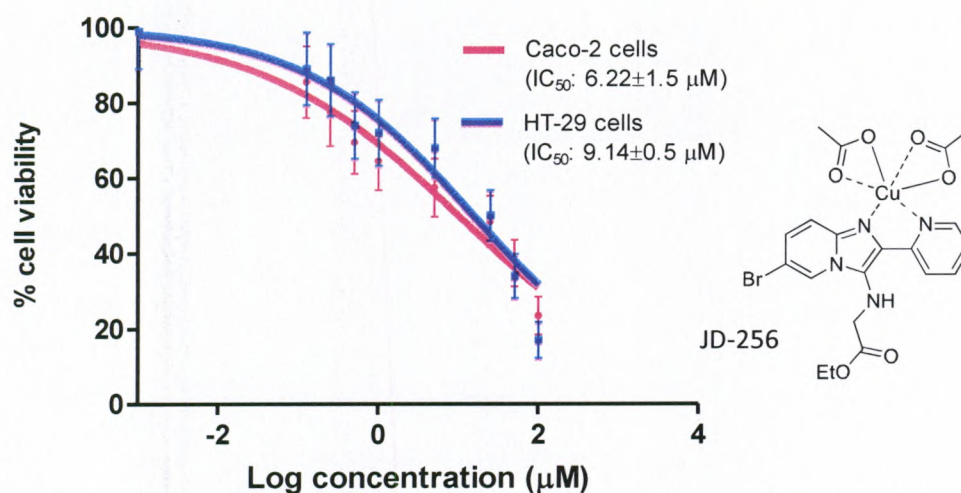


Figure 3.11. Representative dose-response curves and the structure of test compound JD-256 on HT-29 and Caco-2 cells. Cells were treated with a concentration range of 0.125-100 μM for 48 hours. All experiments were repeated three times and each data point was obtained in quadruplicate. Each data point on the curves represents the mean and the standard error of the mean.

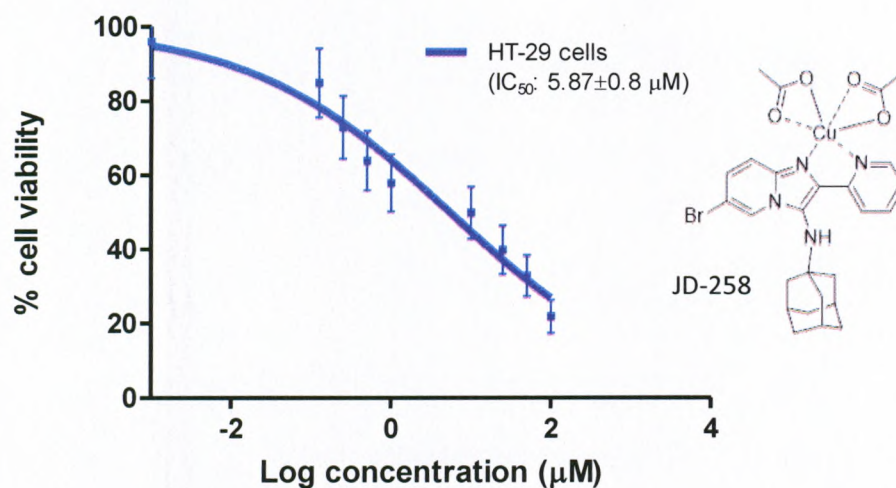


Figure 3.12. Representative dose-response curves and the structure of test compound JD-258 on HT-29 and Caco-2 cells. Cells were treated with a concentration range of 0.125-100 μM for 48 hours. All experiments were repeated three times and each data point was obtained in quadruplicate. Each data point on the curves represents the mean and the standard error of the mean.

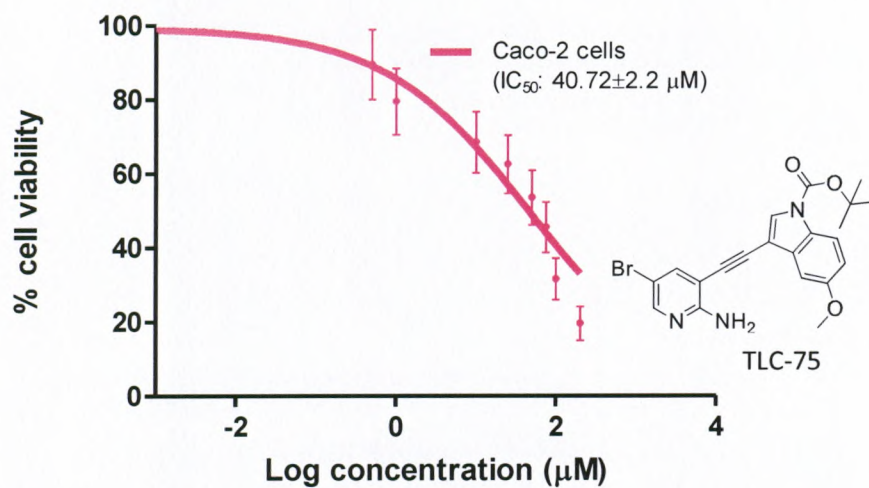


Figure 3.13. Representative dose-response curve and the structure of TLC-75 on Caco-2 cells. Cells were treated with a concentration range of 0.5-200 μM for 48 hours. All experiments were repeated three times and each data point was obtained in quadruplicate. Each data point on the curves represents the mean and the standard error of the mean.

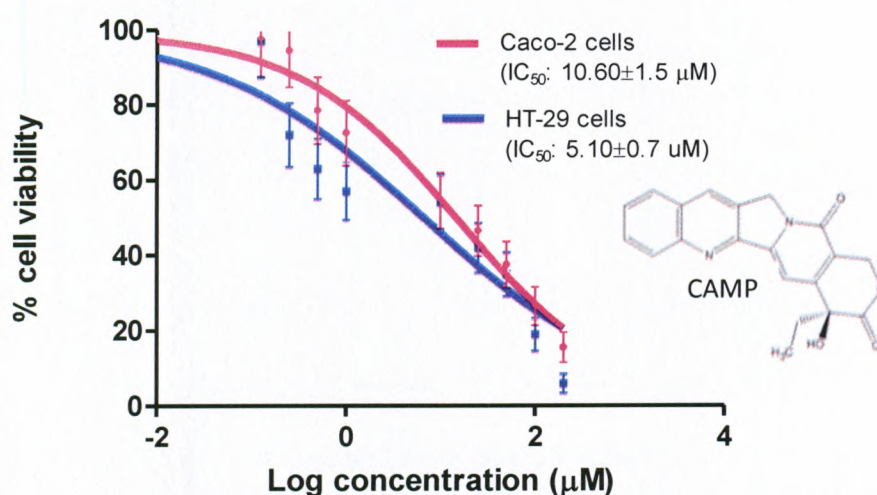


Figure 3.14. Representative dose-response curves and the structure of camptothecin (positive control) on HT-29 and Caco-2 cells. Cells were treated with a concentration range of 0.125- 200 μM for 48 hours. All experiments were repeated three times and each data point was obtained in quadruplicate. Each data point on the curves represents the mean and the standard error of the mean.

3.1.3 Statistical analysis of IC₅₀ values

ANOVA was used to determine if the differences between the average IC₅₀ values obtained for the test compounds were statistically significant between the two cell lines and compared to camptothecin. With a significance threshold of 0.05, the student's t-test, was used to determine if the IC₅₀ values for the HT-29 and Caco-2 cells were statistically equivalent to or better than camptothecin. IC₅₀ values were calculated from at least 3 independent experiments, all data points were done in quadruplicate. The IC₅₀ values of the active test compounds against the HT-29 cells showed that JD-137, JD-253, JD-258 and JD-119 were statistically equivalent in efficacy to camptothecin (Table 3.3)

Table 3.3. Statistical significance of the difference between the IC₅₀ values of the test compounds and camptothecin against the HT-29 cells.

Camptothecin (IC ₅₀ value) compared to the test compounds (IC ₅₀ values)	Two-tailed <i>p</i> value	Difference between IC ₅₀ values
JD-88	0.015	significant
JD-137	0.284	non-significant
JD-253	0.248	non-significant
JD-258	0.502	non-significant
JD-256	0.020	significant
JD-96M	0.011	significant
JD-254	0.009	significant
JD-119	0.133	non-significant
JD-122	0.001	significant
JD-81	0.002	significant

3.1.2 Dose-response curves of active test compounds

IC₅₀ values of the active compounds were calculated from sigmoidal dose response curves constructed by using the data obtained from the MTT assay.

The dose-response curves indicated that some of the compounds showed a preferential selectivity to either HT-29 or the Caco-2 cell line.

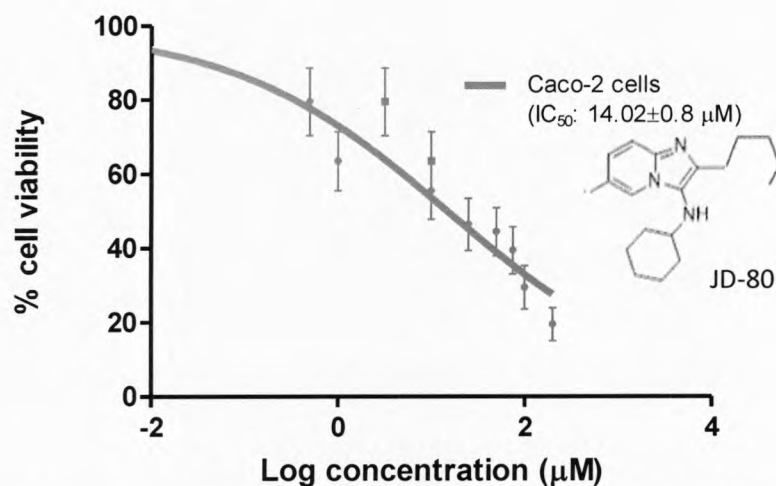


Figure 3.3A. Representative dose-response curves and the structure of test compound JD-80 on the Caco-2 cells. Cells were treated with concentration range of 0.5-200 μM for 48 hours. All experiments were repeated three times and each data point was obtained in quadruplicate. Each data point on the curve represents the mean and the standard error of the mean.

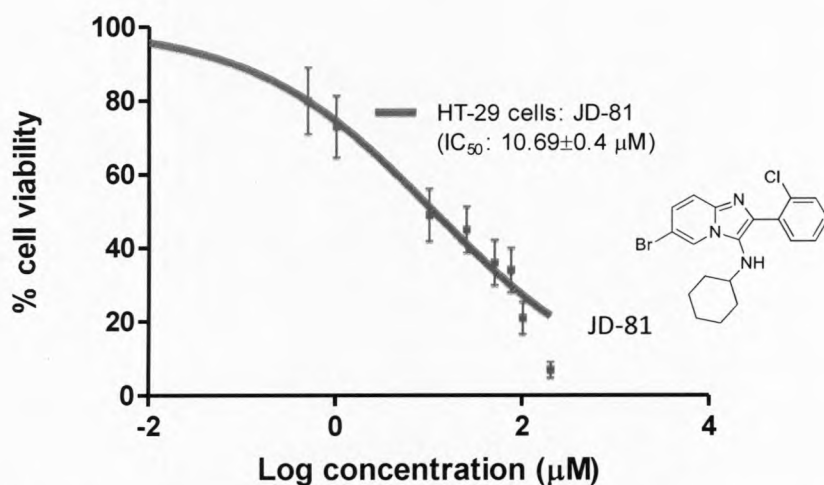


Figure 3.3B. Representative dose-response curves and the structure of test compound JD-81 on the HT-29. Cells were treated with concentration range of 0.5-200 μM for 48 hours. All experiments were repeated three times and each data point was obtained in quadruplicate. Each data point on the curve represents the mean and the standard error of the mean.

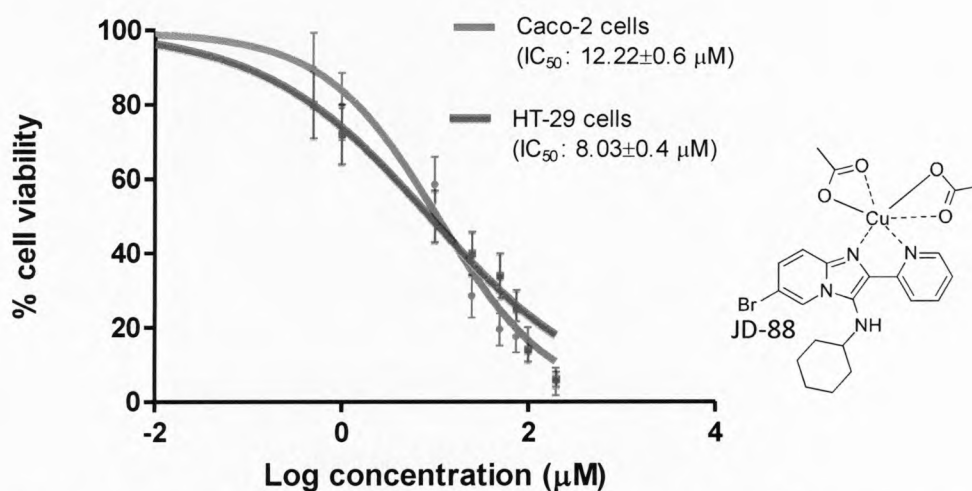


Figure 3.4. Representative dose-response curves and the structure of test compound JD-88 on HT-29 and Caco-2 cells. Cells were treated with a concentration range of 0.5-200 μM for 48 hours. All experiments were repeated three times and each data point was obtained in quadruplicate. Each data point on the curve represents the mean and the standard error of the mean.

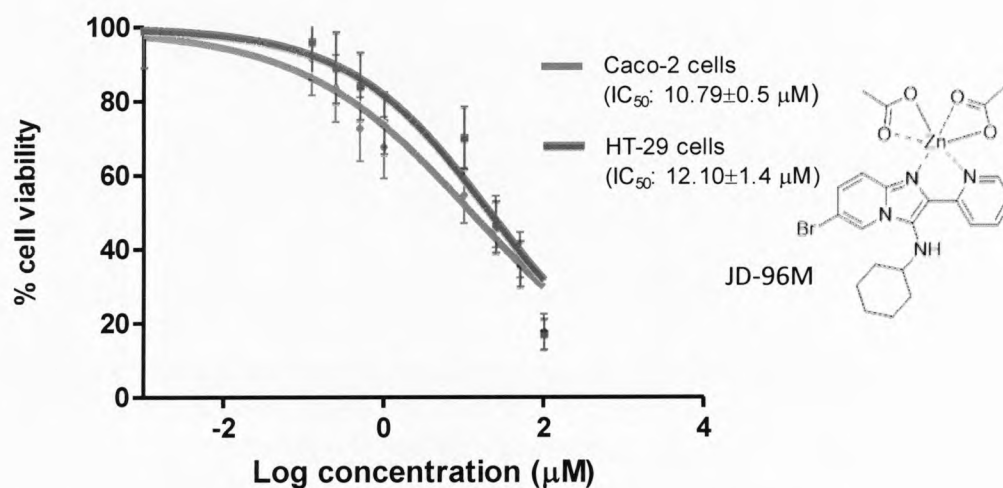


Figure 3.5. Representative dose-response curves and the structure of test compound JD-96M on HT-29 and Caco-2 cells. Cells were treated with a concentration range of 0.125-100 μM for 48 hours. All experiments were repeated three times and each data point was obtained in quadruplicate. Each data point on the curve represents the mean and the standard error of the mean.

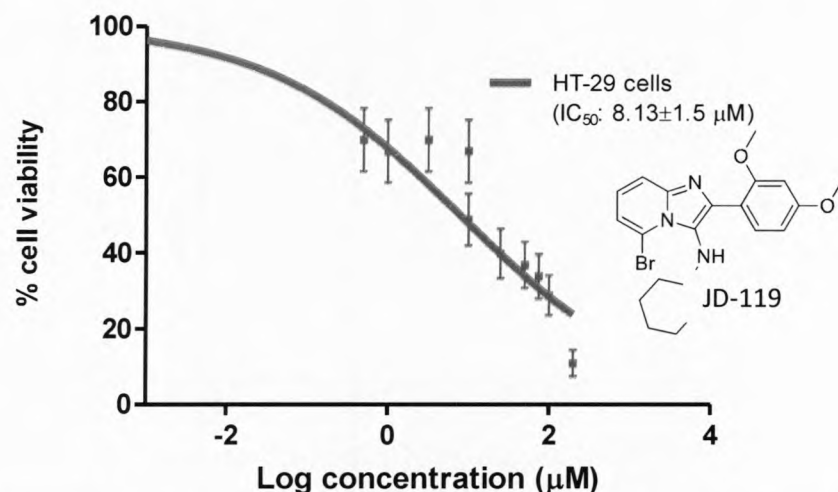


Figure 3.6. Representative dose-response curves and the structure of test compound JD-119 on HT-29. Cells were treated with a concentration range of 0.5- 200 μM for 48 hours. All experiments were repeated three times and each data point was obtained in quadruplicate. Each data point on the curve represents the mean and the standard error of the mean.

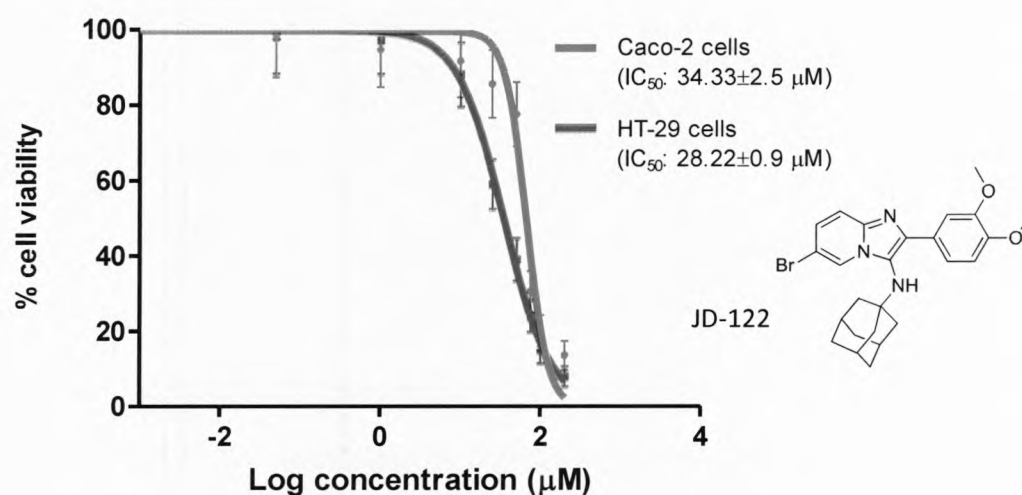


Figure 3.7. Representative dose-response curves and the structure of test compound JD-122 on HT-29 and Caco-2 cells. Cells were treated with a concentration range of 0.5- 200 μM for 48 hours. All experiments were repeated three times and each data point was obtained in quadruplicate. Each data point on the curve represents the mean and the standard error of the mean.

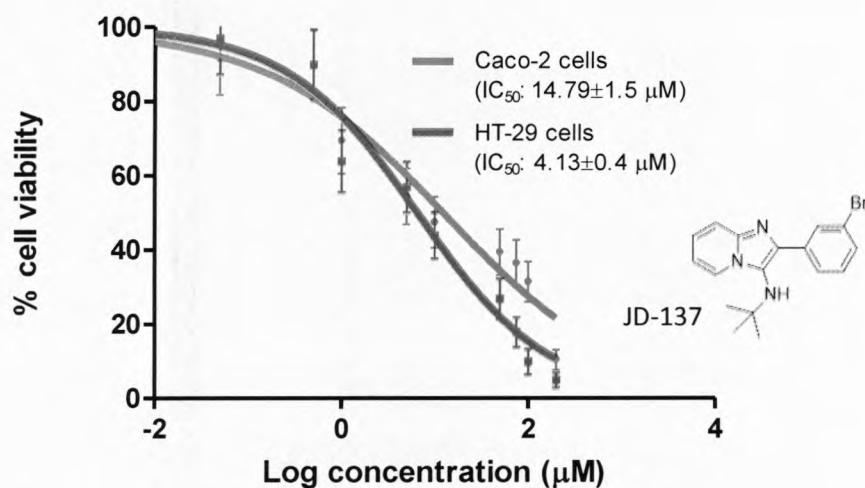


Figure 3.8. Representative dose-response curves and the structure of test compound JD-137 on HT-29 and Caco-2 cells. Cells were treated with a concentration range of 0.5- 200 μM for 48 hours. All experiments were repeated three times and each data point was obtained in quadruplicate. Each data point on the curve represents the mean and the standard error of the mean.

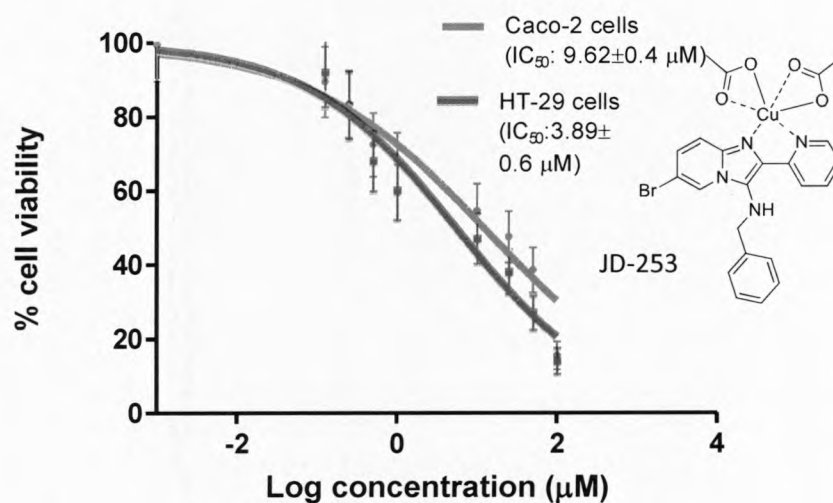


Figure 3.9. Representative dose-response curves and the structure of test compound JD-253 on HT-29 and Caco-2 cells. Cells were treated with a concentration range of 0.125- 200 μM for 48 hours. All experiments were repeated three times and each data point was obtained in quadruplicate. Each data point on the curve represents the mean and the standard error of the mean.

