Insight into the mechanisms by which apigenin, curcumin and sulfasalazine induce apoptosis in colon cancer cells

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ABSTRACT

Two dietary components, curcumin and apigenin along with an anti-inflammatory drug sulfasalazine (SSZ) effect apoptosis in colon cancer cells. However the exact signalling mechanisms involved in this response remain unknown. We investigated their influence, on the ceramide pathway and their effects upon reactive oxygen species (ROS) generation in colorectal cancer (CRC) cell lines.

Initial observations confirmed that each agent dose dependently caused an increase in cell death as indicated by release of cytochrome C, cleavage of pro-caspase 3, PI staining and nuclear condensation, indicative of programmed cell death or apoptosis.

Since ceramide generation has been linked with apoptosis and cell death, we investigated its role in CRC cells. Here for the first time we have shown that all three agents were able to increase ceramide levels in a dose and time dependent manner. Interestingly, SSZ accomplished this increase through the de novo pathway, while the other two dietary agents (curcumin and apigenin) elevated ceramide generation independently of the de novo pathway. This data is supported by inhibition of ceramide generation by both Myriocin and Fumonisin B-1 (FB-1), inhibitors of de novo synthesis. However this was not mechanistically linked to cell death.

Apigenin, curcumin and sulfasalazine also caused an increase in ROS generation in HCT116 cells. Using a series of ROS inhibitors, the route of ROS generation was established. We postulated that apigenin interacted directly with the mitochondrial electron transport chain in order to generate ROS while the other two agents interacted in a more generalized manner in order to generate ROS. Inhibition of ROS in CRC cells treated with each agent lead to increase in cell survival.

This study demonstrates that all three compounds are capable of generating both ROS and ceramide through different mechanisms. Additionally, ROS generation due to treatment with each agent is prominent in mediating apoptosis.
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LIST OF ABBREVIATIONS

A
A, API Apigenin
ADP Adenosine di-phosphate
AIF apoptosis inducing factor
APC Adenomatous polyposis coli
5'-ASA 5'-aminosalicylic acid
Asp Aspartate
ATF-2 Activating transcription factor-2
ATP Adenosine tri-phosphate

B
BHA Butylated hydroxyanisole
BSA Bovine serum albumin
BSO Buthionine sulfoximine

C
C, CURC Curcumin
CARD Caspase recruitment domain
Caspases Cysteinyl-aspartic-acid-proteases
CAT Catalase
CRC Colorectal cancer
CSA Cyclosporin A
Cyt C Cytochrome C
CK2 Casein kinase II

D
DCFH-DA 2', 7'-dichlorofluorescein-diacetate
DD Death domain
DED Death effector domain
DISC Death-Inducing signalling complex
DTT Dithiothreitol

E
ECL Enhanced chemiluminescence
EDTA Ethylene diamine tetraacetate disodium salt
EGTA Ethylene bis (oxyethylenenitrilo) tetraacetic acid
EGFR Epidermal growth factor receptor
ER Endoplasmic reticulum
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>FACS</td>
<td>Fluorescein-activated cell sorter</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associating death domain</td>
</tr>
<tr>
<td>FAP</td>
<td>Familial adenomatous polyposis</td>
</tr>
<tr>
<td>FB-1</td>
<td>Fumonisin B1</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HNPCC</td>
<td>Hereditary nonpolyposis colorectal cancer</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>JNK/SAPK</td>
<td>Jun N-terminal kinase</td>
</tr>
<tr>
<td>M199</td>
<td>Medium 199</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinases</td>
</tr>
<tr>
<td>MMR</td>
<td>Mismatch repair</td>
</tr>
<tr>
<td>MTS</td>
<td>[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetyl cystein</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaF</td>
<td>Sodium fluoride</td>
</tr>
<tr>
<td>NFkB</td>
<td>Nuclear factor κB</td>
</tr>
<tr>
<td>N-Smases</td>
<td>Neutral sphingomyelinase</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly (ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PEN/STREP</td>
<td>Penicillin-streptomycin</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKB/Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PMS</td>
<td>Phenazine methosulfate</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenyl methylsulphonyl fluoride</td>
</tr>
<tr>
<td>PTP</td>
<td>Permeability transition pore</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>R</td>
<td>Receptor interaction protein</td>
</tr>
<tr>
<td>RIP</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>S</td>
<td>Sphingomyelin</td>
</tr>
<tr>
<td>SM</td>
<td>Sphingomyelinase</td>
</tr>
<tr>
<td>SMase</td>
<td>Sphingomyelinase</td>
</tr>
<tr>
<td>SP</td>
<td>Sulfapyridine</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutases</td>
</tr>
<tr>
<td>SODD</td>
<td>Silencer of death domains</td>
</tr>
<tr>
<td>SSZ</td>
<td>Sulfasalazine</td>
</tr>
<tr>
<td>T</td>
<td>Transforming growth factor β activated kinase</td>
</tr>
<tr>
<td>TAK</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumor necrosis factor α receptor</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNF receptor associated death domain protein</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor associated factors</td>
</tr>
<tr>
<td>U</td>
<td>Ulcerative colitis</td>
</tr>
<tr>
<td>UC</td>
<td>Ubiquinone</td>
</tr>
<tr>
<td>UQ</td>
<td>Ubiquinone</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet radiation</td>
</tr>
<tr>
<td>V</td>
<td>Voltage-dependent anion channel</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage-dependent anion channel</td>
</tr>
</tbody>
</table>
ACKNOWLEDGMENTS

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Chapter 1. General Introduction

1.1 Cancer

Cancer is one of the modern plagues of western society. It is estimated that 139,900 new cases of cancer will be diagnosed across Canada and approximately 67,400 Canadians will lose their lives to this disease (Canadian Cancer Society, 2003).

Cancer has traditionally been described as a rapid and uncontrolled proliferation of cells. In general, cancer cells differ from normal neighbouring cells by exhibiting a number of different properties such as, rapid division rate, invasion of surrounding tissue, high metabolic rate and altered shape (Griffiths, 1996).

Today various types of cancers have been identified. One of the major types of cancers plaguing North America today is colorectal cancer (Boland et al., 2000).