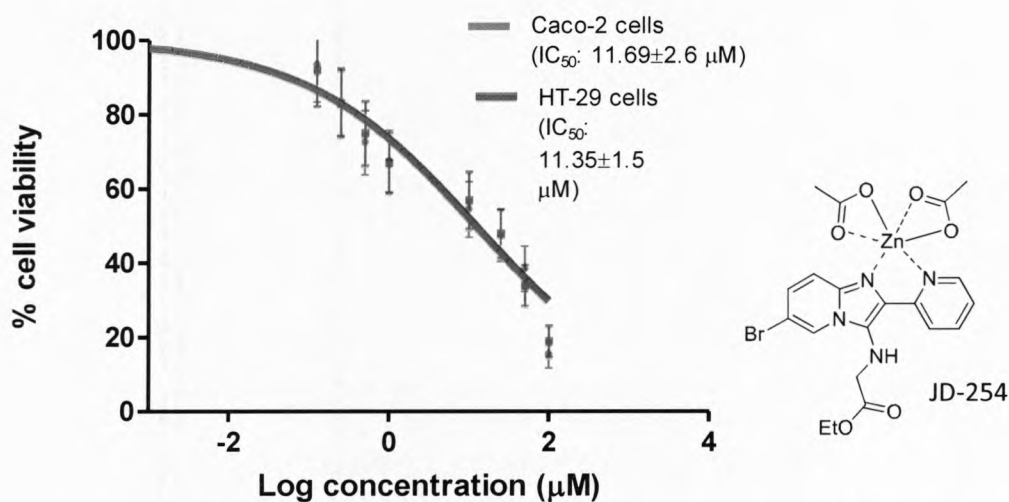


Figure 3.10. Representative dose-response curves and the structure of test compound JD-254 on HT-29 and Caco-2 cells. Cells were treated with a concentration range of 0.125-100 μM for 48 hours. All experiments were repeated three times and each data point was obtained in quadruplicate. Each data point on the curve represents the mean and the standard error of the mean

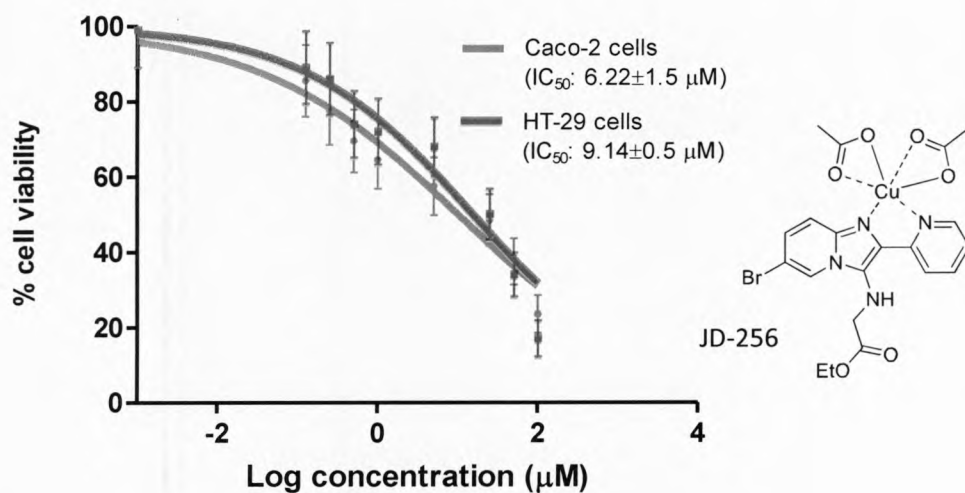


Figure 3.11. Representative dose-response curves and the structure of test compound JD-256 on HT-29 and Caco-2 cells. Cells were treated with a concentration range of 0.125-100 μM for 48 hours. All experiments were repeated three times and each data point was obtained in quadruplicate. Each data point on the curves represents the mean and the standard error of the mean.

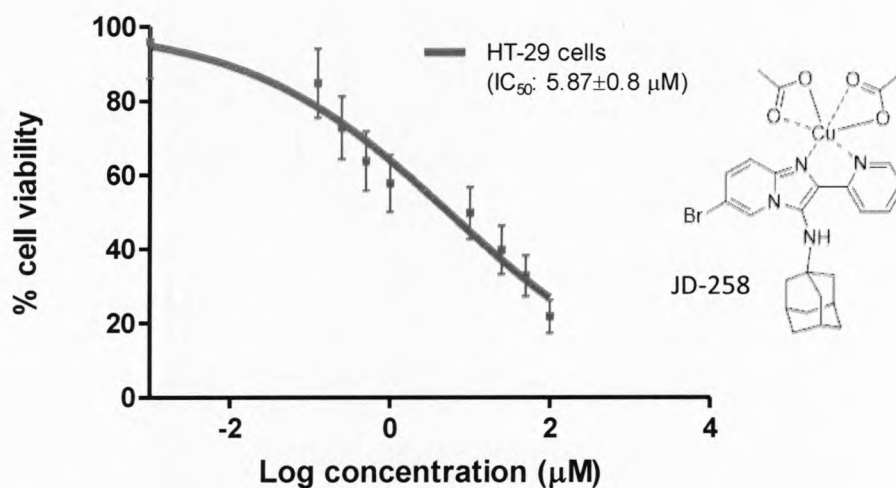


Figure 3.12. Representative dose-response curves and the structure of test compound JD-258 on HT-29 and Caco-2 cells. Cells were treated with a concentration range of 0.125-100 μM for 48 hours. All experiments were repeated three times and each data point was obtained in quadruplicate. Each data point on the curves represents the mean and the standard error of the mean.

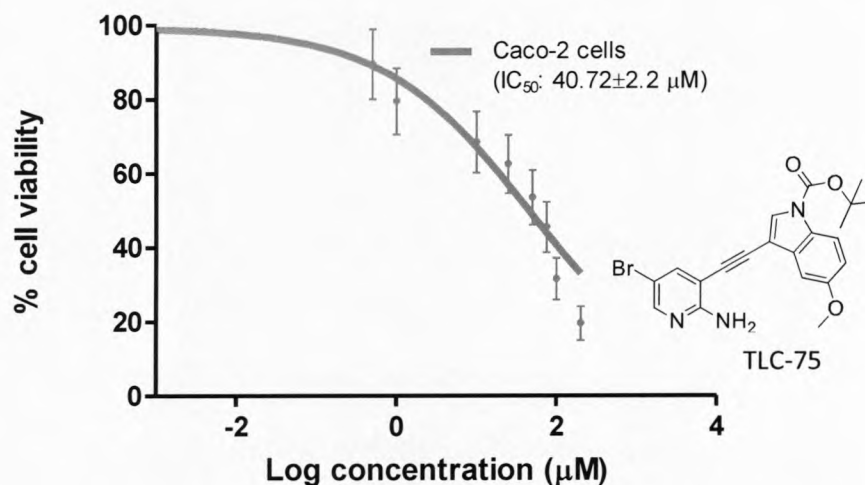


Figure 3.13. Representative dose-response curve and the structure of TLC-75 on Caco-2 cells. Cells were treated with a concentration range of 0.5-200 μM for 48 hours. All experiments were repeated three times and each data point was obtained in quadruplicate. Each data point on the curves represents the mean and the standard error of the mean.

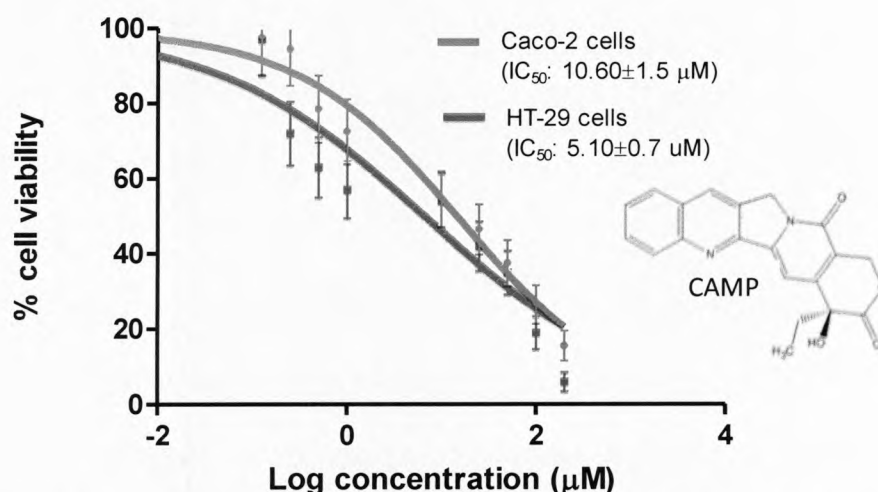


Figure 3.14. Representative dose-response curves and the structure of camptothecin (positive control) on HT-29 and Caco-2 cells. Cells were treated with a concentration range of 0.125- 200 μM for 48 hours. All experiments were repeated three times and each data point was obtained in quadruplicate. Each data point on the curves represents the mean and the standard error of the mean.

3.1.3 Statistical analysis of IC₅₀ values

ANOVA was used to determine if the differences between the average IC₅₀ values obtained for the test compounds were statistically significant between the two cell lines and compared to camptothecin. With a significance threshold of 0.05, the student's t-test, was used to determine if the IC₅₀ values for the HT-29 and Caco-2 cells were statistically equivalent to or better than camptothecin. IC₅₀ values were calculated from at least 3 independent experiments, all data points were done in quadruplicate. The IC₅₀ values of the active test compounds against the HT-29 cells showed that JD-137, JD-253, JD-258 and JD-119 were statistically equivalent in efficacy to camptothecin (Table 3.3)

Table 3.3. Statistical significance of the difference between the IC₅₀ values of the test compounds and camptothecin against the HT-29 cells.

Camptothecin (IC ₅₀ value) compared to the test compounds (IC ₅₀ values)	Two-tailed <i>p</i> value	Difference between IC ₅₀ values
JD-88	0.015	significant
JD-137	0.284	non-significant
JD-253	0.248	non-significant
JD-258	0.502	non-significant
JD-256	0.020	significant
JD-96M	0.011	significant
JD-254	0.009	significant
JD-119	0.133	non-significant
JD-122	0.001	significant
JD-81	0.002	significant

The IC₅₀ values of the active compound against Caco-2 cells showed that JD-88 JD-137, JD-253, JD-96M, JD-81 and JD-256 were statistically equivalent in efficacy when compared to camptothecin (Table 3.4)

Table 3.4. Statistical significance of the difference between the IC₅₀ values of the test compounds and camptothecin against the Caco-2 cells.

Camptothecin (IC ₅₀ value) compared to the test compounds (IC ₅₀ values)	Two-tailed <i>p</i> value	Difference between IC ₅₀ values
JD-88	0.388	non-significant
JD-119	0.004	significant
JD-137	0.135	non-significant
JD-122	0.011	significant
JD-96M	0.913	non-significant
JD-253	0.578	non-significant
JD-256	0.115	non-significant
JD-81	0.691	non-significant
JD-75	0.004	significant

The IC₅₀ values obtained for HT-29 cells were also compared to the IC₅₀ values obtained for Caco-2 cells (Table 3.5). When IC₅₀ values of the active test compounds against the HT-29 cells were compared to the IC₅₀ values of the active test compounds against the Caco-2 cells, the IC₅₀ values of JD-256, JD-96M and JD-81 were statistically equivalent. There was a significant difference in IC₅₀ values when JD-88, JD-253, JD-137 and camptothecin were compared.

Table 3.5. Statistical significance of the difference between the IC₅₀ values of the test compounds against the HT-29 cells vs IC₅₀ values of the test compounds against the Caco-2 cells.

IC ₅₀ values in the HT-29 cells compared to the Caco-2 cells	Two-tailed <i>p</i> value	Difference between IC ₅₀ values
JD-88	0.006	significant
JD-253	0.002	significant
JD-137	0.003	significant
JD-256	0.076	non-significant
JD-96M	0.435	non-significant
JD-81	0.654	non-significant
Camptothecin	0.032	significant

3.1.4 Leucocyte toxicity of the test compounds

The effect of the test compounds on leucocyte viability was performed to determine the cytotoxic potential of the most active test compounds to normal, unstimulated white blood cells as opposed to the cancer cells. Leucocyte viability was evaluated by the MTT assay after the leucocytes were exposed to the test compounds and well as camptothecin at a concentration of 10 μ M for 24 hours. The assay was repeated three times and the results are summarized in Figure 3.15.

Unstimulated normal peripheral white blood cells do not survive *in vivo* for more than 36 hours therefore this assay was carried out at 24 hrs. Camptothecin was observed to be more toxic to the leucocytes than four of the compounds tested (JD-81, JD-96M, JD-137 and JD-254). Treatment with 10 μ M of camptothecin decreased the leucocyte viability to $52.94 \pm 2.9\%$ after 24 hrs. After exposure to JD-256 ($49.38 \pm 0.6\%$) the percentage leucocyte viability was similar to that of camptothecin. Of all the tested compounds JD-258 was the most toxic to the leucocytes with a percentage viability of $29.32 \pm 0.4\%$ followed by JD-253 and JD-88 with viabilities of 32.6 and 37.4% respectively. From the compounds tested JD-254 proved to be the least toxic to the leucocytes with a percentage viability of $85.49 \pm 0.7\%$.

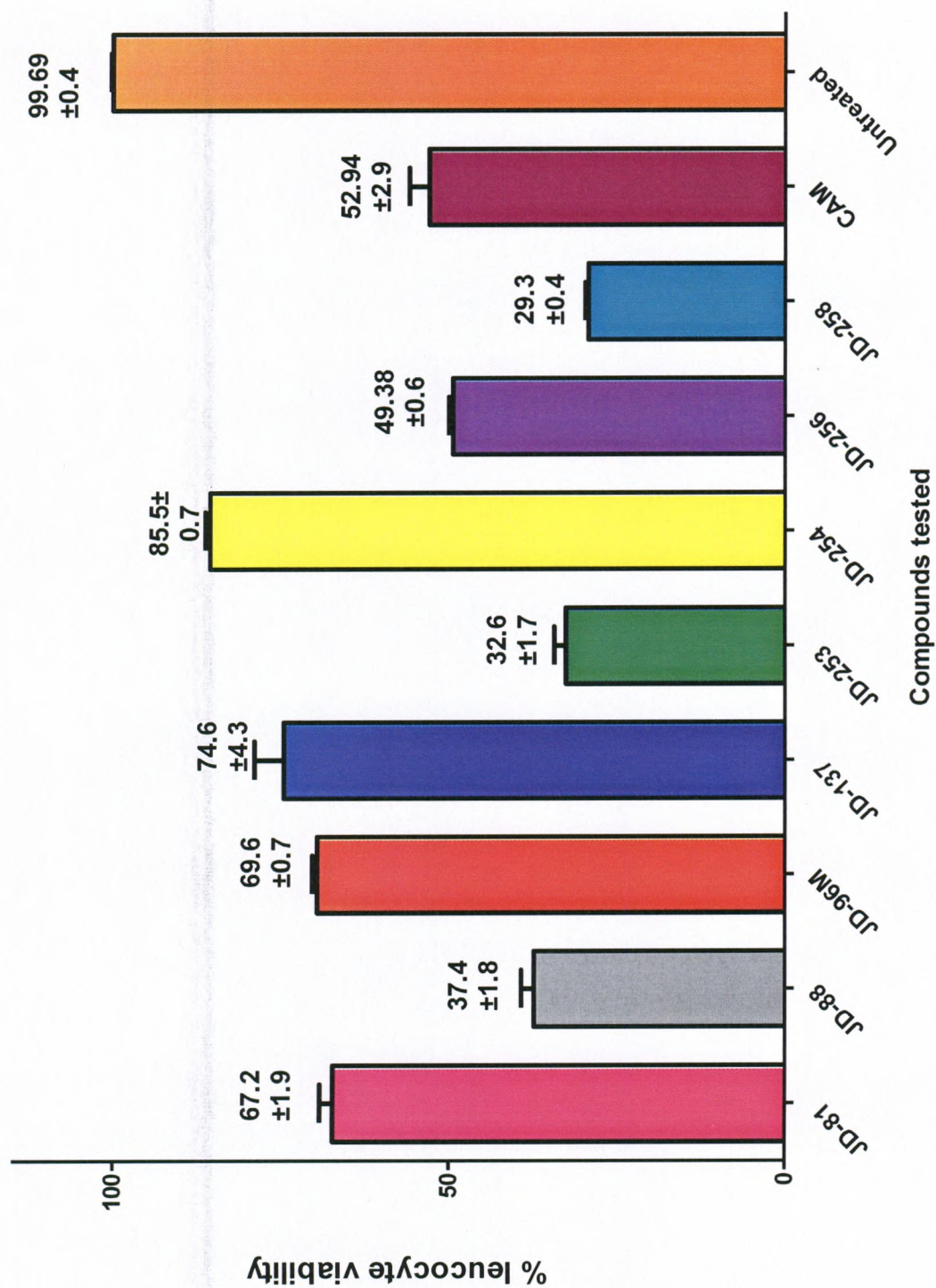


Figure 3.15. Percentage viability of leucocytes after exposure to the active compounds and Camptothecin. Leucocytes were exposed to each compound at a concentration of 10 µM for 24 hours. The bars represent the mean ± SEM of the % leucocyte survival from three separate MTT assays.

3.2 Assessment of apoptosis

3.2.1 Effects of the test compounds on cell morphology

3.2.1.1 Time-course studies of active compounds

Cells were treated with the four most active compounds and were observed after 8, 18, 24 and 48 hours using the Olympus DP21 inverted phase-contrast microscope. Photomicrographs were recorded with an Olympus digital camera. Treated cells were examined for morphological changes relating to cell size, cell membrane integrity and nuclear and cytoplasmic morphology (Figure 3.16 and Figure 3.17).

HT-29 cells

The HT-29 cells were microscopically examined to determine how the morphology of the cells was affected after exposing them to the active test compounds at a concentration of JD-137 (10 μ M), JD-258 (15 μ M) and JD-253 (10 μ M), JD-88 (20 μ M) and camptothecin (15 μ M). Photomicrographs showing the effects on the cells are shown in Figure 3.16.

After an eight hour exposure period to JD-88, JD-137, JD-253, JD-258, and camptothecin, HT-29 cells started dissociate from neighbouring cells. Fewer cells were observed compared to the control (Figure 3.16). Cells appeared spread out except for JD-253 where cells were dense and appeared granular with poorly defined nuclei.

At 18 hours post-exposure, camptothecin treated cells assumed round structures and displayed spindly projections (filopodia). At 18 hours post-exposure, HT-29 cells treated with JD-88, JD-137 and JD-253 appeared granular and cell debris was observed. These cells had cellular shapes with a different morphology compared to the untreated cells. At 18 hours post exposure cells treated with JD-88 and JD-137 detached from the culture surface and formed unusual multi-lobular structures. Cells exposed to JD-88 displayed filopodia. After exposure to JD-137 cells appear multinucleated and appear larger. After exposure to JD-258, HT-29 cells appeared round in shape and appeared swollen.

After 24 hours, exposure to camptothecin, JD-137, JD-253 and JD-258 all cells were round in shape, smaller in size and cell debris was visible. After exposure to JD-258, cells showed signs of membrane blebbing and normal morphology of the cells was lost. After exposure to JD-137, JD-88 and JD-258 vacuoles were present

After 48 hours, HT-29 cells exposed to JD-88, JD-137, JD-253 and JD-258 were round in shape and cells were detached from the surface.

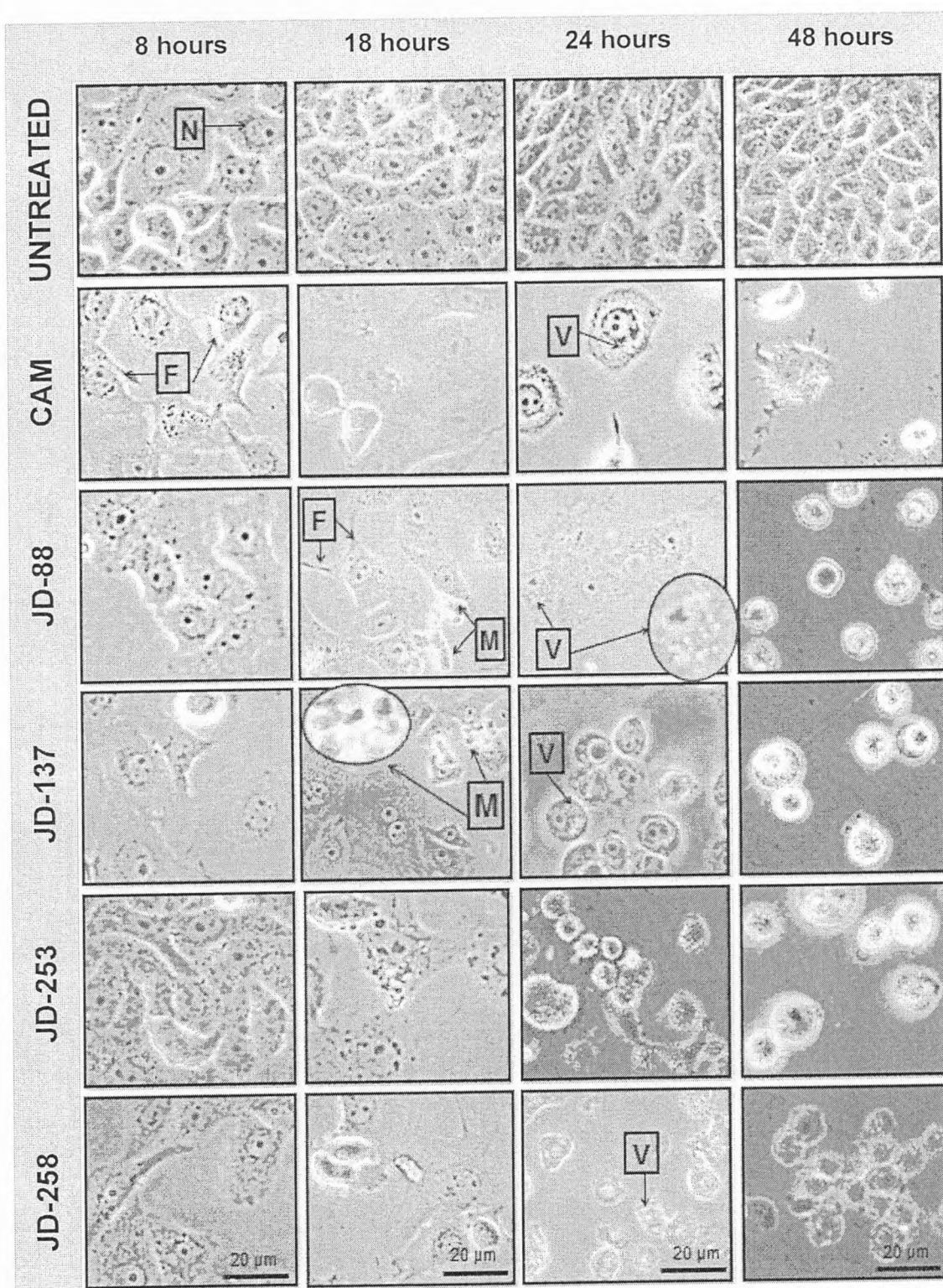


Figure 3.16. The morphology of untreated HT-29 cells compared to treated cells. HT-29 cells were exposed to concentration values of camptothecin (15 μ M), JD-88 (20 μ M), JD-137 (10 μ M), JD-253 (10 μ M) and JD-258 (15 μ M). Cells were treated with the compounds for an 8, 18, 24 and 48 hour exposure periods and were observed using a 400X magnification.
 LEGEND: N- nucleus, V- vacuoles, M- Multi-lobular structures; F- Filopodia; Scale bar: 20 μ M.

Caco-2 cells

The Caco-2 cells were microscopically examined to evaluate the effect of the active compounds on the morphology of the cells (Figure 3.17). Caco-2 cells were exposed to compounds JD-256 (20 μ M), JD-88 (35 μ M), JD-96M (30 μ M) and JD-253 (30 μ M) and camptothecin (30 μ M) and a series of photomicrographs were taken at various time intervals (Figure 3.17).

After eight hours of exposure to the active compounds and camptothecin there was a decrease in cell number as seen in Figure 3.17.

Camptothecin treated cells showed normal cell morphology at 8 hours but at 18 hours cells were enlarged and developed abnormal cell morphology. At 24 and 48 hours post exposure treated camptothecin cells detached from the cells surface and were rounded up.

JD-96M caused some cells to assume an elongated morphology with a small shrunken nucleus. This was followed by a complete loss of normal morphology at 18 hours with cells taking on a more rounded appearance with indistinct nuclei. At 24 hours the cells appeared smaller and at 48 hours only a few cells remained attached to the growth surface. The cells also showed major changes to the structure of the membrane at 24 and 48 hours.

After 8 hours the normal cell morphology was lost and cells began to form filopodia after treatment with JD-88. At 18 hours and 24 hours post-exposure JD-88 treated cells appeared enlarged. After 48 hours cells treated with JD-88 were detached from the cell surface and were rounded up. Elongated cells were visible with exposure to JD-253 at 18 hours and extensive vacuole formation was observed at 24 hours. The cells now had a more rounded appearance and they detached completely after 48 hours.

JD-256 treated cells showed normal morphology at 8 hours but at 18 hours the cells formed filopodia and rounded up but remaining attached to the culture surface by their filopodia. After 24 hours detachment from the cell surface continued and at 48 hours most of the cells were rounded up and detached. At 48 hours vacuoles were present.

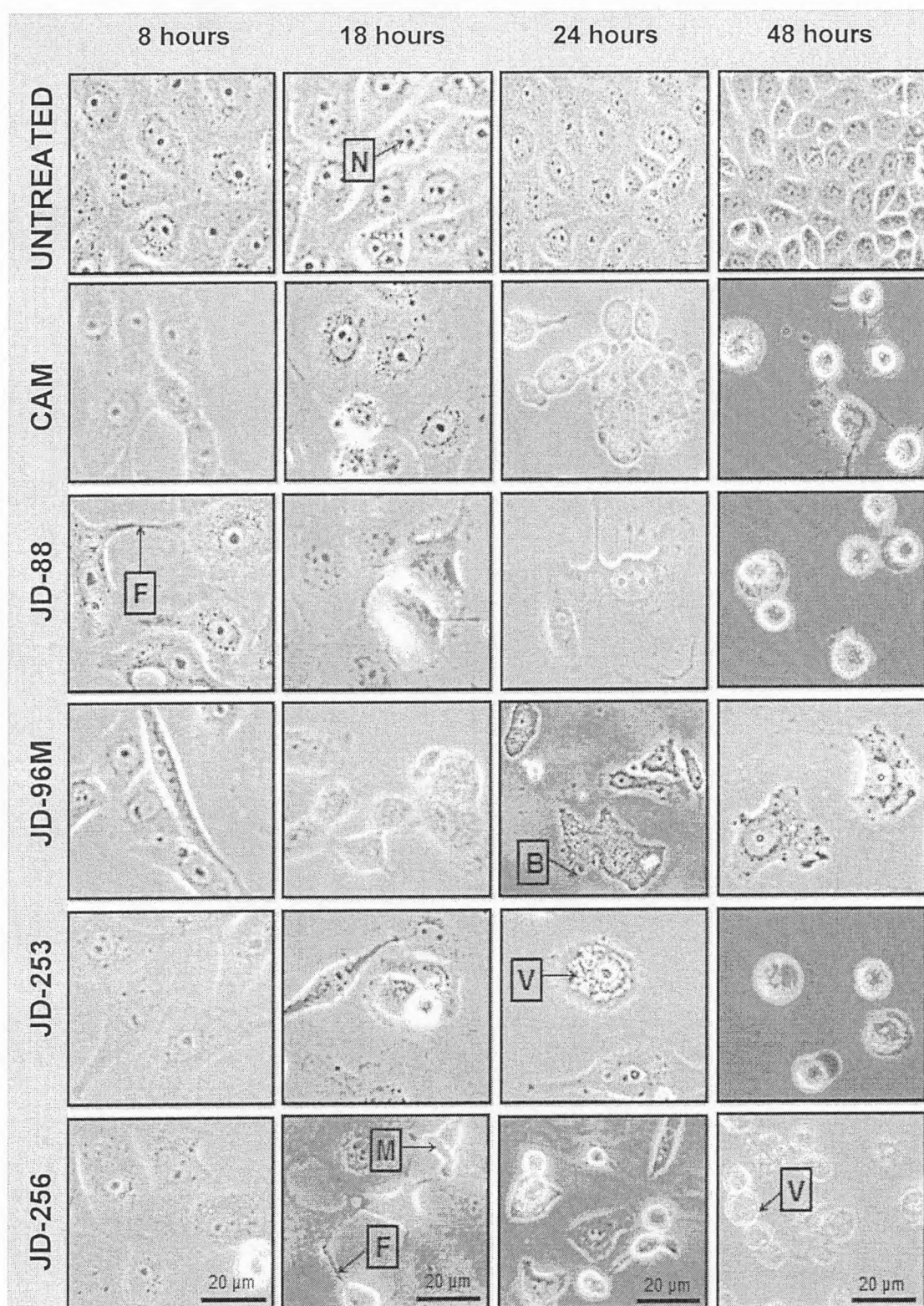


Figure 3.17. The morphology of untreated Caco-2 cells compared to treated cells. Caco-2 cells were exposed to concentration values of camptothecin (30 μ M), JD-88 (35 μ M), JD-96M (30 μ M) and JD-253 (30 μ M) and JD-256 (20 μ M). Cells were treated with the compounds for an 8, 18, 24 and 48 hour exposure periods and were observed using a 400X magnification.

LEGEND: N- nucleus, V- vacuoles, F- filopodia, B- blebbing, M- Multi-lobular structures;
Scale bar: 20 μ M.

3.2.1.2 Fluorescence microscopy

Acridine orange (AO), ethidium bromide (EB) and Hoechst-33342 (HO) were used in combination to further evaluate the morphological changes and effects on subcellular organelles of cells exposed to the four active test compounds. AO is permeable dye that penetrates the cell membrane and stains the nuclei green. Ethidium bromide can only penetrate cells that have lost their membrane integrity and stain their nuclei red. The nuclear DNA of live cells is visualized by staining cells with the DNA-specific dye Hoechst-33342. HT-29 and Caco-2 cells were exposed to the active compounds for a 24 hour period with a concentration corresponding to the IC_{50} concentration of each compound.

HT-29 cells

Cells (Figure 3.18) treated with the active compounds, JD-88, JD-137, JD-253, and JD-258 at a concentration of 8, 4, 3 and 5 μ M respectively showed differences in cellular morphology when compared to untreated cells. Morphological features observed include, irregular shaped nuclei, blebbing, lysosomal aggregation and changes in cell shape and size. The photomicrographs are presented in two sections; Figure 3.18A (control, camptothecin and JD-88) and 3.18B (JD-137, JD-253 and JD-258).

Cells exposed to camptothecin showed cellular stress features, which include rounding of the cells, reduction in size, apoptotic nuclei, lysosomal aggregation around the nuclei, vacuole formation as well as surface granulation (Figure 3.18A, Image E).

AO staining of camptothecin treated cells revealed irregular shaped nuclei and lysosomal aggregation around the nucleus (Figure 3.18A, Image F). AO staining of camptothecin treated cells also stained the nucleus an orange/ yellow colour indicative of pre-apoptotic changes (Mpoke and Wolfe, 1997) (Figure 3.18A, Image F). HO and AO-EB staining revealed irregular shaped nuclei and lysosomal aggregation around the nucleus (Figure 3.18A, Image G).

HT-29 cells were smaller in size after exposure to JD-88. Normal cell shape and cell surface attachments were lost in these cells resulting in round shaped cells with no tight cellular junctions with neighbouring cells (Figure 3.18A, Image I). Vacuoles were also visible (Figure 3.18A, Image I). Staining with AO/EB showed an aggregation of red stained lysosomes around the cell periphery and also stained the nucleus an orange/ yellow colour indicative of pre-apoptotic changes (Figure 3.18A, Image J). Staining with HO revealed irregular shaped nuclei after treatment with JD-88 (Figure 3.18A, Image K).

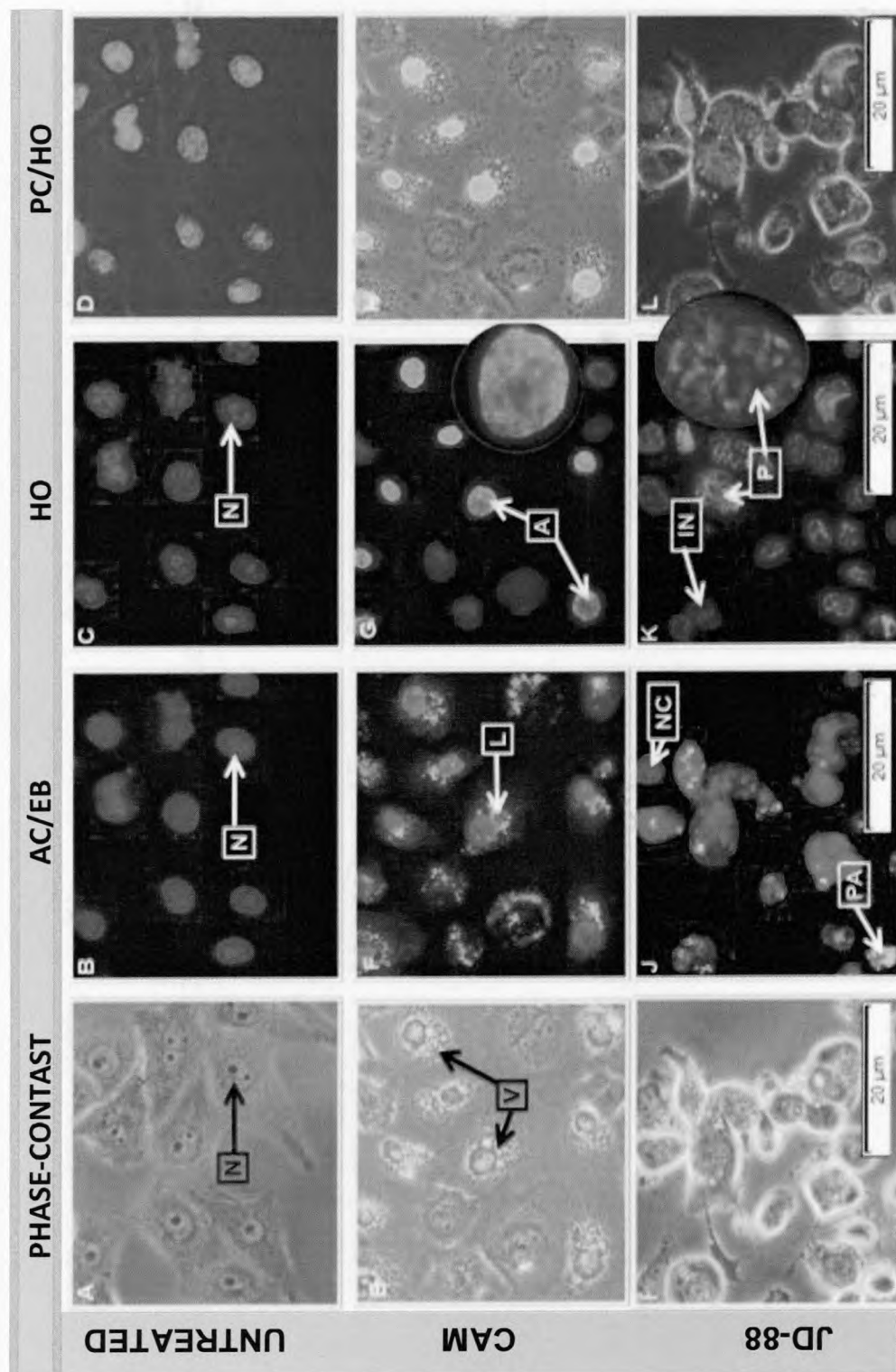


Figure 3.18A. HT-29 cells treated with the IC_{50} concentration of active test compounds for 24 hours. Cells were exposed to camptothecin (5 μ M) and JD-88 (8 μ M). Cells were observed at a 400X magnification.

LEGEND: L= lysosomes; N= nucleus; IN= irregular nucleus; NC= necrotic cell; B=blebbing; V=Vacuoles; PA= pre-apoptotic nucleus P= punctate nucleus
Scale bar: 20 μ m

Cells treated with JD-137 were smaller in size compared to the untreated cells and normal cell shape was lost in these cells (Figure 3.18B Image M). These cells were round with a loss of cell surface attachments and there were also no visible cell-cell junctions with neighbouring cells (Figure 3.18B, Image M). Vacuoles were also visible on the periphery of the cells (Figure 3.18B, Image M). After staining with EB/AO most cells nuclei appear to be smaller and have lost their normal ellipsoid shape. There is also lysosomal aggregation that appears to be found on one side of the cells (Figure 3.18B, Image N). As seen with the HO stain, treated JD-137 cells have lost the normal cell morphology (Figure 3.18B, Image O).

After treating cells to JD-253, cells were round in shape and contracted compared to the untreated cells. Some of the cells appeared to develop elongated cell processes (Figure 3.18B, Image Q). After staining with EB/AO aggregated lysosomes were visible around the nucleus of the cell and some cells stained an orange-yellow colour which is indicative for pre-apoptotic changes (Figure 3.18B, images R). The nucleus of one of the cells was fragmented as revealed by staining with HO (Figure 3.18B, Image S).

HT-29 cells were smaller in size after exposure to JD-258. Normal cell shape and cell surface attachments were lost in these cells resulting cells with no tight cellular junctions with neighbouring cells (Figure 3.18B, Image U). Membrane blebbing was also present and the cytoplasm had a granular appearance (Figure 3.18B, Image U). Staining with AO/EB showed red stained lysosomes (Figure 3.18B, Image V) and the Hoechst stain revealed that the nucleus was similar in shape and appearance to the control cells (Figure 3.18B, Image W).

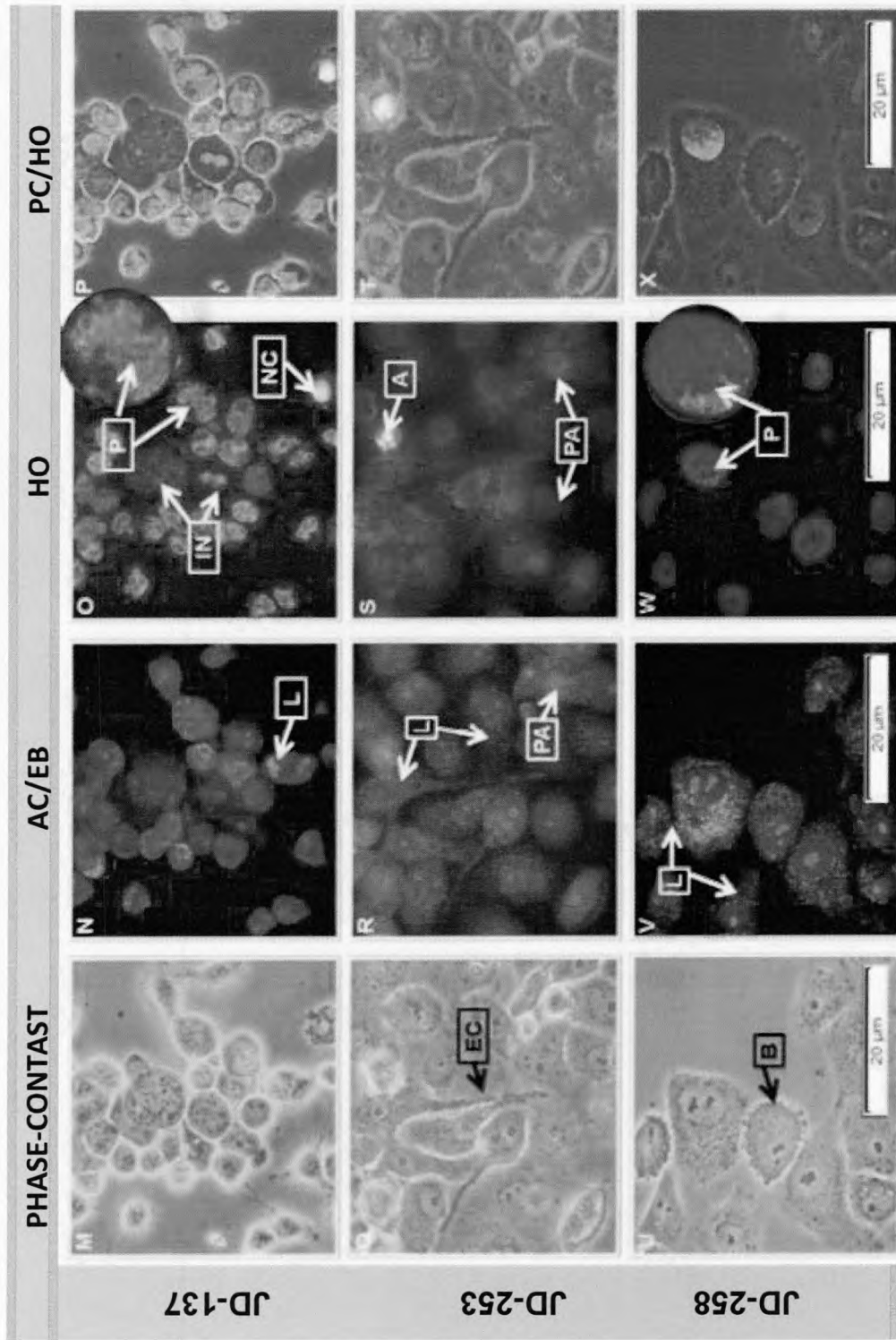


Figure 3.18B. HT-29 cells treated with the IC_{50} concentration of active test compounds for 24 hours. Cells were exposed to JD-137 (4 μ M), JD-253 (3 μ M) and JD-258 (5 μ M). Cells were observed at a 400X magnification.
LEGEND: L= lysosomes; EC= elongated cell; A= apoptotic nucleus; B=blebbing; PA=pre-apoptotic nucleus; P= punctate nucleus; Scale bar: 20 μ m.

The HT-29 cells (50 cells) were evaluated to determine the effect of the test compounds on the size of the nuclei of cells. After exposing HT-29 cells to JD-8, 31 out of the 50 cells had a nuclei diameter of more than 20 μM . Majority of the cells treated with JD-253 had a diameter that was between 10-20 μM . Of all the compounds that were evaluated JD-137 proved to decrease the size of the nuclei as most of the nuclei were less than 15 μM . The nucleus size was measured using the Olympus CellSens software package.