1.2 Colorectal Cancer

Colorectal cancer (CRC) has been dubbed as a major cause of morbidity and mortality in North America. It is the 3rd most common cause of cancer mortality in Canada and United States, and is ranked 2nd next to lung cancer in non-gender specific cancers (Lichtenstein, 2002).

In a study conducted by the National Cancer Institute of Canada (2003) an estimated 18,000 Canadians will be diagnosed and 8,300 (4,400 men and 3,800 women) will die from CRC. In the same study it was indicated that the incidence of CRC increases dramatically with age and is highest amongst 70-79 year olds.

Although the rates of both occurrence and mortality have declined in the past decade it is still one of the leading causes of death in North America.
1.2.1 Morphology of the colon

In order to better understand the pathology of CRC it is important to have a clear knowledge of the normal morphology of the large intestine. On average the large intestine is approximately 150-170 cm in length. It can be divided into ascending colon, transverse colon, descending colon, sigmoid colon, cecum and the rectum (Sanford, 1982). Figure 1.1a depicts a cartoon representation of the large intestine. Embryologically, different parts of the large intestine are derived from different sources. For example, the cecum, ascending colon and the proximal half of the transverse colon are all derived from the mid-gut section and the hind-gut gives rise to the distal colon.

Figure 1.1b shows a cross section of the large intestine. The tissue layers, proceeding from the inside out, are mucosa, the sub-mucosa, muscularis (a combination of inner circular layer and an outer longitudinal layer) and the serosa. The serosa is a continuous layer that wraps around the outer portion of other abdominal organs as well as the large intestine. The serosa is rich in lymphatic vessels. Muscularis is the muscle layer of that allows for peristaltic movements of the luminal content. The sub-mucosa is a connective tissue layer, which contains nerves, blood vessels and lymph tissue. The last layer, epithelium, is composed of uniform finger like indentations named crypts. These crypts are connected to the lamina propria. Areas of the lamina propria that are near the intestinal lumen and between the crypts are covered by a single layer of surface epithelial cells. The upper portion of each crypt contains less vacuolated cells (Figure 1.1c).

Generally, crypts contain three main zones of cells. The first zone, at the base of the crypt is made up of stem cells. The cells present in the lower third of the crypt are involved in proliferation and differentiation. Conversely, the upper two thirds of the crypt consist of a migration zone. A normal colonic cell travels up the crypt during its life cycle and dissociates (anoikis) and then undergoes apoptotic death at the apex (surface epithelium) of the crypt (Shih et al., 2001). Apoptosis is described as programmed cell death and is, further discussed in section 1.3. This migration of colonic epithelial cells from the base to the surface epithelium takes approximately 6-7 days. The normal structure of the crypt is maintained by an existing balance between cell proliferation and apoptosis (Shanmugathasan and Jothy, 2000). This balance is disrupted in colon cancer.
Figure 1.1: Large intestine

A) Diagram of the colon
adapted from: http://www.joelertola.com/grfx/chrt_colonCancer.html

B) Adapted from: http://www.bartleby.com/107/illus1074.html
B) Normal colon tissue.
adapted from http://www.glycoforum.gr.jp/science
1.2.2 Etiology of colorectal cancer

It is estimated that approximately 50% of Western populations will develop a colorectal tumour by the age of 70. In consequence, 10% of these individuals, will progress to a malignant stage (Kinzler and Vogelstein, 1996; Parker et al., 1996).

There are various possible factors that have been linked to CRCs. These factors range from persistent inflammatory bowel disease or ulcerative colitis, to environmental factors, to a positive family history of colon cancer (Ekbon et al., 1990; Kinzler and Vogelstein, 1996; Boland, 2000).

1.2.3 Genetic factors

Studies suggest that approximately 15% of colorectal cancers result from dominantly inherited genetic patterns. There are two identified types of familial forms: FAP and HNPCC (Cannon-Albright et al, 1988; Houlston et al., 1992; Kinzler and Vogelstein, 1996).

Familial Adenomatous Polyposis (FAP) is a rare autosomal dominant disease, which affects approximately 1 in every 10,000 individuals (Goss and Groden, 2000). FAP patients develop multiple polyps during their second and third decades of life. FAP arises from a germline mutation in the Adenomatous Polyposis Coli (APC) gene. The APC gene is located on chromosome 5q21 (Bodmer et al., 1987; Leppert et al., 1987; Kinzler et al., 1991; Kinzler and Vogelstein, 1996). The APC gene plays an important role in degradation of the β-catenin transcription factor. β-catenin is known to interact with the Tcf-4 transcription factor, thus leading to transcription of genes such as PPARγ and c-Myc (Boland et al., 2000).

Hereditary Nonpolyposis Colorectal Cancer (HNPCC) is an autosomal dominant disease, which like FAP predisposes patients to colon cancer at an early age. HNPCC accounts for 3% of all colorectal cancers. This disorder is caused by mutations in DNA mismatch repair (MMR) genes. MMR genes are generally involved in repair of DNA errors that occur during replication. Loss of DNA MMR genes leads to a hyper-mutable state, in which simple repetitive DNA sequences (also known as micro-satellite regions) are particularly unstable during DNA replication. A few of these micro-satellite sequences are located in critical areas which are known to code genes involved in the regulation of cell growth (i.e. TGF-β type II receptor, TCF-4 and β-catenin). Further
mutations may lead to loss of tumour suppressor genes, and thus promotion of tumour growth.

**FAP vs. HNPCC**

In general, FAP is a disease, which is caused by the presence of a faulty gatekeeper gene APC. This allows for formation of thousands of benign tumours. Invariably, these tumours become malignant and progress to cancer (Kinzler and Vogelstein, 1996).

In HNPCC, adenomas form at the same rate as in the general population. Mutations in the MMR genes allow for acquisition of further mutations at a rate of 2-3 times faster than normal cells, and thus result in mutations in oncogenes and tumour suppressor genes leading to a rapid progression to malignancy. This acceleration in malignancy has been observed in mice with mutations in the murine MSH2 gene and also in humans with HNPCC (Kinzler and Vogelstein, 1996; Reitmair et al., 1996).

**1.2.4 Environmental factors**

Like many diseases, environmental factors such as diet and lifestyle play a crucial role in the development of this disease. Since food and its metabolites come into direct contact with the gastrointestinal mucosa, diet seems to play a key role in modulating cellular function. Studies in rats have highlighted the importance of diet in colorectal tumour progression (Zhao et al., 1991; Schatzkin, 1999). Notably, dietary factors such as fat, red meat and over cooked brown meat increase risk of development of colon cancer. Whereas vitamins (A, E, carotene and folate), minerals (calcium and selenium), dietary fibre and vegetables have been linked to decreased risk of colorectal cancer development (Schatzkin, 1999).

**1.2.5 Ulcerative colitis**

Ulcerative colitis (UC) otherwise known as chronic inflammatory bowel disease is typically prevalent in western societies, though its etiology is yet to be determined. An association between UC and CRC has been observed. This association is predominantly linked to presence of chronic inflammation (Seril et al, 2003). There is a morphological difference between UC associated colon cancer and sporadic colon cancer. This is due
to the difference in timing and frequency of similar genetic alterations between UC and CRC. There is evidence indicating that the presence of DNA damage due to oxidative stress contributes to CRC development in UC patients. Thus, various nutrients, such as iron, act as possible risk factors while dietary anti-oxidants play a protective role in UC associated carcinogenesis (Lichtenstein, 2002; Seril et al, 2003).

1.2.6 Colorectal carcinogenesis

CRC is labelled as the silent killer; this is because it is normally not detected until the disease has progressed to a deadly stage. In general cancers arise due to alterations at the genetic level. Colorectal cancers develop from accumulation of mutations of genes that control cell growth or programmed cell death (apoptosis) (Kinzeler and Vogelstein, 1996).

There are currently two pathogenic pathways linked to CRC (Boland et. al. 2000). Each pathway contains distinct forms of genetic deformities, though, whether the pathways diverge before or after an initial gatekeeper mutation (i.e. APC gene) remains unclear. The first pathway depends upon chromosomal instability, which leads to a loss of tumour suppressor genes. This is the most common pathway observed in approximately 80-85% of colorectal tumours. Unfortunately, there is still very little known about the mechanism for this type of genomic instability.

The second pathway (microsatellite instability) is observed in the remaining 15%-20% of colorectal cancers. This category deals with mutation in the mismatch repair genes, which leads to a hyper-mutable phenotype.

The K-Ras gene is one of the most common mutations in colorectal cancers. This mutation may be found in either of the mentioned pathways. Mutations in this gene lead to stabilization of K-Ras protein in the guanosine tri-phosphate bound form thus creating an oncogene, which causes for continuous activation of proliferative signals (Boland et. al. 2000).
1.3 Apoptosis

Apoptosis is the process of self destruction in cells which have been damaged, virally infected or are no longer receiving extra-cellular survival signals. Apoptosis is known to play an integral role in normal development and tissue homeostasis. Apoptosis has also been implicated in various disease processes including cancer (Cotter et al., 1990; Greil et al., 2003).

In general, apoptosis is characterized by a number of distinct phenotypical features such as, membrane blebbing, cytoplasmic shrinkage, and loss of phospholipids asymmetry, chromatin condensation and DNA fragmentation. Upon completion of apoptosis, cells break up into a series of membrane bound bodies called apoptotic bodies which contain normal but condensed chromatin. Ordinarily, these apoptotic bodies are phagocytosed by the adjoining cells. The mentioned features differentiate apoptotic death from other forms of cell death such as necrosis (Granville et al., 1998).

Various signals have been shown to play a key role in apoptosis. Cross talking between death receptors such as TNF α and Fas, serum deprivation, over-expression of certain oncogenes (e.g. c-myc) or tumour suppressor genes (e.g. p53), are all examples of signals involved in the initiation of apoptosis. There are two main mechanisms by which a cell commits suicide by apoptosis:

1. Intrinsic signals
2. Extrinsic signals

1.3.1 Intrinsic apoptotic signals

1.3.1.1 Caspases

Intrinsic signals include a mitochondrial based series of stimuli which lead to the activation of caspase family members. In 1993 researchers discovered similarities between ced-3 gene, known as the death gene in Caenorhabditis elegans and interleukin-1β-converting enzyme (ICE or caspase 1) (Yuan et al., 1993; Thornberry et al., 1992). Subsequent to the 1993 discovery, approximately 14 members (Table 1.1) of the caspase family have been identified (Creagh and Martin, 2001). There is significant evidence demonstrating the importance of caspases in mammalian apoptotic machinery.
In general, caspases (Cysteinyl-aspartic-acid-proteases) are expressed as pro-enzymes. They contain an N-terminal with a large subunit (~20 kDa), which contains an active cysteine site, and small subunits (~10 kDa). In between the pro-domain and the large subunit there is an aspartate cleavage site. Additionally, the small and large sub-units are separated via one or two aspartate cleavage sites (Nicholson and Thornbery, 1997).

Figure 1.2 illustrates caspase activation via proteolysis of the inter-domain linker (Asp-X), allowing for large and small subunits to associate and form a heterodimeric enzyme. Within the caspase family, caspases differ from each other due to their substrate preference, sequences and length of caspase pro-domain (Wolf and Green, 1999; Nicholson and Thornbery 1997).

Caspases are divided into two distinctive groups:

1) Initiator caspases, such as caspases-8 and-9 which in turn promote activation of downstream caspases.

2) Effector caspases, which include caspases-3,-6, and-7. It has been shown that while caspase 8 activates caspase-3 and -7, caspase-3 is itself capable of activating caspase-6 (Hirata et al., 1998; Creagh and Martin, 2001).

Caspase activation has been shown to disrupt phosphatidylserine asymmetry in the plasma membrane, by exposing it to the outer section of the plasma membrane. Generally, this asymmetry is maintained by confining the phosphatidylserine to the inner membrane (Zwall and Schroit, 1997).

Amongst the caspases identified, caspase-3 is regarded as one of the essential executioners in the apoptotic pathway (Faleiro et al., 1997). Caspase-3 is involved in cleaving a variety of proteins such as poly (ADP-ribose) polymerase (PARP) a DNA repair enzyme which is activity in presence of DNA damage. PARP also functions by consuming vast amounts of NAD\(^+\) which, leads to insufficient ATP and thus initiating apoptosis. It is also responsible for cleaving mitotic apparatus protein (NuMA) and therefore mediating DNA fragmentation and chromatin condensation (Hirata et al., 1998). Interestingly, caspase-6 has the capacity to cleave NuMa at different sites than caspase-3 and thus mediating nuclear shrinkage and fragmentation (Lui et al., 1996; Hirata et al., 1998; Creagh and Martin, 2001). Additionally, caspase-3 has been shown to activate neutral sphingomyelinase (N-SMase), a membrane bound enzyme involved in generation of ceramides.
In summary, Caspase-3 activation may take place either within death receptor complexes in a caspase-8 dependent-manner or through a mitochondrial cascade via caspase-9 (Nicholson and Thornberry, 1997).