Table 3.6. Size of the nucleus of untreated and treated HT-29 cells. Cells were exposed for 24 hours at a concentration corresponding to the IC_{50} value (Appendix C1).

COMPOUND	SIZE OF NUCLEUS				
	$\leq 5 \mu\text{M}$	5-10 μM	10-15 μM	15-20 μM	$>20 \mu\text{M}$
Untreated	0 cells	0 cells	23 cells	27 cells	0 cells
Camptothecin	0 cells	2 cells	10 cells	21 cells	19 cells
JD-88	2 cells	5 cells	3 cells	9 cells	31 cells
JD-253	0 cells	7 cells	21 cells	12 cells	10 cells
JD-258	0 cells	11 cells	25 cells	6 cells	8 cells
JD-137	10 cells	15 cells	15 cells	10 cells	0 cells

Table 3.7. Morphological features of HT-29 cells (Figure 3.18) after exposure (24 hours) to compounds

MORPHOLOGICAL FEATURES		COMPOUND				
		Cam (Fig 3.18. E-H)	JD-88 (Fig 3.18. I-L)	JD-137 (Fig 3.18 M-P)	JD-253 (Fig 3.18. Q-T)	JD-258 (Fig 3.18. U-X)
Apoptotic nuclei		✓			✓	
Lysosomal aggregation		✓	✓	✓	✓	✓
Elongated cells		✓		✓		✓
Pre-apoptotic nuclei (AO stain)			✓		✓	
Necrotic cells			✓	✓		
Blebbing						✓

Caco-2 cell morphology

The Caco-2 cells (Figure 3.19) exposed to JD-88, JD-96M, JD-253, and JD-256 for 24 hours at a concentration of 12, 10, 9 and 6 μ M respectively showed changes in the cellular morphology consistent with apoptosis when compared to untreated cells. Morphological changes seen after exposure to test compounds include nuclear fragmentation, irregular shaped nuclei, cell membrane blebbing, lysosomal aggregation and changes in cell's shape and size. The photomicrographs are presented in two sections; Figure 3.19A (control, camptothecin and JD-88) and 3.19B (JD-96M, JD-253 and JD-256).

Caco-2 cells treated with camptothecin for a 24 hour period were rounded up with a loss of cell adherence (Figure 3.19A, Image E). AO stained cells appeared orange/yellow in colour indicating pre-apoptotic nuclei (Figure 3.19A, Image F). The nucleus of a cell stained red which is indicative of cell membrane damage (Figure 3.19A, Image F). After staining with HO camptothecin treated cells appeared apoptotic (Figure 3.19A, Image G)

Cells exposed to JD-88 for 24 hours lost their characteristic morphology and were now rounded up with prominent cell membrane blebbing visible (Figure 3.19A, Image I). When JD-88 treated cells were stained with HO irregular shaped nuclei was visible. Staining with AO-EB also showed the presence of lysosomes that were stained orange and aggregated around the nucleus. Some cells stained an orange/yellow colour which is indicative for pre-apoptotic nuclei. (Figure 3.19A, Images J).

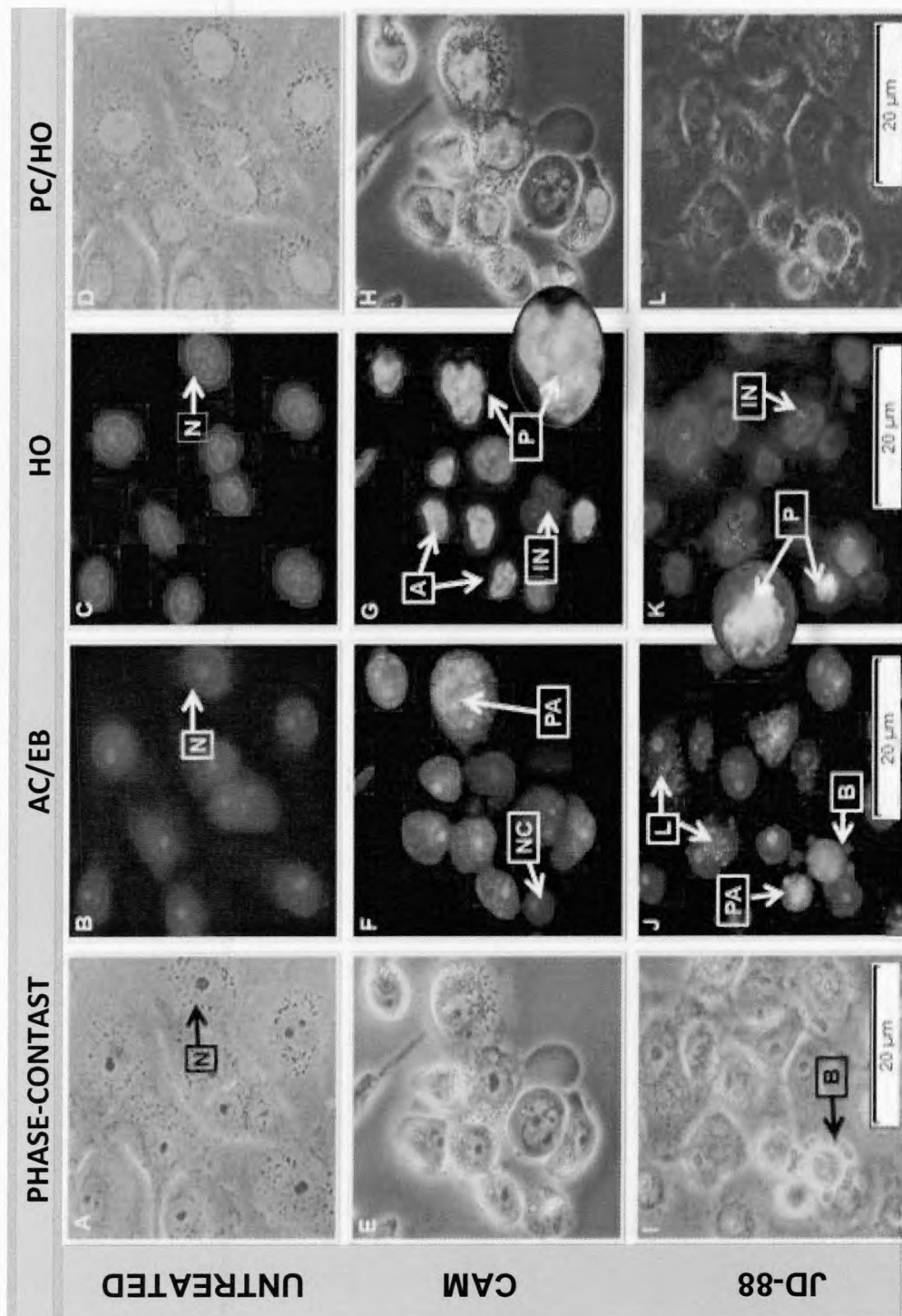


Figure 3.19A. Caco-2 cells treated with the IC_{50} concentration of active test compounds for 24 hours. Cells were exposed to camptothecin ($10 \mu M$) and JD-88 ($12 \mu M$). Cells were observed at a 400X magnification.

LEGEND: L= lysosomes; N= nucleus; A= apoptotic nucleus; IN= irregular nucleus; NC= necrotic cell; EC= elongated cell; B=blebbing; P A= pre-apoptotic nucleus; P= punctate nucleus; Scale bar: $20 \mu m$

After exposing Caco-2 cells to JD-96M for 24 hours, cells developed long elongated processes (Figure 3.19B, Image M). Staining with AO-EB showed a small number of lysosomes that were aggregated around the nucleus (Figure 3.19, images N). HO stained JD-96M treated cells had blue distinct nuclei and one of the cell's nuclei stained red which is indicative of cell membrane damage (Figure 3.19B, Image O).

Cells treated with JD-253 for 24 hours were granular with a cell morphology that was different compared to the untreated cells (Figure 3.19B, Image Q). Lysosomes were aggregated around the nucleus as seen with the AO-EB staining (Figure 3.19B, Image R). AO staining also revealed that the cells appeared an orange/yellow colour which is indicative of pre-apoptotic changes (Figure 3.19B, Image R). Irregular blue nuclei were seen with HO staining (Figure 3.19B, Image S).

Cells treated with JD-256 for 24 hours cells appeared densely packed and appeared granular (Figure 3.19B, Image U). Lysosomes were aggregated around the nucleus as seen with AO-EB staining (Figure 3.19B, Image V). Irregular blue nuclei were seen with HO staining and the nuclei varied in shape. In some cells chromatin condensation was visible with the nucleus appearing to have large spots (Figure 3.19B, Image W).

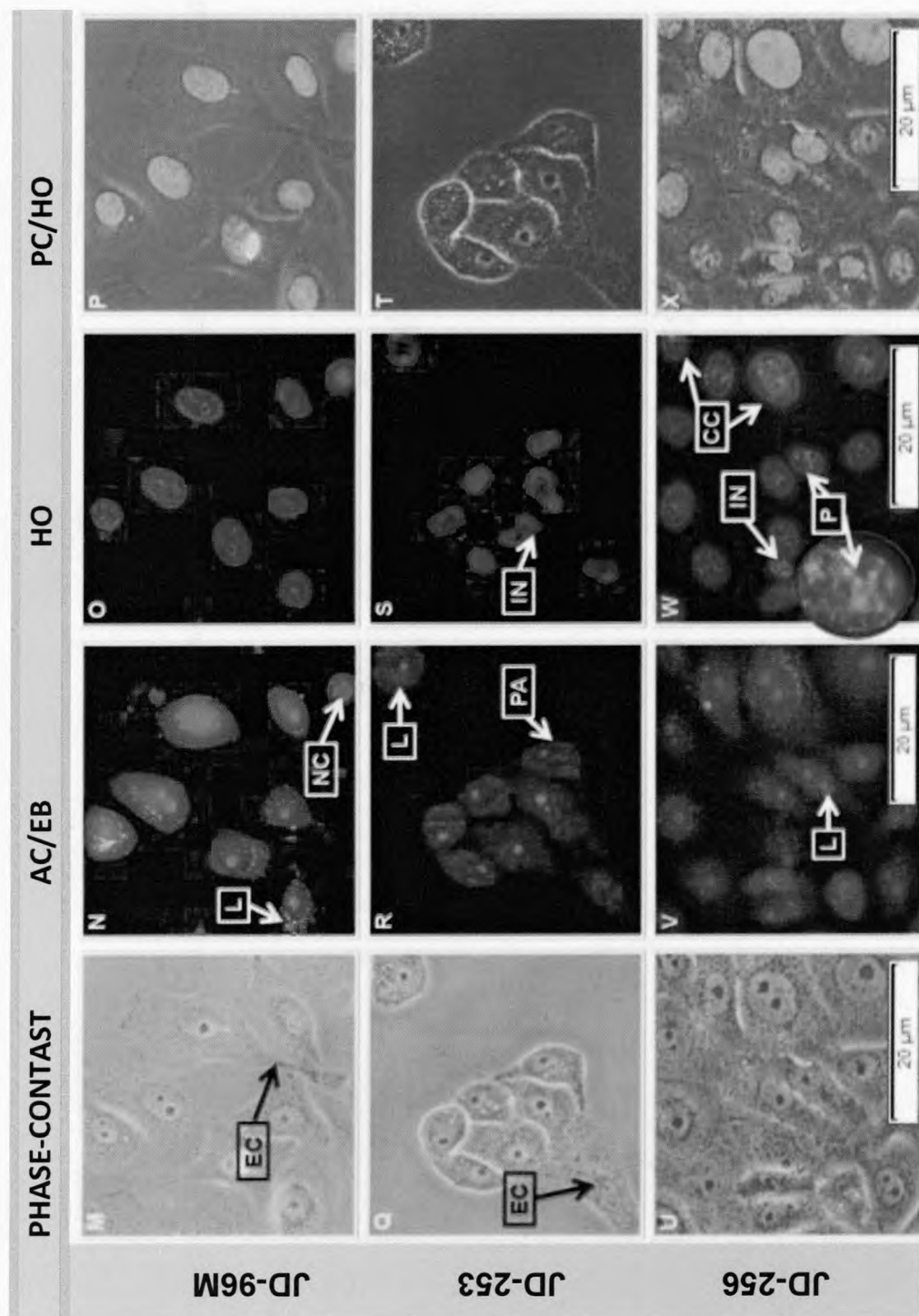


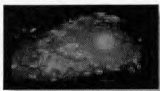
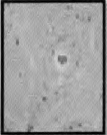
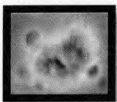
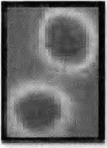
Figure 3.19B. Caco-2 cells treated with the IC_{50} concentration of active test compounds for 24 hours. Cells were exposed to JD-96M (10 μ M), JD-253 (9 μ M) and JD-256 (6 μ M). Cells were observed at a 400X magnification.
LEGEND: L= lysosomes; EC= elongated cell; CC= chromatin condensation; IN= irregular nucleus; NC= necrotic cell; EC= elongated cell; P= punctate nucleus; Scale bar: 20 μ m.

The Caco-2 cells (50 cells) were evaluated to determine the effect of the test compounds on the size of the nuclei of cells. After exposing Caco-2 cells to JD-88, 34 out of the 50 cells had a nuclei diameter of less than 15 μM . Majority of the cells treated with JD-253 had a diameter that was less than 20 μM . The nucleus size was measured using the Olympus CellSens software package.

Table 3.8. Size of the nucleus of untreated and treated Caco-2 cells. Cells were exposed for 24 hours at a concentration corresponding to the IC_{50} values (Appendix C2).

COMPOUND	SIZE OF NUCLEUS				
	$\leq 5 \mu\text{m}$	5-10 μm	10-15 μm	15-20 μm	$>20 \mu\text{m}$
Untreated	0 cells	2 cells	24 cells	24 cells	0 cells
Camptothecin	2 cells	4 cells	10 cells	19 cells	15 cells
JD-88	0 cells	10 cells	24 cells	8 cells	8 cells
JD-253	2 cells	9 cells	10 cells	19 cells	10 cells
JD-256	0 cells	2 cells	22 cells	20 cells	6 cells
JD-96M	1 cells	17 cells	21 cells	6 cells	5 cells

Table 3.9. Morphological features of Caco-2 cells (Figure 3.19) after exposure (24 hours) to compounds

MORPHOLOGICAL FEATURES		COMPOUND				
		Cam (Fig 3.22 E-H)	JD-88 (Fig 3.22 I-L)	JD-96M (Fig 3.3.2 M-P)	JD-253 (Fig 3.3.2 Q-T)	JD-256 (Fig 3.3.2 U-X)
Pre-apoptotic nuclei		✓	✓		✓	
Lysosomal aggregation			✓	✓	✓	✓
Elongated cells				✓	✓	
Cell membrane blebbing			✓			
Loss of cell-cell junctions		✓	✓			
Necrotic cells		✓		✓		

The HT-29 and Caco-2 cells (50 cells) were observed and analysed to determine the changes in the nuclear morphology after exposing cells to active compounds. Changes in the nuclear morphology were determined by observing the Hoechst staining pattern and the appearance of punctate nuclei.

Table 3.10. Analysis of cell nuclei with irregular morphology. Cells were exposed for 24 hours at a concentration corresponding to the IC₅₀ values (Appendix C2).

COMPOUND	% of cell nuclei with irregular morphology	
	HT-29 cells	Caco-2 cells
Camptothecin	20%	28%
JD-88	40%	8%
JD-96M	-----	20%
JD-253	60%	-----
JD-256	-----	40%
JD-258	24%	-----
JD-137	36%	24%

3.2.2 Caspase-3/7 activity

Caspases are important regulators of apoptotic cell death. Caspase-8, which is an initiator caspase, cleaves and activates the executioner caspase-3. Caspase-3 cleaves proteins in apoptotic cells. Caspase-3 and Caspase-7 have similar substrate specificity and the assay used in this study did not distinguish between the two caspases.

Caspase-3/7 activity was measured after a 2 hour, 4 hour, 8 hour, 12 hour and 24 hour exposure period. The caspase-3/7 activity is expressed in relation to the amount of protein in samples since toxic effects on cells decrease the protein content of cells. Caspase-3/7 activity is therefore expressed as specific activity. Measurement of the protein concentration was performed according to the Bradford method. Standardized bovine serum albumin (BSA) (BIO-RAD) was used to construct a standard curve (Appendix B4) and the protein concentration was measured from the standard curve. Caspase-3 activity was normalised against the protein concentration in the HT-29 and Caco-2 cell lysates.

Appendix D1 indicates the effect that the active compounds had on the amount of protein found in the lysate of treated HT-29 cells. HT-29 cells were treated with JD-137 (10 µM), JD-258 (15 µM) and JD-253 (10 µM), JD-88 (20 µM) and camptothecin (15 µM).

Appendix D2 indicates the effect that the active compounds had on the amount of protein found in the lysate of the Caco-2 cells. Caco-2 cells were treated with JD-256 (20 µM), JD-88 (35 µM), JD-96M (30 µM) and JD-253 (30 µM) and camptothecin (30 µM).

The results of specific caspase-3/7 activity are presented as graphs showing fold increase versus time (Figure 3.20 – Figure 3.21). The effect of camptothecin on the caspase-3/7 activity is shown in Figure 3.20 for the HT-29 cells and Figure 3.21 for the Caco-2 cells.

3.2.2.1 Effect of the compounds on caspase-3/7 activity in HT-29 cells

Figure 3.20 illustrates the induction of caspase-3 activity in the HT-29 cells after treatment with the IC_{75} concentration of compounds JD-137 (10 μ M), JD-258 (15 μ M) and JD-253 (10 μ M), JD-88 (20 μ M) and camptothecin (15 μ M). After a two hour exposure period, all compounds caused an increase in caspase-3/7 activity relative to the control with JD-258 being the highest. At two hours JD-88 had the lowest caspase-3/7 activity, although JD-88 and camptothecin were the only compounds that showed more activity than the control after 12 hours. With the exception of JD-258 all the compounds showed the highest caspase-3/7 activity at 4 hours albeit not as high as that of camptothecin. At 24 hours, after the start of treatment, caspase-3 levels were similar to the control for all the test compounds.

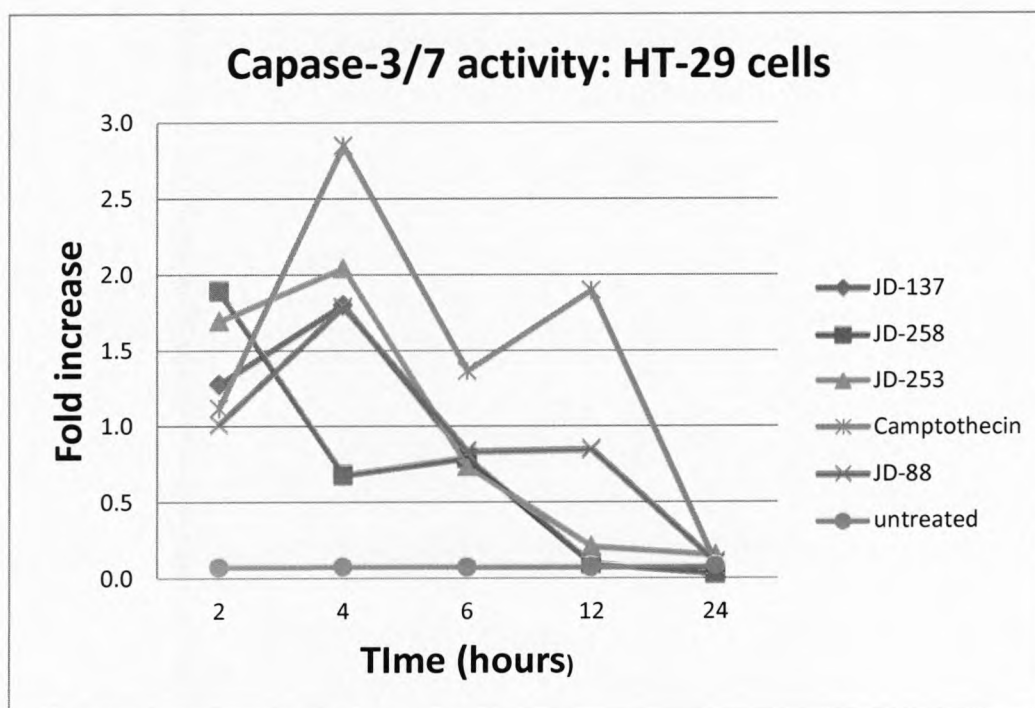


Figure 3.20. Time-dependent induction of caspase-3/7 activity after exposure to the most active compounds in the HT-29 cells. Cells were treated with JD-137 (10 μ M), JD-258 (15 μ M) and JD-253 (10 μ M), JD-88 (20 μ M) and camptothecin (15 μ M). Each data point was done in duplicate and represents the specific caspase-3/7 activity after exposing cells to the compounds for a 2 hour, 4 hour, 6 hour, 12 hour and 24 hour period. Each experiment was repeated three times.

3.2.2.2 Effect of compounds on caspase-3/7 activity in Caco-2 cells

Figure 3.21 illustrates the time-dependent induction of caspase-3/7 activity in the Caco-2 cells after treatment with the test compounds at an IC_{75} concentration (JD-256 (20 μ M), JD-88 (35 μ M), JD-96M (30 μ M) and JD-253 (30 μ M) and camptothecin (30 μ M)). After a two hour exposure period, all the compounds caused an increase in caspase-3/7 activity relative to the control with JD-256 being the highest. The highest caspase-3/7 activity was observed at four hours for all the compounds except camptothecin. By 12 hours only JD-256 and JD-88 showed a higher caspase activity relative to the control. Of significance here is that the caspase activity is higher than that of camptothecin. After 24 hours of treatment with the compounds and camptothecin, caspase-3/7 levels decreased to levels similar to that of the control.