**Table 1.1:** List of the fourteen caspases and their various names.

<table>
<thead>
<tr>
<th>Caspase-1</th>
<th>ICE</th>
<th>Caspase-8</th>
<th>FLICE, MACH, Mch 5</th>
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<td>Caspase-9</td>
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<td>Mch 3,ICE-LAP 3, CMH-1</td>
<td>Caspase-14</td>
<td>MICE</td>
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</tbody>
</table>
Figure 1.2  Activation of caspases
Adapted from: http://home.attbi.com/~xoanon/apo101.html

Pro-enzyme is cleaved at caspase cleavage sequences (AspX)

2 large and 2 small subunits combine to form the active tetrameric enzyme.
1.3.1.2 Mitochondrial pathway

Next to respiration, regulation of apoptosis is one of the main functions preformed by mitochondria (van Gurp et al., 2003). Mitochondria participate in apoptosis by releasing apoptogenic factors (APAF) and cytochrome C (Liu et al., 1996; Kluck et al., 1997). In addition to the above, a series of proteins referred to as the Bcl-2 family interact with mitochondria to regulate apoptosis.

The Bcl-2 family of proto-oncogenes were first discovered in human B-cell lymphomas at chromosomal breakpoint of t (14:18) (Tsujimoto et al., 1985). In general Bcl-2 was initially found to inhibit cell death induced by lack of IL-3 (Interleukin 3) stimulation in lymphoid and myeloid cell-lines (Vaux et al., 1988). The Bcl-2 multi-gene family is characterized by four highly conserved domains called Bcl-2 homology domains (BH1, BH2, BH3 and BH4) (Adams and Cory 1998; Kelekar and Thompson 1998; Reed 1998). Currently they can be divided into two different groups:

1. Pro-apoptotic members: The members of this group facilitate apoptosis. Additionally, this group is further divided into two sub-groups.
   a. Multi-domain: This group consists of Bax, Bak, Bok, Bcl-Xs. The members of this group all contain the four BH sites.
   b. BH3 domain: This group consists of Bad, Bid, Bik, Bim. As the name implies, this group of proteins contain sequence homology within the BH3 domain. BH3 domain has been shown to function as a death domain in the pro-apoptotic Bcl-2 family members.

2. Anti-apoptotic members: This group includes members such as Bcl-2, Bcl-Xl, Bcl-w, Mcl-1, A1 (Bfl-1) and Boo. The members of this group all have BH1 through BH4 domains. This group of proteins function in a pro-survival-manner, disabling the pro-apoptotic groups' effort especially in absence of death signals.

   In the absence of death signals, pro and anti-apoptotic Bcl-2 family members localize to separate intracellular compartments. By and large, anti-apoptotic proteins tend to act as integral membrane proteins in cellular compartments such as mitochondria, endoplasmic reticulum (ER), and/or nuclear membranes (Hockenbery et al., 1990; Krajewski et al., 1993; de Jong et al., 1994; Gross et al., 1999). Conversely, pro-apoptotic proteins tend to converge within the cytosol in the absence of apoptotic signals.
There are various mechanisms of action for each pro and anti-apoptotic molecule. For example, Bax, a multi-domain pro-apoptotic molecule, upon receiving death signals dimerizes and integrates within the mitochondrial membrane as an integral protein. This leads to activation of two major executionary programs: activation of the caspase cascade and mitochondrial dysfunction. Mitochondrial dysfunction comprises a change in membrane potential ($\Delta \psi_m$), production of reactive oxygen species (ROS), opening of permeability transition pore (PTP) and release of cytochrome C, an inter-membrane space protein (Gross et al., 1999). Release of cytochrome C activates Apaf-1 which in turn is responsible for activation of caspase cascades leading to cellular demise (Figure 1.3). The pro-apoptotic protein Bax and anti-apoptotic molecule Bcl-2, work upon similar mitochondrial pathways, conducting opposing roles. Bcl-2 and similar anti-apoptotic proteins are known for their role in "guarding the mitochondrial gates" thus disabling pro-apoptotic molecules to gain access to the mitochondria. The ratio between Bcl-2 and Bax determines cellular fate; thus an increased Bcl-2: Bax ratio leads to cell survival, even in presence of apoptotic stimuli (Gross et al., 1999).

Bad, an example of a BH3-domain-only pro-apoptotic protein is generally localized within the cytoplasm. This is accomplished via phosphorylation of the Bad protein at two serine (112,136) sites, to which 14-3-3 protein binds thus sequestering the Bad protein in the cytoplasm (Zha et al., 1996). In the presence of death signals (e.g. FasL), Bad is dephosphorylated and moves towards mitochondria where it interacts, via its BH3 domain, with Bcl and Bcl-XL (Kelekar et al., 1997; Ottile et al., 1997; Zha et al., 1997). Thus, Bad is regulated through phosphorylation of serines 112 and 136. Various kinases such as Akt/PKB and/or PKA have been shown to be involved in phosphorylation of Bad. PKB has been shown to favour ser 136 (Datta et al., 1997; Blume-Jensen et al., 1998) and PKA targets ser 112 for phosphorylation (Harada et al., 1999) hence inactivating the Bad protein (Gross et al., 1999).
Figure 1.3: Mitochondrial effect on caspase activation
Adapted from: http://home.attbi.com/~xoanon/apo101.html
1.3.2 Extrinsic apoptotic Signals or Death receptor pathway

1.3.2.1 Tumour necrosis factor α pathway

Tumour necrosis factor α (TNFα), is one of the most important and well documented cytokines to date. TNFα, first isolated in 1975 by Carswell et al., is a pleiotropic cytokine involved in a wide variety of physiological events. TNFα is member of larger family of ligands, called the TNF ligand family. TNFα has been shown to act as a cytotoxic cytokine in various malignant cell lines (Munker et al., 1987). Generally, it is produced by activated macrophages. TNFR1 and TNFR2 are specific receptors that are capable of binding the TNFα ligand.

In general, apoptotic signalling is primarily mediated by TNFR1 (55kDa). TNFR1 operates via recruitment of cellular proteins in order to relay the death signal message. Ordinarily, a protein named SODD (Silencer Of Death Domains) is associated with TNFR1 receptor. Upon binding of trimeric TNFα to the TNFR1 receptor, SODD dissociates from the receptor and cytoplasmic adaptor protein TRADD (TNF Receptor Associated Death Domain protein) interacts with the receptors through its death domain. This interaction leads to further recruitment of proteins such as FADD (Fas Associated Death Domain protein). FADD contains a DED (Death Effector Domain) which plays a role in recruitment of pro-caspase 8. Pro-caspase 8 contains two DED or CARD (Caspase Recruitment Domain) domains which are capable of binding FADD at its DED. This ultimately results in activation of caspase 8 and thus initiation of the caspase cascades leading to cellular apoptosis. Conversely, RIP (Receptor Interaction Protein) may also bind to either TRADD or straight to the receptor, causing for activation of various pathways.

TNFR2 (75kDa) also plays a role in apoptosis. Though, due to lack of death domain signalling through TNFR2 is more difficult. Proteins such as TRAF-2 (TNF Receptor Associated Factors), RIP as well as casein kinase and P80TRAK kinases have been shown to directly interact with TNF22 (Aggarwal, 2000). TNF22 is thought to increase local TNFα, therefore leading to further activation of TNFR1 (Tartaglia et al., 1993).

TNFα has various cellular effects including the activation of transcription factors such as NFκB and AP-1 (Activating Protein 1). AP-1 is a transcription factor which is
known to be regulated by three mammalian MAPK (mitogen activated protein kinases). One of these kinases is called JNK (c-Jun N-terminal Kinase also referred to as SAPK1).

1.3.2.2 c-Jun N-terminal kinase (JNK/SAPK1)

JNK (c-Jun N-terminal kinase) is a serine threonine protein kinase which phosphorylates c-Jun and AP-1. JNK is the member of the MAPK family of kinases. Three different JNK genes have been identified in humans. Due to different splice variants there are a total of 10 isoforms of JNK genes. JNK activity has been shown to play a critical role in both immune response and apoptosis (Bennett et al., 2001; Gupta et al., 1996). In general, JNK is a stress-activated protein kinase that can be induced by various stimuli such as bacterial endotoxin, inflammatory cytokines, UV radiation and hypoxia. Activation of JNK is through the small GTPases (i.e. Rho family) which signal for SAPK/JNK to translocate to the nucleus. With in the nucleus JNK is capable of regulating activity of several transcription factors such as c-Jun, ATF-2, and p53. These transcription factors may lead to varied cellular responses from apoptosis to cellular survival (Leppa and Bohmann, 1999).

1.3.2.3 Fas/CD95 pathway

Fas/CD95, a type-I trans-membrane protein, is another receptor-mediated apoptotic pathway, which may be included in the list of extrinsic apoptotic signals (Ashikhenazi and Dixit, 1998; Timmer et al., 2002).

Upon ligation of Fas ligand (FasL) to Fas receptor or cross-linking of anti-Fas antibody forces receptor trimerization, which, leads to recruitment of a series of molecules such as the adaptor molecule FADD (Fas-Associating Death Domain) protein to the receptor (Chinnaiyan et al., 1995). The N-terminal region of FADD protein acts as a docking site for DISC (Death-Inducing Signalling Complex) which binds to pro-caspase 8 (Muzio et al., 1996; Poulaki et al, 2001). Cells which recruit sufficient amount of pro-caspase 8 to DISC tend to signal for apoptotic death through activation of Caspase-3 (Schlottmann and Coggeshall, 1996).
Conversely, cells which have a limited amount of caspase-8 recruited at the DISC site, depend on the mitochondrial pathway in order to induced apoptotic death (Scaffidi et al., 1998). This is conducted through cleavage of the pro-apoptotic protein Bid (a BH3 domain containing cytoplasmic member of the pro-apoptotic Bcl-2 family) which translocates to the mitochondria, thus releasing cytochrome C (Li et al., 1998; Luo et al., 1998). Cytochrome C along with APAF1 leads to activation of caspase-9 and in turn caspase-3 and thereby causing for apoptosis (Zou et al., 1999).

Thus, the above outlines mechanisms involved in Fas and TNF induced apoptosis. Both Fas and TNF are examples of two extraneous signals which induce apoptotic death mediated through surface cellular receptors.

1.4 Reactive oxygen species (ROS)

Reactive Oxygen Species (ROS) are typically generated during aerobic respiration. During aerobic respiration, oxygen is partially reduced and thus produces superoxides, small amounts of O$_2^\cdot$, OH$^\cdot$ and H$_2$O$_2$. These species along with unstable intermediates in lipid peroxidation, are referred to as Reactive Oxygen Species (ROS). ROS are toxic metabolites that are metabolized by specialized enzymes such as catalase, peroxidases and superoxide dismutases (SOD).

There are a variety of diseases linked to damage caused by ROS, or otherwise referred to as oxidative stress, such as Parkinson’s, atherosclerosis, and some cancers. ROS have been implicated in the regulation of cellular death.

Due to a suggested role for ROS in various diseases plus the discovery of existence of enzymes such as superoxide dismutase (SOD) in all mammalian cells (McCord and Fridovich, 1969) further studies on ROS activity were conducted. SOD is an essential enzyme involved in elimination of superoxide radicals, which is considered to be an important anti-oxidant in aerobic cells (Hileman et al., 2001).