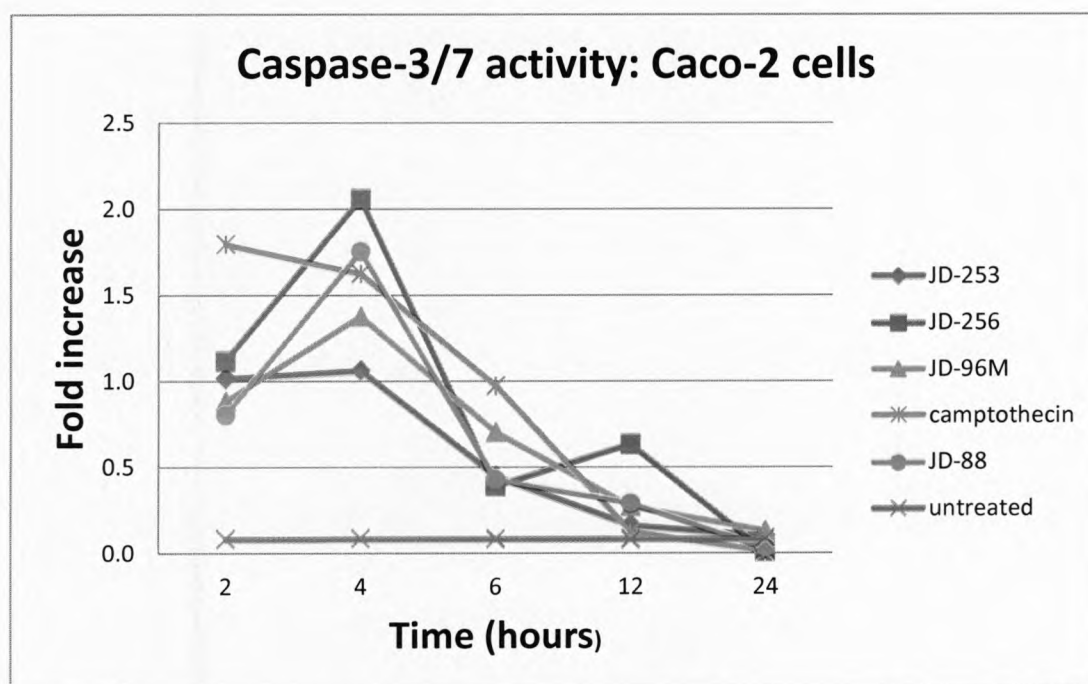


Figure 3.21. Time-dependent induction of caspase-3/7 activity after exposure to the most active compounds in the Caco-2 cells. Cells were treated with JD-256 (20 μ M), JD-88 (35 μ M), JD-96M (30 μ M) and JD-253 (30 μ M) and camptothecin (30 μ M). Each data point was done in duplicate and represents the specific caspase-3/7 activity after exposing cells to the compounds for a 2 hour, 4 hour, 6 hour, 12 hour and 24 hour period. Each experiment was repeated three times.

3.2.3 Annexin-V binding assay

In cells, phosphatidyl serine is normally found on the cytoplasmic surface of the cell membrane. In early apoptotic cells, phosphatidyl serine is translocated from the inner to the outer leaflet of the plasma membrane. This translocation allows the exposure of phosphatidyl serine to the external cellular environment. Treated HT-29 and Caco-2 cells were stained with a combination of fluorescein isothiocyanate (FITC) conjugated annexin-V and propidium iodide. Annexin-V stains the cell membrane of apoptotic cells green. Annexin-V-FITC stains the cell membrane due to the affinity of annexin-V for phosphatidyl serine. Propidium iodide which is nucleic acid binding dye that is impermeable to living and early apoptotic cells, but enters and stains the nuclei of dead and late apoptotic cells red when viewed with the appropriate filters. The cells that were exposed to the active test compounds were observed using the Olympus BX41 fluorescence microscope under a 400X magnification. Photomicrographs were captured using the Olympus DP72 digital camera and analyzed with the Olympus CellSens software package.

3.2.3.1 Annexin-V binding assay in HT-29 cells

Fluorescence was observed in all cells treated with the active test compounds. After staining the HT-29 cells with FITC conjugated annexin-V and PI, apoptotic cells showed green fluorescence, dead cells showed red and green fluorescence, and normal cells showed no fluorescence (Figure 3.22).

Untreated control cells did not show any fluorescence after staining with Annexin-V/PI indicating that there were small amounts of phosphatidyl serine on the outer surface of the cell membrane. HT-29 cells treated with compound JD-137 (10 μ M) fluoresced green after four hours showing annexin-V binding. Close to 100% of the cells fluoresced green. HT-29 cells exposed to JD-258 (15 μ M), showed a green fluorescence after a four hour exposure period. JD-253 (10 μ M) treated cells showed a green fluorescence after a four hour exposure period and almost 100% of these cells fluoresced green. HT-29 cells treated with the JD-88 (20 μ M) showed a green fluorescence after a six hour exposure period showing annexin-V-FITC binding.

After a six hour exposure period, some cells treated with camptothecin (15 μ M) fluoresced red after incubation with FITC conjugated annexin-V and PI which is indicative of cell membrane damage i.e. necrosis.

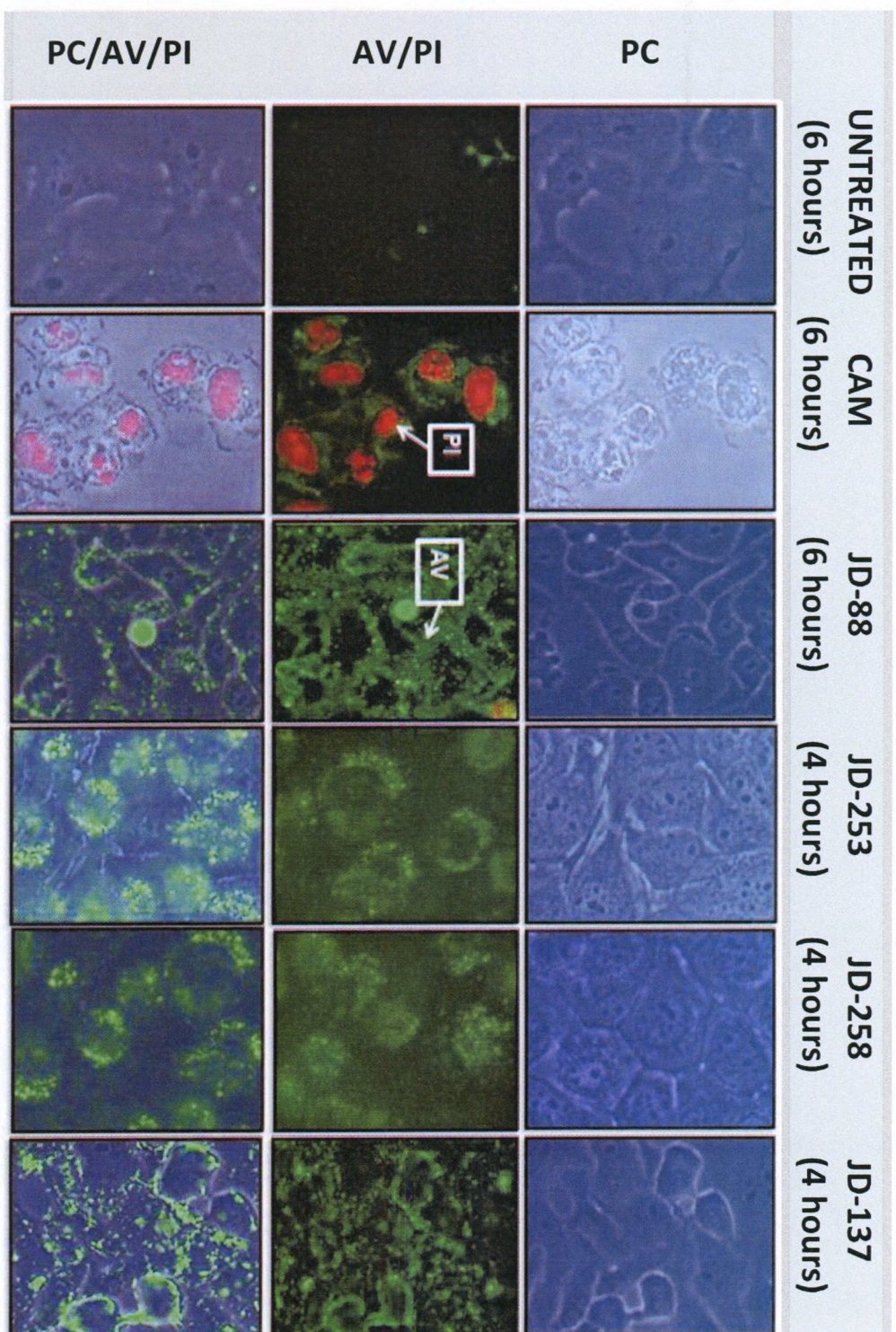


Figure 3.22. Exposure of phosphatidyl serine in HT-29 cells after treatment with active compounds. HT-29 cells were exposed to JD-137 (10 μ M), JD-258 (15 μ M) and JD-253 (10 μ M), JD-88 (20 μ M) and camptothecin (15 μ M). Cells were exposed for 4 hours (JD-137, JD-253 and JD-258) and 6 hours (JD-88 and camptothecin). Cells were observed at a 400X magnification. Images were captured with an ISO of 200 and a constant exposure time of 530 milliseconds for the FITC channel. **LEGEND:** PC= phase-contrast AV= Annexin-V; PI= Propidium iodide, Scale bar: 20 μ m

3.2.3.2 Annexin-V binding assay in Caco-2 cells

Fluorescence activity was expressed in all cells treated with the active test compounds (Figure 3.23). After staining the Caco-2 cells with FITC conjugated annexin-V and PI, apoptotic cells showed green fluorescence, necrotic (dead) cells showed red and green fluorescence, and live cells showed no fluorescence (Figure 3.23). Untreated cells did not show any fluorescence when stained with the FITC conjugated annexin-V.

Cells exposed to compound JD-256 (20 μ M) showed fluorescence after a six hour exposure period. At six hours post exposure, 100% of treated Caco-2 cells fluoresced green which is an indication that the apoptotic process was induced in the treated cells.

Caco-2 cells treated with compound JD-88 (35 μ M), fluoresced green after an eight hour exposure period.

Caco-2 cells treated with JD-96M (30 μ M) fluoresced green after an eight hour exposure period. Caco-2 cells treated with JD-253 (30 μ M) showed fluorescence after an eight hour exposure period. Most of the treated cells expressed fluorescence activity.

After a six hour exposure period, cells exposed to camptothecin (30 μ M) fluoresced red which is indicative of necrosis.

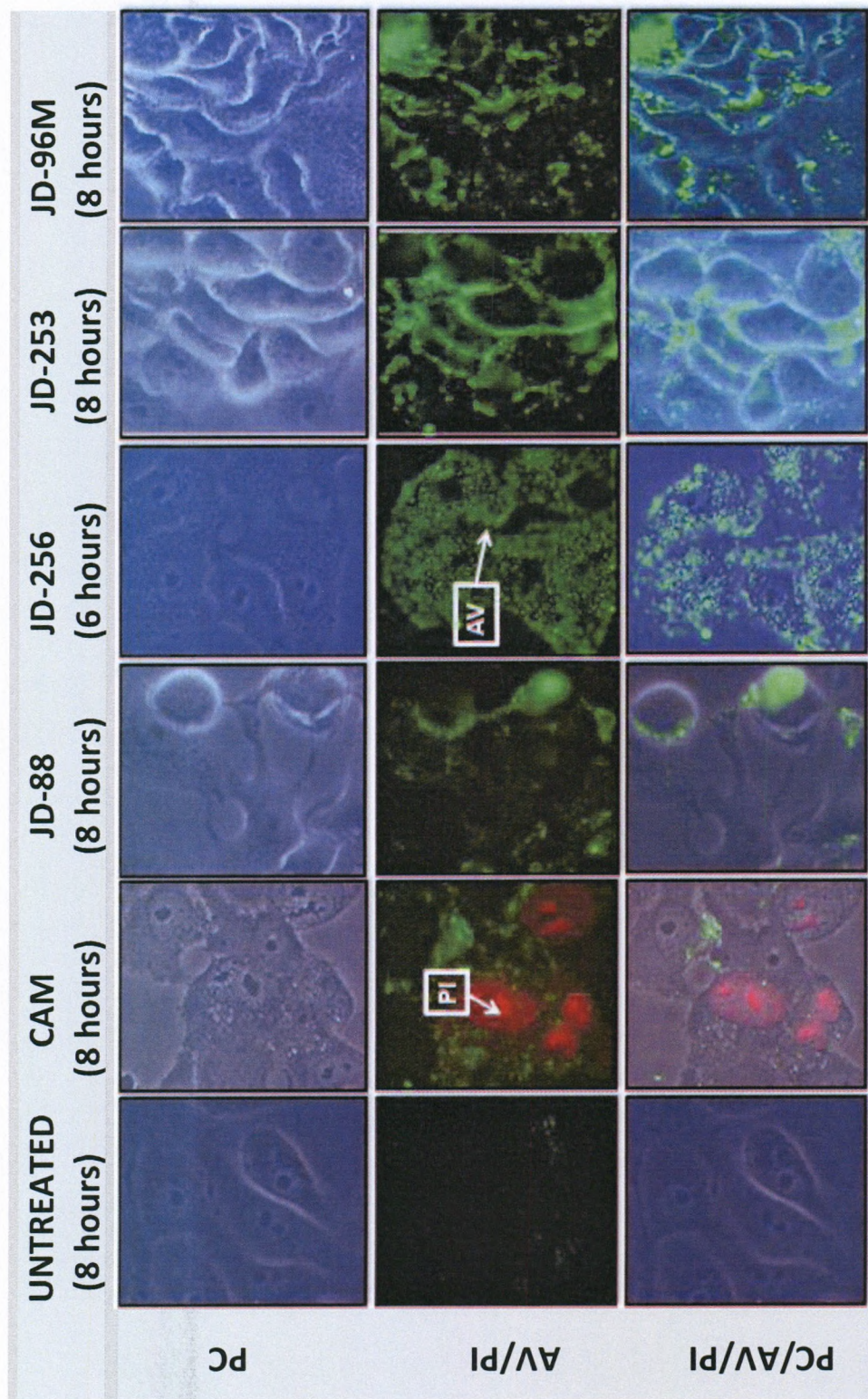


Figure 3.23. Exposure of phosphatidyl serine after Caco-2 cells were treated with active test compounds. Caco-2 cells exposed to JD-256 (20 μ M), JD-88 (35 μ M), JD-96M (30 μ M) and JD-253 (30 μ M) and camptothecin (30 μ M). Cells were exposed for 6 hours (JD-256) and 8 hours (camptothecin, JD-96M, JD-88 and JD-253). Cells were observed at a 400X magnification Images were captured with an ISO of 200 and a constant exposure time of 530 milliseconds for the FITC channel. **LEGEND:** PC= phase-contrast; AV= Annexin-V; PI= Propidium iodide, Scale bar: 20 μ m

3.2.4 The release of Cytochrome c from mitochondria

The mitochondrion is important for the maintenance of cell homeostasis and energy production. The mitochondria play an important role in the control of cell death via the intrinsic apoptotic pathway by releasing pro-apoptotic factors such as cytochrome c. During the apoptotic process, cytochrome c is released from the mitochondria to the cytosol which then activates a caspase cascade. The mitochondrial (intrinsic) apoptotic pathway originates from within the cell and is characterised by mitochondrial dysfunction or endoplasmic reticulum stress. The subcellular distribution of cytochrome c (Figure 3.24) was determined for the HT-29 cells as they were more sensitive to the compounds than the Caco-2 cells. Cytochrome c levels were determined by a human cytochrome c protein Elisa assay as described in the Materials and Methods section 2.7. A standard curve (Appendix B3) was constructed and this curve was used to calculate Cytochrome c concentrations.

3.2.4.1 Cytochrome c subcellular distribution in the HT-29 cells

The total amount of cytochrome c and the distribution between the mitochondrial and cytoplasmic fractions are shown in Figure 3.24. The untreated control cells showed that 90% of cytochrome c protein was found in the mitochondrial fraction and 10% of the total cytochrome c was found in the cytosolic fraction.

After exposure to the JD-137 a total of 295.50 ± 11.5 pg/mL of the cytochrome c protein was measured with 45% of the cytochrome c found in the mitochondrial fraction and 55% was found in the cytosolic fraction. With compound JD-253 a total of 355.70 ± 0.3 pg of the cytochrome c protein was measured of which 81% was found in the mitochondrial fraction and 9% of the cytochrome c was found in the cytosolic fraction.

After exposure to compound JD-258 a total of 690.8 ± 8.7 pg of the cytochrome c protein was measured of which 87% was found in the mitochondrial fraction and 13% of the cytochrome c was found in the cytosolic fraction. After exposure to compound JD-88 a total of 651.4 ± 10.8 pg of the cytochrome c protein was measured of which 91% was found in the mitochondrial fraction and 9% of the cytochrome c was found in the cytosolic. This was similar to the total amount of cytochrome c measured in the untreated cells.

Of all the compounds tested only JD-137 caused a meaningful redistribution of cytochrome c to the cytoplasm albeit less than camptothecin (67%) which showed the highest percentage of cytochrome c leakage to the cytoplasm indicating apoptotic changes to the mitochondrial membrane.

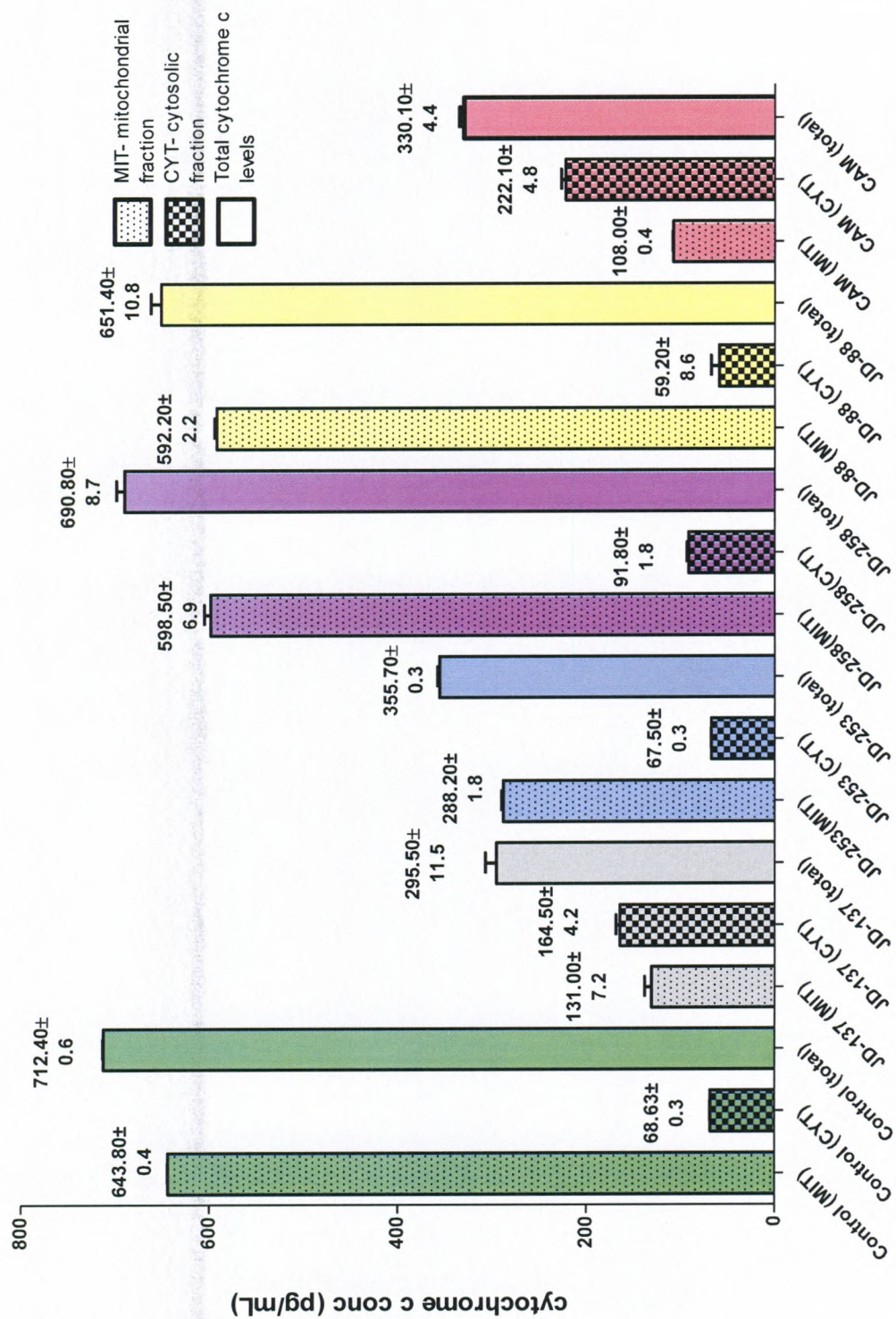


Figure 3.24. The effect of the active compounds on the subcellular distribution of cytochrome-c in HT-29 cells. Cells were exposed to concentration values of camptothecin (15 μ M), JD-88 (20 μ M), JD-137 (10 μ M), JD-253 (10 μ M) and JD-258 (15 μ M) for 24 hours. Each data point was obtained in duplicate and the experiment was repeated twice.