Although traditionally, ROS are considered a toxic by-product; their role as intra-cellular signalling molecules effecting cell growth/death pathways have also been documented. Most importantly, ROS have been shown to induce apoptosis. ROS’s role in apoptosis has been illustrated in different ways; first, addition of peroxide or lipid peroxide directly lead to apoptotic death. Also, addition of nitric oxide and other oxidants have been implicated as inducers of apoptosis in macrophages and monocytes (Albina et al., 1993; Andrieu-Abadie et al., 2001). Secondly, cellular exposure to physical or
chemical agents (e.g. ionizing radiation) leads to an increase in intra-cellular ROS generation and ultimately apoptosis (Andrieu-Abadie et al., 2001). Thirdly, various cellular antioxidants block apoptosis. Some examples of these antioxidants are: N-acetylcysteine (NAC) which is a thiol antioxidant plus a glutathione precursor, catalase, superoxide dismutase (SOD). Furthermore, it has been illustrated that Bcl-2 gene products are capable of inhibiting apoptosis, through interaction with SOD (Andrieu-Abadie et al., 2001). Figure 1.4 demonstrates some reactions that lead to ROS generation.

1.4.1 Mitochondria and ROS

One of the major sites of endogenous ROS generation is mitochondria (Raha and Robinson, 2000). In general, mitochondria are eukaryotic organelles which contain two membranes. The outer membrane is permeable to small molecules (up to 5 kDa) and ions due to presence of pore protein such as porin or VDAC (Voltage-Dependent Anion Channel) (Mannella et al., 1992; Szweczyk and Wojtczak, 2002). Conversely, the inner membrane is impermeable to most small molecules except water O₂, CO₂, and NH₃ (Kuan and Saier, 1993; Sluse, 1996). Additionally, the inner membrane contains the respiratory electron chain carriers known as complexes I-IV plus ADP-ATP translocases and ATP synthase (FₒF₁). Each of the four complexes contains various enzymes which allow for electron transfer. Complexes I and II catalyze electron transfer to ubiquinone from two different electron donors NADH (Complex I) and Succinate (Complex II). Complex III transfers electron from ubiquinone (UQ) to cytochrome C. Complex IV is involved in transferring electrons from cytochrome C to O₂. ROS is typically generated at both complexes III and IV sites within the inner mitochondrial membrane. ROS generation in complex III is generally due to proton cycling between ubiquinone, cytochromes B and C, and iron-sulphur protein (Sugioka et al., 1988). Thus, complex III is considered to be one of the major sites within the respiratory chain, which leads to production of superoxide anion (O₂⁻) due to transfer of a single electron to molecular oxygen (Andrieu-Abadie et al., 2001).

In the case of complex IV, sometimes a portion of the O₂ may be involved in one electron reduction processes, and therefore generate free radical oxygen which is a ROS constituent (Sugioka et al., 1988; Lehninger et al., 1993).
Although mitochondria are one of the major sites of ROS generation, they are not the only intra-cellular oxidative stress organelle. The endoplasmic reticulum is another site of electron transport; therefore, $O_2^*$ as a result of electron leakage from NADPH cytochrome P450 reductase (Andrieu-Abadie et al., 2001). Additionally, in the cytosol, hypoxanthine/xanthine oxidase, lipoxygenase and cyclooxygenase all lead to ROS generation (Andrieu-Abadie et al., 2001). Figure 1.4 demonstrates the interaction between the various complexes within the mitochondrial inner membrane.
Figure 1.4  Electron chain transfer system in mitochondria
Adapted from Lehninger et al. (1993)
1.5 Ceramide Generation and Metabolism

Ceramides are lipid second messengers which are involved in an array of functions from proliferation and differentiation to growth arrest and apoptosis (Mathias et al., 1999). Ceramide interactions within the cell have remained conserved during evolution from yeast to humans (Hannun and Bell, 1989; Mathias et al., 1998; Cinque et al. 2003). The magnitude of the effect of ceramide generation depends on the site of activation along which signalling modules have been affected, and thus ultimately with which enzymes have been activated (Mathias et al., 1998).

There are two potential sources of ceramide generation:

1) Sphingomyelin (SM) pathway: hydrolysis of SM by SMase (sphingomyelinase).

2) De novo synthesis: condensation of serine and palmitoyl-CoA through a series of steps to generate ceramides.

The sphingomyelinase catabolic pathway for ceramide generations involves action of various SMases. In general SMases are sphingomyelin specific forms of phospholipase C enzymes which are capable of hydrolyzing the phosphodiester bond in SM and thus producing ceramide and phosphocholine. Currently there are a number of different SMase isoforms which are distinguished based on their pH. Hence they are referred to as acidic, neutral and alkaline SMases. Neutral and acidic SMases are thought to be activated rapidly upon stimulation via exogenous stimuli (Hannun and Bell, 1989; Mathias et al., 1998). Numerous extra-cellular stimuli lead to activation of the SMase pathway. A few examples of such stimuli are the following: TNF super family of ligands and activation of TNF family of receptors, or other stress stimuli such as ionizing radiation all lead to SMase-ceramide progression (Kolesnick and Fuks, 1995; Mathias et al., 1998).

The de novo pathway is an alternate pathway that leads to ceramide synthesis in cells through activation of a series of enzymes. The first step of de novo synthesis consists of serine palmitoyl transferase's role in condensation of serine and palmitoyl-CoA to form 3-ketosphinganine. This 3-ketosphinganine is further reduced to form dihydrosphingosine with the help of a reductase. Conversely, dihydrosphingosine is then converted into dihydroceramide with the help of ceramide synthase. At this point a trans-4,5 double bond is introduced by dihydroceramide desaturase and therefore leads to ceramide generation (Mathias et al., 1998).
Upon generation, ceramides are capable of undergoing metabolic transformation to form other molecules. One such metabolite is known as ceramide 1-phosphate, which results from the phosphorylation of ceramide by ceramide kinase. Another metabolite is sphingosine which results from deacylation which in turn can be phosphorylated into sphingosine 1-phosphate. Ceramides are also capable of reverting back to sphingomyelin. This may be done upon addition of phosphocholine form phosphatidylcholine by sphingomyelin synthase (Gomez-Munoz, 1998). Figure 1.6 summarizes the two different pathways which lead to ceramide generation.

**Figure 1.5** Ceramide generation and metabolism
Adapted from Mathias et al. 1998.
1.5.1 Ceramides and apoptosis

As discussed earlier TNFα and Fas receptors are involved in initiating apoptosis by signalling through their death domains (DD). Ceramide is a second messenger which has been shown to be involved in the early stages of apoptosis. Ceramides are also generated subsequent to TNFα signalling (Datta et al., 1997), however, this TNFα induced ceramide generation is independent of DD signalling involvement. Although apoptosis can occur in the absence of ceramide generation, its presence seems to cause for optimal apoptotic effect (Mathias et al., 1998).

Ceramides regulate apoptosis through two variable pathways; one by alteration of mitochondrial function (Geley et al., 1997; Siskind et al., 2002; Darios et al., 2003) and the other by transcriptional activation of JNK pathway (Verhij et al., 1996; Huang et al., 1997).

Recently researchers have shown that ceramide interacts with mitochondria. For example, ceramide synthase has been found in mitochondrion rich fractions. Furthermore, ceramide has been detected in the outer mitochondrial membranes; all indicative of ceramides direct interaction with oxidative stress in mitochondria.

Addition of ceramides or TNFα leads to a morphological change in the mitochondria. Also, addition of various short chained ceramide analogues such as C2-ceramides (N-acetyl-D-erythro-sphingosine) and C6-ceramides (N-hexanoyl-D-erythro-sphingosine) have been linked with release of cytochrome C. Over-expression of cells with Bcl-2 prevents this cytochrome C release (Geley et al., 1997; Siskind et al., 2002). Recent studies have indicated that ceramides cause the formation of channels in the outer mitochondrial membrane. These channels enable the release of cytochrome C and thus initiate the apoptotic cascade (Siskind et al., 2002).

1.5.2 Ceramides and JNK

As stated in the last section, one of the pathways ceramides choose in order to cause apoptosis is activation of SAPK/JNK pathway. Typically, the JNK pathway is activated in response to cellular stress such as serum deprivation, ionizing radiation, UV (ultraviolet radiation) and cytokine stimulations (Mathias et al., 1998). Upon activation of
the JNK pathway, a series of transcription factors such as c-Jun and activating transcription factor-2 (ATF-2) and AP-1 are activated (Mathias et al., 1998).

Numerous studies have established a link between ceramide and JNK pathway. For example in a study conducted by Verheij et al. (1996) they were able to demonstrated that upon stimulation of BAEC cells (bovine aortic endothelial cells) and U937 (lymphocytes) with TNFα, ceramides were generated rapidly which in turn lead to JNK activation. Currently, it is thought that ceramide generation may lead to JNK activation via two potential mechanisms. This can occur through either TGFβ (Transforming Growth Factor β) activated kinase (TAK1) or through small G-protein Rac-1 (Mathias et al., 1998). Different studies have indicated that either TAK1 or Rac-1 or both are activated upon treatment with exogenous ceramides. Their activation leads to a series of steps which ultimately activate JNK.

JNK has been illustrated to play an integral role in induction of apoptosis (Bezombes et al., 2003). JNK is capable of translocating to mitochondria and inactivating anti-apoptotic molecules Bcl-2 and Bcl-XL by phosphorylating them at an inhibitory site (Fan et al., 2000).

1.5.3 Ceramides and ROS

Recent studies have established a link between ceramide and oxidative stress. Various chemicals and physiological treatments which are known to induce apoptosis have been shown to activate both SM-ceramide pathway and ROS generation. TNFα, an example of such apoptotic inducers, has been reported to activate both ceramide and ROS generation (Thannickal and Fanburg, 2000).

Further investigation of the relationship between ROS and ceramides reveals ROS’s capability to activate ceramide generation. Using pro-oxidant H2O2 in various cell types, it has been shown that apoptosis is mediated through the sphingomyelinase pathway. Current studies indicate neutral SMases as ROS targets. For example there is a marked decline in magnesium dependent neutral SMase activity observed in glutathione peroxidase (GPx) expressing cells (Gouaze et al., 2001). This suggests that GSH regulates ceramide generating enzymes. Interestingly, there have been very few observations of activation of acidic SMase in connection with oxidative stress (Andrieu-Abadie et al., 2001).
Since mitochondria are one of the sites of ROS generation and initiation of apoptosis, the effect of ceramides on this organelle have been studied. Mitochondria are considered as the main site for ROS generation most probably due to electron leakage in the electron transport chain thus resulting in formation of $\text{O}_2^-$ (Raha and Robinson, 2000). Cell permeable ceramide analogs have been shown to exert their direct effects on mitochondria by inhibiting the mitochondrial respiratory chain. Experimentally, ceramides effect on mitochondria have been demonstrated by addition of short chain ceramide on isolated mitochondria or alternatively by addition of ceramides to mitochondrial respiration deficient cells, which resulted in inhibition of ceramide induced $\text{H}_2\text{O}_2$ production (Garcia-Ruiz et al., 1997; Gudz et al., 1997; Quillet-Mary et al., 1997; Andrieu-Abadie et al., 2001).

Furthermore, studies on mitochondrial complexes (respiratory chain) have substantiated the role of ceramides on the respiratory electron chain system. Inhibition of complexes I and II resulted in reduction of ceramide induced $\text{H}_2\text{O}_2$ production. Interestingly, inhibition of complex III by antimycin A (a specific complex III inhibitor) leads to potential activation of ceramide. Complex III is also affected by molecular triggers of ceramide production which lead to apoptosis such as TNFα or other anticancer drugs (Schulze-Osthoff et al., 1992; Tuquet et al., 2000; Andrieu-Abadie et al., 2001).

This study analyzes role of two dietary components (apigenin and curcumin) and a therapeutic drug’s (Sulfasalazine) role in induction of death in colon cancer cell lines. The effect of these three agents on ceramide, ROS and apoptotic pathways are evaluated.
Chapter 2. Materials and Methods

2.1 MATERIALS:

2.1.1 Reagents and laboratory supplies

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### Antibodies

#### Antibody

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2.2 METHODS

2.2.1 Cell culture

HCT-116 colorectal carcinoma cells were grown and maintained using 10% FBS (fetal bovine serum) and McCoy's 5A media which was supplemented with penicillin and streptomycin (0.1 mg/ml). These adherent epithelial cells were a gift from B. Vogelstein.

DLD-1 colorectal cells were grown and maintained in 10% FBS containing RPMI media supplemented with penicillin and streptomycin. DLD-1 cells were obtained from Tissue Type Culture (ATTC).

HT-29 colorectal cells were grown and maintained in M199 media containing 10% FBS and supplemented with penicillin and streptomycin. These cells were obtained from ATTC.