Table 3.11. Percentage of the total cytochrome c found in the mitochondrial fraction and in the cytosolic fractions

	% Mitochondrial fraction	% Cytosolic fraction
Untreated	90%	10%
Camptothecin	33%	67%
JD-88	91%	9%
JD-137	45%	55%
JD-253	81%	19%
JD-258	87%	13%

CHAPTER 4: Discussion

Current chemotherapy of metastatic colorectal cancer is plagued by adverse drug reactions, drug resistance and therapeutic failure. There is thus a need to discover new treatment options. Apoptosis is compromised in CRC, therefore, compounds that can induce the apoptotic process offers a potential advantage for clinical applications (Hassan *et al*, 2014). Abnormalities in the normal apoptotic process contribute directly to both the pathogenesis of colorectal cancer as well as its resistance to chemotherapy and radiation therapy (Ho *et al*, 2009).

In the present study, HT-29 and Caco-2 cells were exposed to two groups of compounds, the azaindoles and imidazo[1,2-*a*]pyridines, with the aim of investigating the cytotoxic effects on the cells and the mode of cell death. Azaindoles and imidazo[1,2-*a*]pyridines are essential subunits in many pharmaceutically important compounds. This includes compounds like anti-inflammatory, antibacterial and anticancer agents (Lalit *et al*, 2012). Studies have shown that azaindole derivatives have an inhibitory effect on angiogenesis and tumour growth in breast cancer grafts (Prudent *et al*, 2013). In this study the cytotoxic effects of the azaindole derivatives on both tested cell lines was minimum. Therefore the apoptotic inducing effects of the azaindole derivatives on both cell lines was not evaluated.

Imidazo[1,2-*a*]pyridine derivatives are of great importance for their biological properties and many of them have been used as inhibitors of various target enzymes and are used in medicines, herbicides and fungicides (Jianchao and Qiyuan, 2010; reviewed in Bagdi *et al*, 2014). The imidazo[1,2-*a*]pyridine derivatives have shown a wide range of biological activities such as antifungal, anti-inflammatory, antitumor, antiviral, antibacterial, antiprotozoal, antipyretic, analgesic, anti-apoptotic, hypno-selective, and anxio-selective activities (Kamal *et al*, 2014, Lacerda *et al*, 2009).

In the present study fourteen azaindole and twenty-seven imidazo[1,2-*a*]pyridine derivatives were initially screened to identify compounds that decreased the cell viability of the HT-29 and

Caco-2 cells. Thereafter, IC_{50} values were obtained for compounds showing 80% inhibition of cell growth at a 100 μ M concentration.

Camptothecin was used as the positive control in this study due to its ability to initiate apoptosis in a dose dependent manner *in vitro* (Morris and Geller, 1996). Irinotecan, parent compound of camptothecin plays an important role in the management of metastatic CRC. Camptothecin induces apoptosis through both the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway (Wang *et al*, 2008). Camptothecin derivatives are plant alkaloids that kill malignant cells by inhibiting the activity of the enzyme DNA topoisomerase I, a key enzyme responsible for cutting and religating DNA strands. Inhibition of the re-ligating function of this enzyme results in DNA strand breakage and leads to apoptotic cell death (Katzung *et al*, 2013; Fulda, 2010).

4.1 The cytotoxic effects of the imidazo[1,2-a]pyridine derivatives on the HT-29 cells, Caco-2 cells and leucocytes

Compared to the imidazo[1,2-a]pyridine derivatives, the azaindoles showed poor activity against the HT-29 and Caco-2 cells. In the preliminary evaluations of the imidazo[1,2-a]pyridine and azaindoles, at 100 μ M, only one azaindole compound, TLC-75 was shown to be active (> 80% decrease in cell viability) against the Caco-2 cells but not against the HT-29 cells. In contrast, a number of imidazo[1,2-a]pyridine compounds inhibited the growth of both cells lines by 80%. These compounds were further evaluated to determine their IC_{50} values.

The active imidazo[1,2-a]pyridine compounds identified in the preliminary screen all inhibited the growth of the HT-29 and Caco-2 cells in a dose-dependent manner with IC_{50} values mostly below 15 μ M. The azaindole derivative had an IC_{50} value higher than 40 μ M (JD-75) against the Caco-2 cells. This high IC_{50} value indicated that the azaindoles were not viable candidates for future studies and no further investigations were carried out on this compound.

Compound JD-253 showed the highest activity against the HT-29 cells with an IC_{50} value of 3.89 ± 0.6 μ M and compound JD-256 was the most active against the Caco-2 cells with an IC_{50} value of 6.22 ± 1.5 μ M which is lower than that of camptothecin in these two cell lines.

Compounds were considered active if their IC₅₀ values against either cell line were lower than 15 μ M. Some of the active compounds displayed selective sensitivity to either the HT-29 cells or Caco-2 cells (Table 4.1).

Table 4.1. IC₅₀ values of active compounds against the HT-29 and Caco-2 cells

COMPOUNDS	IC ₅₀ value (μ M)	
	HT-29 cells	Caco-2 cells
JD-80	Inactive	14.02 $\pm$ 0.8
JD-81	10.69 $\pm$ 0.4	Inactive
JD-88	8.03 $\pm$ 0.4	12.22 $\pm$ 0.6
JD-96M	12.10 $\pm$ 1.4	10.79 $\pm$ 0.5
JD-119	8.13 $\pm$ 1.5	Inactive
JD-122	28.22 $\pm$ 0.9	34.33 $\pm$ 2.5
JD-137	4.13 $\pm$ 0.4	14.79 $\pm$ 1.5
JD-253	3.89 $\pm$ 0.6	9.62 $\pm$ 0.4
JD-254	9.14 $\pm$ 0.5	11.69 $\pm$ 2.6
JD-256	11.35 $\pm$ 1.5	6.22 $\pm$ 1.5
JD-258	5.87 $\pm$ 0.8	Inactive

A statistical comparison of the IC₅₀ values of the active test compounds against the HT-29 cells showed that JD-88, JD-137, JD-253, JD-258 and JD-119 showed equivalent efficacy when compared to camptothecin (Table 3.3). With the Caco-2 cells, the IC₅₀ value of compounds, JD-96M, JD-253, JD-256 and JD-81 showed equivalent efficacy compared to camptothecin (Table 3.4). This comparison proves that these test compounds were active as cytotoxic agents against the cells.

A study conducted by Dahan-Farkas *et al*, (2011) studied the cytotoxic ability of a group of imidazo[1,2-a]pyridines derivatives by conducting a MTT viability cell assay. A major criticism of the study was the short exposure time (24 hours) to the test compounds compared the 48 hours in the current study. Dahan-Farkas *et al*, (2011) observed that imidazo[1,2-a]pyridine derivatives

were significantly cytotoxic against the HT-29 and Caco-2 cells. Dahan-Farkas *et al*, (2011) observed that four of the compounds had IC₅₀ values lower than 10 µM against the HT-29 cells and against the Caco-2 cells there were four compounds with IC₅₀ values less than 10 µM (Dahan-Farkas *et al*, 2011).

Martínez-Urbina *et al*, (2010) evaluated 6-Substituted 2-(N-trifluoroacetyl-amino) imidazopyridines for cytotoxic activity, via the MTT assay, *in vitro* against various human cell lines (glia carcinoma U251, prostate PC-3, leukemia K-562, colon HCT-15, human breast MCF7 and lung carcinoma SK-LU-1). An initial screen with concentration of 50 µM induced cytotoxicity against all cell lines with growth inhibition activity greater than the positive control, a known CDK inhibitor. From the tested six compounds one compound displayed an IC₅₀ value below 10 µM (Martínez-Urbina *et al*, 2010).

The cytotoxic effect of five novel chromene derivatives fused with the imidazo[1,2-*a*]pyridine nucleus was determined against the colorectal cancer cell line, HCT116 by Lima *et al*, (2015). The study evaluated the effect of test compounds (in 48 h incubations) on cell growth and was evaluated by estimating the number of metabolically active cells via the MTT assay in 24-multiwell culture plates. Lima *et al*, (2015) observed that at a concentration of 20 µM, the effect of the two of the tested compounds on growth inhibition for 48 h was significant. Two compounds showed significant inhibitory activity and had IC₅₀ values of 15 µM and 11 µM, respectively and the most potent compound tested had an IC₅₀ value of 5 µM (Lima *et al*, 2015).

Garamvolgyi *et al*, (2016) investigated a number of diarylamide and diarylurea compounds that had either the imidazo[1,2-*a*]pyridine or the imidazo[1,2-*a*]pyrazine scaffold. The study evaluated the cytotoxicity of the compounds *in vitro* against melanoma cells using the CellTiter-Glo® Luminescent Cell viability Assay. The results showed that seven of the sixteen diarylamide derivatives that contained an imidazo[1,2-*a*]pyridine scaffold had IC₅₀ values lower than 10 µM against the melanoma cell line (Garamvolgyi *et al*, 2016).

The cytotoxic potential of the imidazo[1,2-*a*]pyridine derivatives tested in this study confirms the results obtained from previously reported studies. The above mentioned studies observed that

the imidazo[1,2-*a*]pyridine derivatives are effective in inducing cell death in various cancer cells with promising IC₅₀ values.

The imidazo[1,2-*a*]pyridine evaluated in this study shows promise for further investigations especially since some of the compounds display some selectivity towards either the Caco-2 or HT-29 cell line. JD-80 displayed high selectivity towards the Caco-2 cells while JD-81, JD-119 and JD-258 displayed selectivity against the HT-29 cells. Most of the imidazo[1,2-*a*]pyridine compounds that were evaluated proved to be highly toxic to both the HT-29 and Caco-2 cells with IC₅₀ values comparable to the positive control, camptothecin.

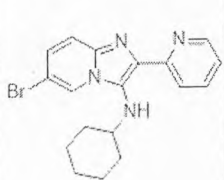
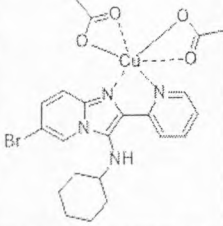
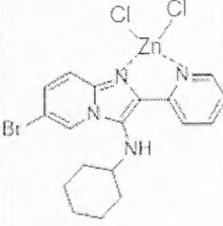
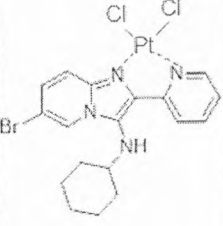
Most cancer chemotherapeutic agents generally have low therapeutic indices and affect other types of proliferating cells. Most chemotherapeutic agents are cytotoxic to normal cells such as lymphocytes, bone marrow cells, foetal cells, germ cells, hair follicle cells and intestinal cells. *In vitro* technology to determine drug toxicity is an evolving science that has the potential to revolutionize drug toxicity to determine the tolerable threshold levels (Kaempf *et al*, 2008). Krishna *et al*, (2009) determined the effect of seven anti-cancer agents on lymphocyte cultures from peripheral blood of non-smoking donors under *in vitro* conditions by using the MTT assay (Krishna *et al*, 2009). The investigation by Krishna *et al*, (2009) determined the lethal concentrations and the dosages at which individual anti-cancer agents cause cytotoxicity in human lymphocytes. The MTT assay is a reliable *in vitro* technique for the measurement of drug toxicity (Suman and Jamil, 2006; van Meerlo *et al*, 2011). Therefore the current study used peripheral lymphocytes as a preliminary indicator for toxicity against normal cells. Compounds were tested at 10 µM and the cytotoxicity potential of the imidazo[1,2-*a*]pyridine derivatives was determined after a 24 hour exposure period.

As shown in Figure 3.10 camptothecin was significantly more toxic to the leucocytes than compounds JD-81, JD-254, JD-96M and JD-137. After exposure to camptothecin the percentage leucocyte survival was 53%. The results indicate that the toxicity of JD-81 (67%), JD-254 (86%), JD-96M (70%) and JD-137 (75%) was lower than that of camptothecin with JD-258 being the most toxic to the leucocytes with a percentage viability of 9%. The toxic profile of compound JD-258 suggests that this compound is not selective towards cancer cells.

4.2 The structure-activity analysis of test compounds

The Department of Chemistry at the University of the Witwatersrand supplied 40 novel compounds of azaindoles and imidazo[1,2-*a*]pyridines derivatives. The compounds were synthesized using a multi-component reaction system. The imidazo[1,2-*a*]pyridine amine base structure did not cause a significant decrease in cell viability of the HT-29 or Caco-2 cells. A second set of compounds were synthesized by the Department of Chemistry, with structural modifications to the base structures in order to enhance the cytotoxic activity of the compounds against the HT-29 and Caco-2 cells. One such modification involved the incorporation of various metal complexes to the imidazo[1,2-*a*]pyridine nucleus (Table 4.2). The incorporation of a copper complex to the base structure increased the cytotoxic activity of compound JD-88 against both the HT-29 and Caco-2 cells. Compound JD-88, had a copper complex attached to the amine base structure and cell viability assays showed that the compound was active against both the HT-29 and Caco-2 cells.

Table 4.2. Addition of a metal containing complex to the imidazo[1,2-*a*]pyridine amine base structure

COMPOUNDS STRUCTURES	JD-60	JD-88	JD-142	JD-PtCl ₂
				
HT-29 IC ₅₀ Caco-2 IC ₅₀	Inactive	8.03±0.4 µM 12.22±0.6 µM	Inactive	Inactive
Comments	• The imidazo[1,2-<i>a</i>]pyridine amine base structure was not active against either cell line. • The addition of a copper complex increased the activity of the compound against both cell lines • Substituting the copper complex with a zinc/chloride complex inactivates the compound against the HT-29 and Caco-2 cells • Substituting the copper complex with a platinum/chloride complex inactivates the compound against both cell lines			

The chemical properties of copper make it possible for copper to function in biological processes such as electron transfer, catalysis, and structural shaping (Santini *et al*, 2014). Copper is essential for the functioning of living organisms and is a very important element in redox chemistry, growth and development. Copper plays a key role in energy metabolism, respiration and DNA synthesis (Tardito and Marchio, 2009). With the exception of platinum (II) compounds, there is a limited amount of information regarding how metal anticancer drugs function. Copper being an essential element, may also be less toxic than non-essential metal such as platinum. The ability of copper complexes to kill cancer cells occurs due to the induction of an oxidative stress, but recently their ability to inhibit the proteasome, a protein complex whose proteolytic activity is needed by several cellular processes has gained interest (Santini *et al*, 2014).

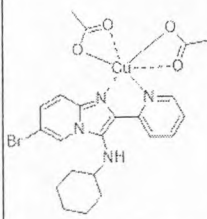
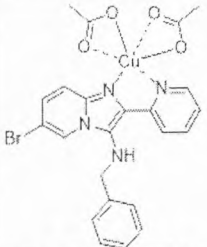
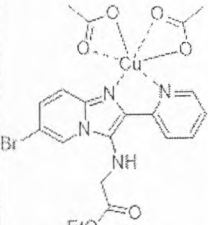
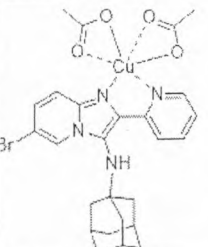
Jany *et al*, (2015) investigated newly developed complexes that contained two copper centres. These complexes were developed for the targeting of two neighbouring phosphates on the DNA backbone which provides active sites for metallo-enzymes such as nucleases. These copper containing compounds inhibited DNA synthesis and induced cell death of cancer cell types with a much higher potency than cisplatin (Jany *et al*, 2015). In order to overcome the chemoresistance seen with cisplatin, Pivetta *et al*, (2014) evaluated the anticancer effect of three copper compounds in combination with cisplatin. The study observed a synergistic effect with the combination therapy, even against cells that were identified as being resistant to cisplatin. Pivetta *et al*, (2014) also observed that when administered in combination each drug could be used at a reduced dose in comparison to when used as single agents (Pivetta *et al*, 2014). Commonly the toxic effects of copper complexes can result in cell death either by necrosis or through the induction of apoptosis. Studies have been conducted to determine the ability of some copper compounds to activate an alternative non-apoptotic form of programmed cell death. Studies also show that several cancer cell lines have a marked sensitivity to copper toxicity (Tardito and Marchio, 2009).

A third set of compounds was synthesized by the Department of Chemistry, in addition to the incorporation of copper to the amine base structure further modifications were employed and

the efficacy of the compounds was evaluated. The incorporation of a benzyl to the amidogen group (JD-253) significantly increased the activity of the compound against both the HT-29 and Caco-2 cells in the present study. Another modification to the set of imidazo[1-2a]pyridine derivatives was the incorporation of an amantadine group to the amidogen group (JD-258). The IC_{50} value of JD-258 ($5.87 \pm 0.8 \mu M$) shows that the compound was active against the HT-29 cells.

In the HT-29 cells JD-253 was the most active compound, the structure included a benzyl which was added to the amidogen group (Table 4.3). Addition of an ethoxide to the amidogen group (JD-256) did not affect the activity against the HT-29 cells but it did increase the activity of the compound against the Caco-2 cells. The addition of the amantadine group significantly increased the activity of the compound against HT-29 cells although the compound was inactive against the Caco-2 cells.

Table 4.3. The addition of an R-group to the amidogen structure

COMPOUNDS STRUCTURES	JD-88	JD-253	JD-256	JD-258
				
HT-29 cells	$8.03 \pm 0.4 \mu M$	$3.89 \pm 0.6 \mu M$	$9.14 \pm 0.4 \mu M$	$5.87 \pm 0.8 \mu M$
Caco-2 cells	$12.22 \pm 0.6 \mu M$	$9.62 \pm 0.4 \mu M$	$6.22 \pm 1.5 \mu M$	Inactive
Comments	- Addition of a benzyl to the amidogen group significantly increases the activity of the compound against both cell lines. - Addition of an ethoxide to the amidogen group did not affect the activity against the HT-29 cells but it did increase the activity of the compound against the Caco-2 cells. - Addition of an amantadine group to the amidogen group significantly increases the activity of the compound against the HT-29 cells although the compound was inactive against the Caco-2 cells.			

For each cell line four of the most active compounds were selected to further investigate their ability to induce apoptosis.

4.3 Assessment of apoptosis

Apoptosis is very important for the regulation of cell turnover of the intestinal epithelium. In colorectal cancer the intrinsic as well as the extrinsic apoptotic pathways are compromised which results in an increase in cell proliferation (Wong *et al*, 2011). Various experimental techniques such as DNA fragmentation, changes in cell morphology, activation of caspases and the detection of the subcellular location of cytochrome c levels have been developed for the detection of cells undergoing apoptosis. Three fluorochromes, acridine orange, ethidium bromide and Hoechst were used to determine changes in cell morphology after treatment with test compounds and to differentiate between live, apoptotic and necrotic cells. The caspases are believed to be the most common executors of the apoptotic process (Weber *et al*, 2008; Lamkanfi *et al*, 2007). In this study, the activity of caspase-3 was measured via a colorimetric assay. Apoptosis can be seen as a stage-dependent process from its induction to early, intermediate and late-stage apoptotic events. Therefore the detection of apoptosis at early stages, before gross morphological changes, is critical for understanding the pathways of programmed cell death. A common used method to detect apoptosis relies on the detection of phosphatidyl serine on the surface of the plasma membrane which is an early event in apoptosis. Phosphatidyl serine redistribution occurs earlier than DNA-associated changes during apoptosis (Zhang *et al*, 1997). In this study annexin-V was used to detect the exposure of phosphatidyl serine on the cell surface. Cytochrome c levels in the mitochondria and cytosol were quantified via a human cytochrome c Elisa assay.

4.3.1 The effects of test compounds on the cell morphology

4.3.1.1 Changes in cell morphology

To determine whether the imidazo[1,2-a]pyridine induced cell death is associated with apoptosis, the cellular morphology of the treated HT-29 and Caco-2 cells was investigated in a time-dependent manner (Figure 3.16-3.17). The onset of apoptosis is characterised by morphological cell features such as the shrinkage of the cell and a change in the size of the nucleus including condensation of nuclear chromatin. Another key feature that is found on apoptotic cells is the formation of cell membrane protrusions called blebs. As apoptosis progresses; the nucleus condenses and round circular fragments are visible. Lastly the cells

start to round up, detach from the cell surface and form convoluted extensions (Kroemer *et al*, 2009).

The data obtained illustrates that the morphology of cells after exposure to the selected active test compounds was markedly different when compared to that of the untreated HT-29 and Caco-2 cells (Fig 3.16-Fig 3.17). Morphological characteristics of apoptotic cells such as plasma membrane blebbing, cell shrinkage, cell detachment, development of elongated processes and loss of normal cell shape was observed in the treated HT-29 and Caco-2 cells. Moreover the severity of morphological features increased with an increase in exposure times. As early as 8 hours post-exposure HT-29 and Caco-2 cells treated with the selected compounds displayed cell morphology that was markedly different when compared to that of the untreated cells. After 48 hours of exposure to the selected compounds the treated HT-29 and Caco-2 cells were completely rounded up and lost adherence to the cells surface.

After an eight hour exposure period to camptothecin, JD-88, JD-137, JD-253 and JD-258, HT-29 cells started dissociate from neighbouring cells as shown in Figure 3.16. After an 18 hour exposure period HT-29 cells exposed to JD-88 and JD-137 formed unusual multi-lobular structures while HT-29 cells exposed to JD-88 started to form filopodia (Figure 3.16). After a 24 hour exposure period, HT-29 cells treated with JD-258, showed signs of cell membrane blebbing. After a 24 hour exposure to JD-137, JD-88 and JD-258 vacuoles were present.

After an 8 hour exposure period treated Caco-2 cells began to form filopodia after treatment with JD-88. As seen in Figure 3.17 compound JD-253 was able to induce cytoplasmic vacuole formation in the Caco-2 cells after a 24 hour exposure period. Caco-2 cells exposed to JD-256 developed filopodia. Cytoplasmic vacuolisation was present in Caco-2 cells treated with JD-256 for 48 hours. Cytoplasmic vacuolisation is not usually associated with apoptosis, but rather with a non-apoptotic cell death (Gozuacik and Kimchi, 2001).

All the compounds selected caused a decrease in cell number and a loss of adherence to the growth surface most likely indicating cell death after an eight hour exposure period.

4.3.1.2 Morphological changes in treated HT-29 and Caco-2 cells after staining with acridine orange, ethidium bromide and Hoechst-33342

Apoptosis can be recognized by several features that include loss of cell volume, cell membrane blebbing, lysosomal aggregation and condensation of the nucleus. The differential uptake of fluorescent DNA binding dyes such as ethidium bromide, acridine orange and Hoechst-33342 staining is a method for the distinguishing between live, apoptotic and necrotic cells. This method is used due to its simplicity (Kamal *et al*, 2014).

HT-29 and Caco-2 cells exposed to the test compounds displayed morphological features that are not usually observed in normal, untreated cells (Figure 3.18 and Figure 3.19). Morphological changes observed in treated cells included cell shrinkage, changes in cell shape, detachment from the growth surface, loss of intercellular attachments, development of elongated processes, lysosomal aggregation and membrane blebbing, irregular nuclei morphology (presence of punctate nuclei, difference in pattern of Hoechst stain and changes in nuclei size (Mpoke and Wolfe, 1997; Hajrezaie *et al*, 2014; Chen *et al*, 2015).

Fluorescence microscopy of HT-29 and Caco-2 cells stained with Hoechst showed that the nuclei of untreated cells were typically oval in shape, and stained blue with a uniform distribution of the dye (Figure 3.18A and Figure 3.19A). After staining with Hoechst the nuclei of HT-29 cells treated with JD-88, JD-137 and JD-253 appeared irregular shaped and showed variations in the sizes (Figure 3.18A and Figure 3.18B). This was also seen in Caco-2 cells treated with JD-88, JD-253 and JD-256. After staining with Hoechst, the nuclei of HT-29 cells treated with JD-253 appeared fragmented and this was not observed in Caco-2 cells treated with JD-253.

The percentage of cells with irregular nuclei morphology of the treated HT-29 (Table 3.10) and Caco-2 (Table 3.10) cells was determined by observing the staining pattern of the Hoechst stain and by the presence of punctate nuclei. Hoechst staining of HT-29 cells indicated that a high percentage of cells had irregular nuclei (60%) after treating cells with compound JD-253. Caco-2 cells treated with JD-256 indicated that 40% of the cells had nuclei with an irregular morphology. The observed changes in the pattern staining of the Hoechst stain of the nuclei of treated HT-29 and Caco-2 is indicative of a pre-apoptotic state as seen in Appendix C

Fluorescence microscopy of HT-29 and Caco-2 cells stained with the AO/EB stain showed lysosomal aggregation around the nucleus, which is indicative of a degenerating nucleus (Mpoke and Wolfe, 1997; Ubah and Wallace, 2014). This was observed in both HT-29 and Caco-2 cells, when the cells were exposed to the active test compounds. Acridine Orange also stained the nuclei of the cells an orange/yellow colour instead of the usual bright green colour and this is another indication of a pre-apoptotic state (Mpoke and Wolfe, 1997). This was observed in HT-29 cells treated with JD-253, and JD-88 (Figure 3.18) and with the Caco-2 cells, treated with JD-253 and JD-88 (Figure 3.19).

4.3.2 The effect of the test compounds on caspase-3/7 activity

The evaluation of caspase-3/7 activity is considered to be one of the most important biochemical indicators of apoptosis (Takahashi, 1999). Caspase-3 and caspase-7, the key executioners of apoptosis, are important in the cleavage and degradation of a number of cellular components that are associated with DNA repair and regulation of apoptosis (Kroemer *et al*, 2009; Shen *et al*, 2010). Caspase-3 is the most commonly activated death protease and it catalyses the cleavage of many key cellular proteins which ultimately leads to the execution-phase of apoptosis. Caspase-3 is activated in the apoptotic cell by both the intrinsic and extrinsic pathways (Earnshaw *et al*, 1999).

In the present study the level of caspase-3/7 activity was determined to provide evidence of apoptotic cell death as shown in Figure 3.20 and Figure 3.21. Caspase-3/7 activity was detected after two hours of exposure with the compounds in the HT-29 cells and Caco-2 cells. JD-258 caused the greatest increase in activity at two hours in the HT-29 cells. In contrast in the Caco-2 cells compound JD-256 caused the greatest increase in caspase-3/7 activity after two hours. At four hours caspase-3 activity was the highest in both cell lines. Compound JD-253 was the most potent and induced caspase-3 activity in the HT-29 cells that was similar to camptothecin and with the Caco-2 cells JD-88 and JD-256 induced caspase-3/7 activity to a similar level of camptothecin. The high levels of caspase-3/7 activity that was observed after four hours shows that apoptosis was initiated soon after exposure to the imidazo[1,2-a]pyridines. The increased levels of caspase-3/7 activity indicates that the mode of cell death induced by the active imidazo[1,2-a]pyridines compounds was caspase-dependent. An increase in caspase-3/7

activity provides further evidence for apoptosis induction. After a 24 hour exposure period caspase-3/7 levels in both cells was lower than the untreated cells. The low levels caspase-3/7 activity after a 24 hour exposure in the tested cell lines could occur due to the cytotoxic effects of the active test compounds (Kumar *et al*, 2007).

4.3.3 Annexin-V

Annexin-V is a calcium-dependent, phospholipid-binding protein that interacts with phosphatidyl serine. The Annexin-V assay, gives an indication of the number of cells that have phosphatidyl serine exposed on the outer leaflet of the cell membrane (Fadok *et al*, 1992; Chen *et al*, 2008). Annexin-V in conjunction with propidium iodide is widely used to determine if cells are viable, apoptotic or necrotic. Propidium iodide is an intercalating and fluorescent molecule that is used as a DNA stain to evaluate cell viability. Propidium iodide's ability to enter a cell is dependent on membrane integrity and it does not stain living or early apoptotic cells (Rieger *et al*, 2011)

The conventional Annexin-V/PI assay done by cytometry assay, can lead to a various false positives which can occur as result of trypsinization. It is well known that the trypsinization of adherent cells can cause changes to the cell membrane (Bundscherer *et al*, 2013). Zhang *et al*, (1997) demonstrated an altered Annexin-V/PI protocol which did not include the trypsinization of cells. This assay was used to detect apoptotic cells in culture, using fluorescence microscopy (Zhang *et al*, 1997).

In this study the binding of a FITC-conjugated annexin-V molecule was used to detect phosphatidyl serine exposure to the outer membrane of cells exposed to active test compounds in both HT-29 and Caco-2 cells. The HT-29 and Caco-2 cells exposed to the four selected compounds expressed fluorescence activity although exposure time varied in certain compounds. In the HT-29 cells exposure to JD-253, JD-258 and JD-137 expressed fluorescence activity as early four hours after exposure to the compounds while HT-29 cells exposed to JD-88 expressed fluorescence activity after six hours (Figure 3.22). This result corresponds to the increase of caspase-3/7 that was observed after exposure to the compounds.

After exposure to JD-256, stained Caco-2 cells showed an increase in fluorescence activity after six hours (Figure 3.23). This indicated some differences in terms of the time it takes for phosphatidyl serine translocation between different cell lines. Caco-2 cells treated with JD-88, JD-96M and JD-253 expressed fluorescence activity after an eight hour exposure period (Figure 3.23)

The exposure of phosphatidyl serine on the outer leaflet of the cell membrane after exposure to active compounds provides further evidence that the test compounds were able to induce apoptosis in the HT-29 and Caco-2 cells. Camptothecin treated cells showed a high percentage of cells in late apoptosis or necrosis which was not seen in HT-29 or Caco-2 cells treated with the active test compounds. The HT-29 cells were exposed to camptothecin for 6 hours while the Caco-2 cells were exposed to camptothecin for 8 hours.

4.3.4 Evaluation of the subcellular distribution of Cytochrome c

Mitochondria play a key role in the apoptotic process. The intrinsic apoptotic pathway is initiated in response to death stimuli that includes DNA damage, chemotherapeutic agents, serum starvation, and UV radiation. The inter-membrane space of the mitochondria contains a number of proteins, including cytochrome c that promotes cell death via apoptosis once they are redistributed in the cytosol (Smith *et al*, 2008). Several pro-apoptotic stimuli induce the permeabilization of the outer cell membrane, facilitate the communication between intermembrane and intercristae spaces and promote the mobilization of cytochrome c (Chipuk *et al*, 2007).

The anti-apoptotic proteins Bcl-2 and Bcl-xL reside in the outer mitochondrial wall and prevent cytochrome c release by inactivating the inner mitochondrial permeability transition pore (Wang and Youle, 2009). The pro-apoptotic Bcl-2 proteins Bad, Bid, Bax, and Bim are found in the cytosol and translocates to the mitochondria following cellular stress, where they promote the release of cytochrome c. The interaction between the pro-apoptotic and the anti-apoptotic Bcl-2 proteins disrupts the normal functioning of the anti-apoptotic Bcl-2 proteins and promotes the formation of pores in the mitochondrial membrane. The formation of pores initiates the apoptotic process by releasing cytochrome c into the cytosol (Czabotar *et al*, 2014).

Once in the cytosol, cytochrome c binds to the Apaf-1 molecule and ATP, which results in the activation of pro-caspase-9 in the presence of dATP or ATP. Caspase-9 is responsible for the cleavage and activation of pro-caspase-3 and caspase-7 (Ow *et al*, 2008; Campos *et al*, 2006).

In this study the distribution of cytochrome c in the mitochondria and cytosol was determined for the HT-29 cells since they were more sensitive than the Caco-2 cells to the test compounds. The results obtained from Figure 3.24 shows that JD-88, JD-137 and JD-253 increased the cytosolic cytochrome c fraction but not as much as camptothecin.

Camptothecin caused the greatest increase in cytosolic cytochrome c protein with 71% of the total measured cytochrome c protein being found in the cytosol. From the test compounds that were tested, JD-137 caused the greatest increase in the cytosolic cytochrome c protein with 38% of the cytochrome c found in the cytosol albeit not as high as camptothecin. Therefore the changes seen in the distribution of cytochrome c in the HT-29 cells treated with JD-137 indicate that apoptotic changes to the mitochondrial membrane did occur.

With the exception of JD-137, data collected in this study does not support apoptosis via intrinsic pathway. However, this could be a function of exposure time or that the cells would only enter apoptosis at a later stage when a different mechanism triggers the cell stress initiating the mitochondrial leakage of cytochrome c. The current study did not conduct enough assays to exclude intrinsic pathway therefore more assays are needed to draw further conclusions.

The apoptotic inducing effects seen after treating the HT-29 cells with JD-253, JD-88 and JD-258 may occur via the extrinsic apoptotic pathway. The extrinsic apoptotic pathway involves transmembrane death receptors that are members of the tumour necrosis factor receptor family and is initiated via the binding of the death ligands to their death receptors (Wong, 2011).

Data obtained in this study indicates that JD-88, JD-96M, JD-137, JD-253, JD-256 and JD-258 are able to induce apoptosis with minimal evidence of necrosis. The determination of the subcellular distribution of cytochrome c indicates that JD-137 is likely to activate the intrinsic mitochondrial pathway of apoptosis. This study shows that the imidazo[1,2-a]pyridine derivatives have the potential for the testing in further pre-clinical studies. These derivatives

were able to induce apoptosis in CRC cell lines with minimal cytotoxicity against normal cells such as leucocytes.

CHAPTER 5: Conclusion

Colorectal cancer is the third most common cancer in the world, with nearly 1.4 million new cases diagnosed in 2012. Colorectal cancer remains a major health problem in world. According to GLOBOCAN statistics, colorectal cancer accounts for more than 600,000 patient deaths every year (www.globocan.iarc.fr). The prevalence of colorectal cancer in Sub-Sahara Africa for both sexes was found to be 4.04 per a 100 000 population and shows an increased incidence with increasing age (World Cancer Report, 2014). The highest incidence was in Southern Africa, particularly in South Africa. Studies have estimated that Africa will have an 85% increase in the cancer burden in the next 15 years therefore it is imperative that new and improved treatment options are discovered (Graham *et al*, 2013). In African countries there is a shortage of colorectal screening programs and poor access by the underprivileged population to standard treatment for this disease (Laiyemo *et al*, 2015).

Approximately 25% of colorectal cancer patients present with synchronous metastases. More than 40% of colon cancer sufferers develop metastases for which the five-year survival rate is estimated at only 13% (Saletti *et al*, 2015). Increased knowledge regarding the molecular features of colorectal cancer has led to the development of targeted therapy with monoclonal antibodies such as cetuximab and bevacizumab. However this treatment path is prohibitively expensive and have limited efficacy since it is only effective in a subset of patients with a specific molecular signature patients presenting with KRAS, NRAS and PI3k mutations do not respond to cetuximab (Saletti *et al*, 2015). Drugs that can induce apoptosis would be an ideal treatment option since apoptotic cell death evades the inflammatory immune response seen with necrotic cell death and shows a milder adverse effect profile.

In this study a series of azaindole and imidazo[1,2-*a*]pyridine derivatives were synthesized by the School of Chemistry, University of Witwatersrand. Their purity and structures were confirmed by NMR and mass spectroscopy. The compounds were evaluated against two colorectal cancer cells lines in order to identify new lead compounds with potential anticancer effects. After the identification of active compounds, compounds were evaluated to determine if the compounds induced apoptosis or necrosis in colorectal cancer cells. The cytotoxic activity of

the azaindole derivatives against both the HT-29 and Caco-2 cell lines was poor, therefore the azaindoles were not considered to be viable candidates for further investigations in this study.

However, the current study showed that the imidazo[1,2-*a*]pyridines derivatives induced cytotoxic activity in the HT-29 and Caco-2 cells with the effects of some of the imidazo[1,2-*a*]pyridine compounds being comparable to and better than that of camptothecin. HT-29 cells were more sensitive than the Caco-2 cells to the cytotoxic effects of the imidazo[1,2-*a*]pyridine derivatives. This is an important observation since Caco-2 cells display a more wild type phenotype than the HT-29 cells. Three compounds, JD-137, JD-253 and JD-258 showed IC_{50} values of around 5 μ M against the HT-29 cells which were better than camptothecin. In the Caco-2 cells JD-253 and JD-256 had IC_{50} values of 9.62 ± 0.4 and 6.22 ± 1.5 μ M which was lower than camptothecin (10.60 ± 1.5 μ M). Four of the most active compounds against each cell line were selected for further studies to determine their mechanism of action.

JD-81 displayed selectivity for the HT-29 cells in that it inhibited the HT-29 cells with an IC_{50} value of 10.69 ± 0.4 μ M but was completely inactive against the Caco-2 cells. JD-80 displayed selectivity towards the Caco-2 cells in that it inhibited the Caco-2 cells with an IC_{50} value of 14.02 ± 0.8 μ M but was completely inactive against the HT-29 cells. The selectivity of the compounds to either the HT-29 cells or the Caco-2 cells could be the result of the different mutations that is observed in colorectal cancer cells. The HT-29 cells are positive for genetic mutations of the KRAS, BRAF, PIK3CA and the TP53 (p53 tumour suppressor) gene while the Caco-2 cells are positive for a genetic mutation of the TP53 gene (Ahmed *et al*, 2013).

An investigation into the toxicity of the compounds towards peripheral leucocytes indicated that the test compounds showed a degree of selective cytotoxicity to the cancer cell lines as compared to normal unstimulated white blood cells. JD-81, JD-96M, JD-137 and JD-254 were significantly less toxic the leucocytes than camptothecin. On the other hand JD-88, JD-253, JD-256 and JD-258 were significantly more toxic to the leucocytes than to camptothecin. Therefore these compounds may have a detrimental adverse effect profile. However, this does require confirmation by further preclinical studies.

This study showed that in HT-29 cells four compounds, JD-88, JD-137, JD-253 and JD-258 were able to induce apoptosis. In the Caco-2 cells three compounds, JD-88, JD-253 and JD-256 were able to induce apoptosis. This was supported by specific apoptotic morphological features that were observed in the cells treated with the compounds. The main differences observed in cells exposed to the test compounds for 24 hours were fragmented nuclei, lysosomal aggregation, appearance of pre-apoptotic nuclei and blebbing of cell membranes. After exposing Caco-2 cells to JD-256 chromatin condensation was observed. Data collected in this study indicates that the JD-88, JD-137, JD-253, JD-258, JD-256 are able to induce apoptosis with minimal evidence of necrotic cell death in the HT-29 and Caco-2 cells.

The evaluation of caspase-3/7 activity showed that after a two hour exposure period to JD-137, JD-253 and JD-258 there was a greater increase in caspase-3/7 activity compared to camptothecin in the HT-29 cells. In the Caco-2 cells after a 4 hour exposure period, JD-88 and JD-256 caused an increase in caspase-3/7 activity when compared to camptothecin.

This together with the translocation of phosphatidyl serine to the cell surface of the HT-29 and Caco-2 cells indicates that these compounds induced apoptosis in a caspase-dependent manner within a short time following exposure (2 to 4 hours). In the HT-29 cells JD-253, JD-137 and JD-258 were able to translocate phosphatidyl serine from the inner to the outer leaflet of the plasma membrane after four hours and JD-88 after six hours. In the Caco-2 cells JD-256 was able to translocate phosphatidyl serine from the inner to the outer leaflet of the plasma membrane after six hours and JD-88, JD-253 and JD-96M was able to translocate phosphatidyl serine after eight hours. This implies that the caspase-3/7 activity increase occurs before the phosphatidyl serine switch which is generally seen as an early event in the apoptotic process. This is an important finding and due to the early changes seen for both these parameters hints that the cytotoxic effect probably does not involve specific cell cycle events.

After exposing HT-29 cells to JD-137, cytochrome c leaked from the mitochondria to the cytoplasm and may be an indicator that the intrinsic apoptotic pathway was activated. The results obtained after treating the HT-29 cells with JD-88, JD-253 and JD-258 indicated that the cytosolic cytochrome c release from mitochondria is not responsible for the induction of the apoptotic process by these compounds. However, the release of cytochrome c from the

mitochondria may be time-dependent or may require another event to take place first. The data obtained in this study indicates that the apoptotic inducing effects of compound JD-137 could be a result of the activation of the intrinsic pathway in HT-29 cells. The evaluation of caspase-9 activity and /or the mitochondrial membrane potential will further clarify this observation.

The development of analogues to the imidazo[1,2-*a*]pyridine amines similar in structure to the most active compounds indicates that the base amine structure of the imidazo[1,2-*a*]pyridine derivative plays a key role in the cytotoxic activity of these compounds. The addition of a copper group to the base structure significantly increased the activity of the compound (JD-80) against both the HT-29 and Caco-2 cells. Copper complexes in JD-253 significantly increased the activity against the HT-29 cells while the copper complex in JD-256 significantly increased the activity in the Caco-2 cells compared to the zinc and platinum complexes. The addition of a benzyl group to the amidogen group significantly increased the activity of the compound against both the HT-29 and Caco-2 cells. In contrast the addition of an amantadine group to the amidogen group significantly increased the activity of the compound against the HT-29 cells but the compound was inactive against the Caco-2 cells. The structure of compound JD-137 did not include a metal complex and behaved differently in that it was able to cause a leakage of cytochrome c from the mitochondria to the cytoplasm. Moreover the cytotoxic activity of the compound was higher in the HT-29 cells compared to the Caco-2 cells.

The imidazo[1,2-*a*]pyridine derivatives are cytotoxic compounds against colorectal cancer cell lines with promising cytotoxic properties. JD-253 and JD-137 have both shown promising activity against colorectal cancer cell lines and data from this study has shown the ability of these compounds to induce apoptosis in these cells. Before these derivatives can be safely used as anti-cancer agents they have to be subjected to further evaluation in terms of toxicity and efficacy. The exact mechanism of the induction of apoptosis needs to be further evaluated. An investigation into the death-receptor proteins, initiator caspases, the Bcl-2 and Bax proteins and the activity and expression of the p53 gene needs to be conducted to determine their potential role in the induction of apoptosis by the imidazo[1,2-*a*]pyridine derivatives.

The evaluation of toxicity/safety profiles of newly designed agents can be performed on cancer cell lines but requires validation in animal models. It is of utmost importance that these

molecules are evaluated for efficacy in an *in vivo* setting with an animal model of colorectal cancer. This is commonly done with nude mice studies. In addition, this will provide a histological evaluation of acute and chronic toxicity to the kidneys, liver, and heart (Schuh, 2004).

In this study the aim of evaluating the cytotoxic effects of the imidazo[1,2-*a*]pyridine and azaindole derivatives was achieved. In addition, the ability of the compounds to induce apoptosis was studied. Six imidazo[1,2-*a*]pyridine compounds, JD-88, JD-96M, JD-137, JD-253, JD-256 and JD-258 showed cytotoxic effects comparable to camptothecin against the HT-29 and Caco-2 cells. Four of these imidazo[1,2-*a*]pyridines proved to be inducers of apoptosis and are therefore worthy of further investigation. Imidazo[1,2-*a*]pyridine amines are cytotoxic compounds against both the HT-29 and Caco-2 cells with promising cytotoxic properties. Manipulation of the base structure of these compounds have given rise to new copper containing configurations with improved activity against both cell lines.

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APPENDICES

Appendix A

Human Research Ethics Committee (Medical)
(formerly Committee for Research on Human Subjects (Medical))

Secretariat: Research Office, Room SH10005, 10th floor, Senate House • Telephone: +27 11 717-1234 • Fax: +27 11 939-5708
Private Bag 3, Wits 2050, South Africa

University
of the Witwatersrand,
Johannesburg



Ref: W-CJ-130614-1

14/06/2013

TO WHOM IT MAY CONCERN:

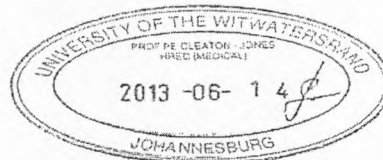
Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: Dr L Harmse, Ms S Ragie (student no 362832).

Project title: The effects of azaindoles and imidazopyridines on colon cancer cell lines.

Reason: This is an *in vitro* laboratory study using HT29 and Caco-2 cell lines. No humans are involved.

A handwritten signature in black ink, appearing to read 'Peter Cleaton-Jones'.



Professor Peter Cleaton-Jones
Chair: Human Research Ethics Committee (Medical)

copy: Anisa Keshav, Zanele Ndlovu, Vits Research Office



R14/49 Dr Robyn L van Zyl

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M140669

NAME: Dr Robyn L van Zyl
(Principal Investigator)

DEPARTMENT: Pharmacy & Pharmacology
Medical School

PROJECT TITLE: The Chemotherapeutic Properties of Novel Synthetic
and Natural Compounds (Renewal previously M090532)

DATE CONSIDERED: 29/05/2009 (Initial Approval) 26/06/2014 (Renewal)

DECISION: Approved unconditionally

CONDITIONS:

SUPERVISOR:

APPROVED BY: 
Professor PE Cleaton-Jones, Chairperson, HREC (Medical)

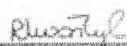
DATE OF APPROVAL: 26/06/2014

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. I agree to submit a yearly progress report

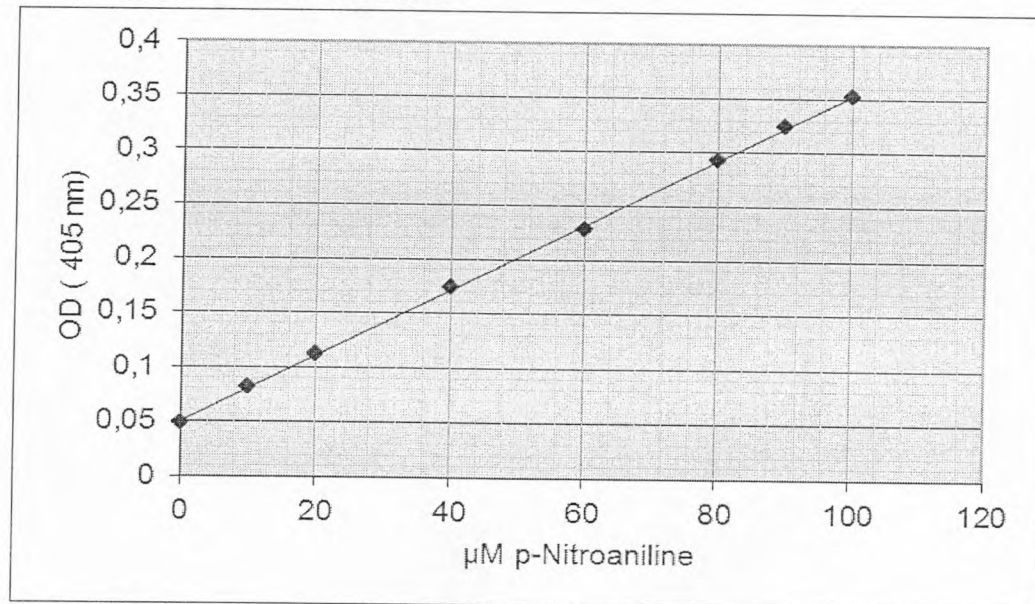

Principal Investigator Signature

Date 21-07-2014

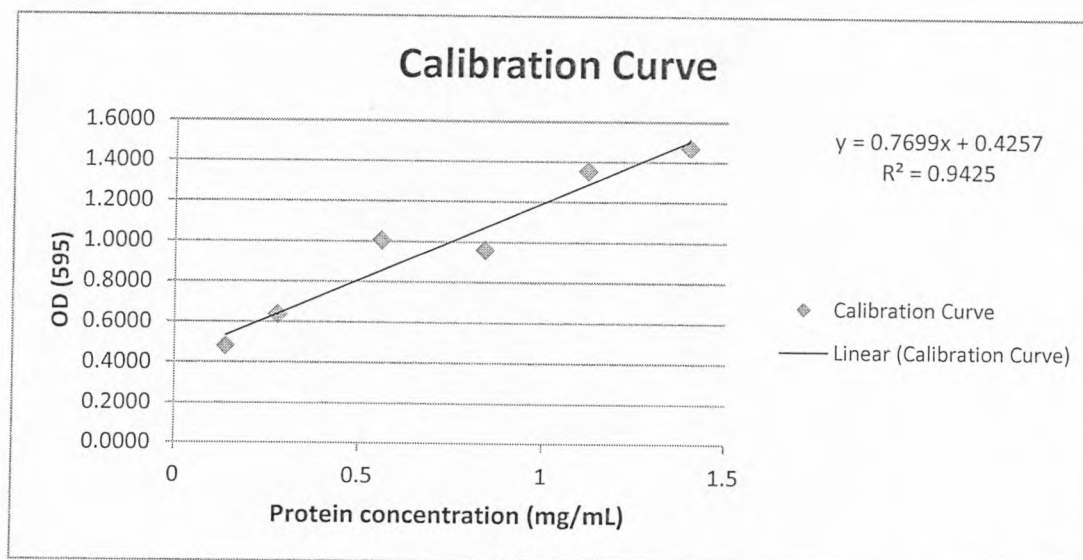
PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix B: Calibration Curves

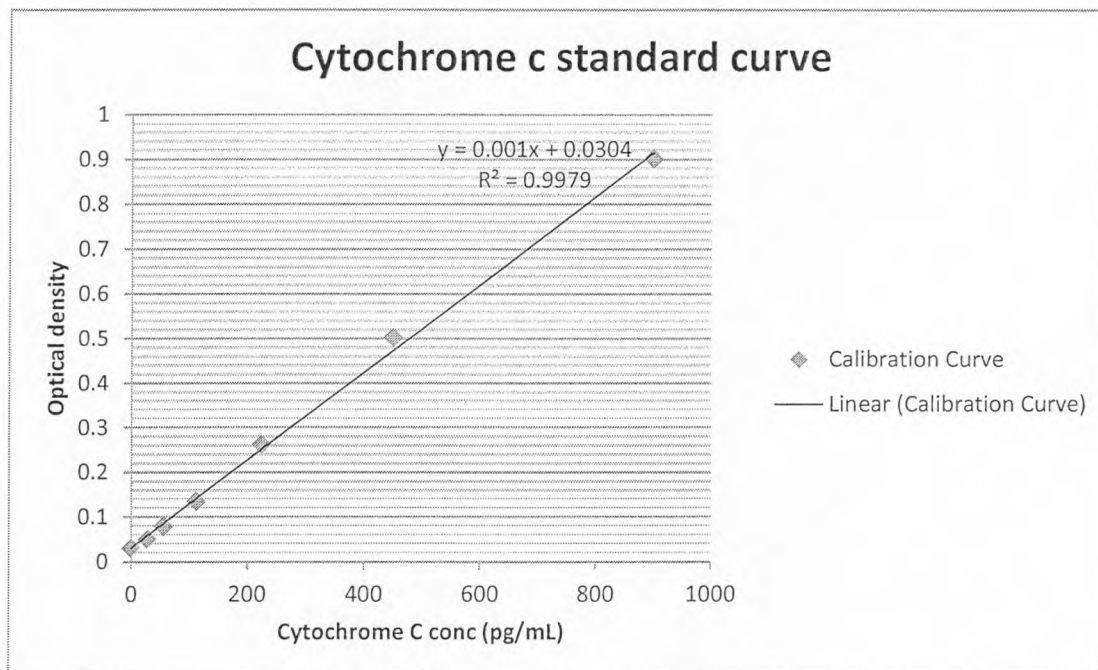
Appendix B1. pNA Calibration Curve



Appendix B2. A representative of the Bio-Rad Protein Assay Calibration Curve (Calibration curve used to calculate protein concentration in test samples)

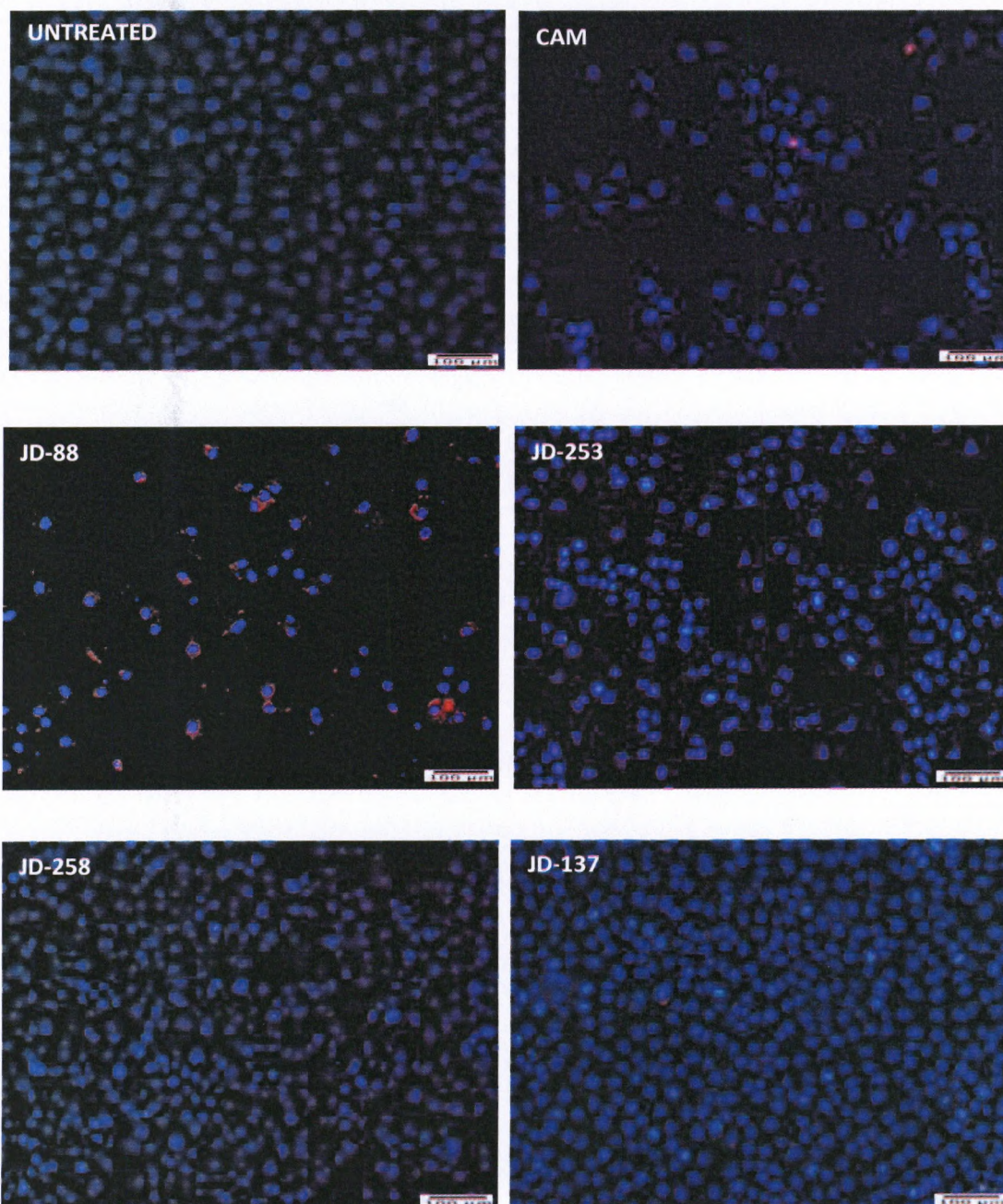


Appendix B3. Cytochrome c standard curve (calibration curve used to calculate cytochrome c activity in test compounds)

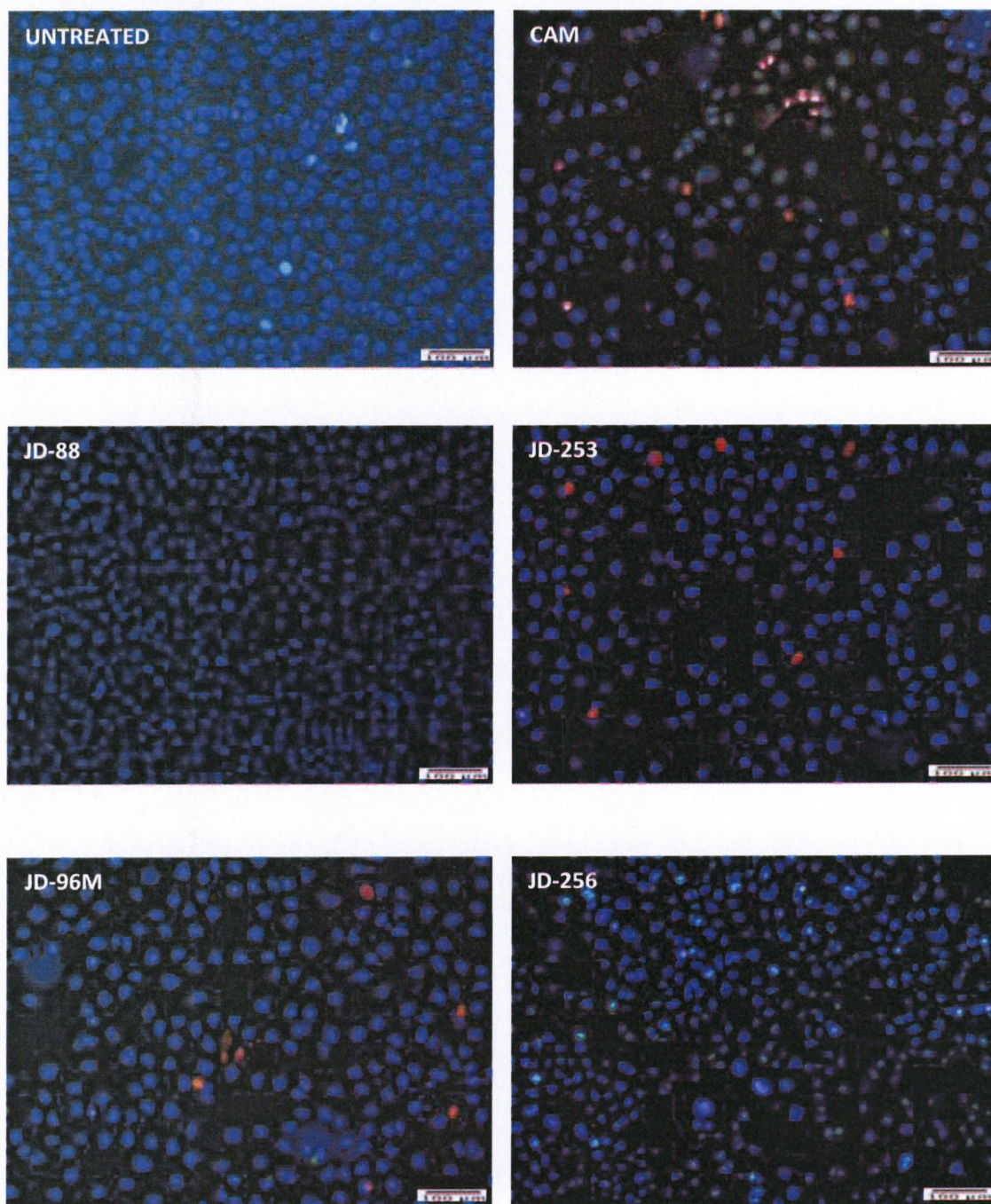


Appendix C: Fluorescence microscopy

Appendix C1. HT-29 cells exposed (24hrs) to the most active compounds and camptothecin

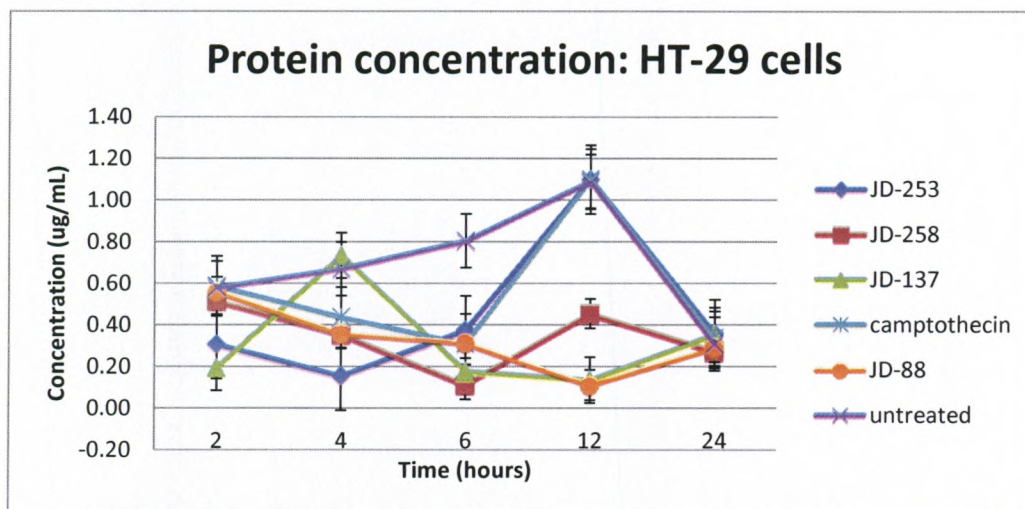


Appendix C2. Caco-2 cells exposed (24 hrs) to the most active compounds and camptothecin

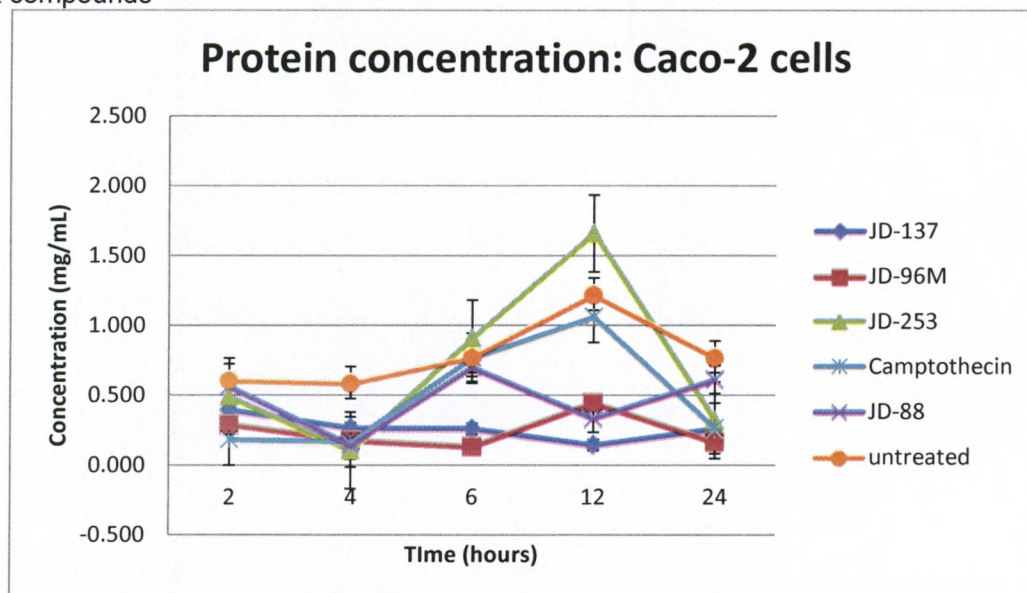


Appendix D: Protein concentration after exposure to test compounds

Appendix D1. Protein concentration (mg/mL) found in the HT-29 cell lysates after exposure to test compounds



Appendix D2. Protein concentration (mg/mL) found in the Caco-2 cell lysates after exposure to test compounds



Appendix E: Percentage growth of HT-29 cells after exposure to JD-119 for various concentrations

Concentration (μM)	Percentage growth (%)		
200	11	12	12
100	29	28	28
75	34	35	36
50	37	38	39
25	40	44	45
10	49	48	49
1	67	67	65
0.5	70	71	72
0	101	104	102

Appendix F: Request to use Figure

Re: Permission to use figure

Thursday, April 28, 2016 2:15 AM

[Mark as Unread](#)

From:

"Rebecca Wong" <rebeccawongsy@gmail.com>

To:

"Somayya Ragie" <somayyaragie@yahoo.com>

[Full Headers](#) [Printable View](#)

Dear Somayya,

You may include the figure in you dissertation.

Regards,

Dr. Rebecca Wong

Associate Professor

Faculty of Medicine

SEGi University

On Tue, Apr 26, 2016 at 8:50 PM, Somayya Ragie <somayyaragie@yahoo.com> wrote:

Good morning Ms. Wong

I am an MSC (med) student studying at the University of witwatersrand. I am currently writing up my dissertation and I would like to obtain your permission to use your figure on the apoptotic pathways that was used in the journal article titled "Apoptosis in cancer: from pathogenesis to treatment" in my dissertation.. Your approval would be highly appreciated.

Regards

Somayya Ragie

MSC (med) student