2.2.2 MTS cell viability assay

HCT-116 cells were plated into 96-well plates at $1 \times 10^4$/well density. Cells were grown for 24 h before being serum deprived in 1% FBS containing media for 3 h. Cells were then stimulated with different concentrations of anti-cancer drugs for 24 h and 48 h. 250 μg/ml of MTS [3-(4,5-dimethylthiazol-2-yl) 5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt] plus 20 μg of PMS solutions were mixed together and added to each well for 1.5 h. Using an ELISA plate reader colorimetric reading at absorbance of 490 nm was recorded. Each condition was plated in quintuplicate.

2.2.3 Fluorescein-activated cell sorter (FACS) analysis for PI staining

HCT-116 cells were seeded in 12-well plates and grown to 80% confluence. Cells were incubated for 3 h in 1% FBS containing media, and then exposed to anticancer agents (i.e. Apigenin, Curcumin, and Sulfasalazine) for 4 h duration. Cells were then mechanically lifted by repeated pipetting and washed twice with cold phosphate-buffered saline (PBS). After centrifugation at 1000 rpm, cell pellets were re-
suspended in ice cold 70% ethanol for 1 h. The cells were then washed twice with ice-cold PBS and re-suspended in 50 μg/ml propidium iodide and 25 μg/ml RNAase. At least 10,000 cells were counted on the Flow cytometer FL3 gate.

2.2.4 Apoptotic dyes
2.2.4.1 PI staining

HCT-116 colon cancer cells were plated on 12-well plates, and grown to 80% confluence. Cells were than incubated in 1%FBS containing McCoy's 5A media for the duration of 3 h. They were than exposed to various drugs (Apigenin, Curcumin and Sulfasalazine) for approximately 4 h. Cells were mechanically lifted by repeated pipetting, and washed twice with cold PBS. The pellets were than re-suspended in ice-cold 70% ethanol for1h. The cells were then washed twice with ice cold PBS and re-suspended in 50 μg/ml propidium iodide and 25 μg/ml RNAase. A 5 μl aliquot was removed from the cell suspension and placed on a slide. A cover slip was added and sealed using crazy glue. Cells were observed under a light microscope under two different magnifications. Using a digital camera the pictures were taken. At least four different fields of view were photographed and the representative photo is displayed.

2.2.4.2 Hoechst staining

Colon carcinoma cells (HCT116, HT29 and DLD-1) were seeded on sterile cover slips in 6-well plates. Upon reaching 80% confluency, cells were starved in 1% FBS containing media for 1 h. Cells were than incubated with 1 mM SSZ, 100 μM curcumin and 80 μM of Apigenin or no drugs for the control over a 4 h period. Media was then removed and washed twice with PBS. Cells were fixed using 1 ml of 90% ice cold media for 1 h. 1 ml of diluted Hoechst 33285 (1 μg/ml) in PBS was added to each well for 1 h. Cover slips were removed and placed on slide and pictures were taken from at least 3 different fields of view.
2.2.5 Protein analysis

2.2.5.1 Evaluation of protein concentration

The protein concentration for each sample was determined using the Bradford assay. Initially BSA standards ranging from 0-30 μg were prepared in 2.5 ml of Bradford reagent (100mg Coomassie Blue G, 50 ml ethanol, 100 ml H₃PO₄, 850 ml dH₂O). The standards were mixed via light vortexing. Each standard was read at 595 nm with TYPE spectrophotometer. The absorbance values were used to plot a linear trace plot.

In case of the samples to be evaluated for their protein content, 5 μl of stock sample was diluted in 2.5 ml of Bradford reagent. These samples were mixed by vortexing and the absorbance was read at 595 nm. The concentrations were determined by using linear regression analysis.

2.2.5.2 Western immunoblotting

HCT-116 cells were seeded in 6 well plates. After incubation in 1%FBS containing media for 3 h, the cells were treated with various anticancer agents for 24 h. Cells were washed with ice cold PBS, and re-suspended in homogenization buffer (20 mM MOPS, 50 mM β-glycerophosphate, 5 mM EGTA, 50 mM NaF, 1 mM DTT, 1 mM sodium vanadate, and 1 mM PMSF) for 30 min on ice. Samples were then sonicated for 15 s and centrifuged at 14,000 rpm for 20 min and the supernatant was collected. Protein concentrations were determined by Bradford assay. Aliquots of the samples were resolved on 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane. The nitrocellulose membrane was blocked in 5% skim milk in TBST (20 mM Tris-HCl pH 7.4, 250 mM NaCl, 0.05% Tween-20) for 1 h. The primary antibody (1:1000 dilution) was then applied to the membrane and incubated overnight at room temperature. Blots were washed three times for 10 min with TBST, and then incubated with appropriate secondary antibody conjugated to horseradish peroxidase (HRP) were added and 1:5000 dilution for 1 h in TBST. Blots were then washed three times with TBST and twice with TBS only at 10 min. Signals were detected by incubation of blots in ECL for 1 min, and then visualized by exposing to film.
2.2.5.3 Stripping Blots

In order to re-probe nitrocellulose blots with new antibodies, the blots were stripped. Pre-probed nitrocellulose blots were kept moist in TBS. These blots were then incubated at 50°C in stripping solution (60 mM Tris-HCl, pH 6.7, 100 mM β-mercaptoethanol, 2% SDS) for 30 min. The nitrocellulose was then washed with TBST and re-blocked in 5% skim milk for 1 h. The blots were then re-probed with primary antibody (1:1000 dilution) overnight. Blots were then washed three times for 10 min with TBST, and then incubated with appropriate secondary antibody conjugated to horseradish peroxidase (HRP) were added and 1:5000 dilution for 1 h in TBST. Blots were then washed three times with TBST and twice with TBS only at 10 min. Signals were detected by incubation of blots in ECL for 1 min, and then visualized by using film.

2.2.6 Mitochondrial preparation and western blot analysis of cytochrome C

Cells were plated in 6 well plates and grown to 80% confluency. Cells were then starved in 1% FBS containing media and treated with appropriate agent (apigenin, curcumin or SSZ). Cells were harvested by centrifugation at 1000 x g for 5 min at 4°C. The pallets obtained were washed twice with ice cold phosphate buffer saline (PBS). Mitochondrial and cytosolic fractions were separated by adding 100 μl of mitochondrial buffer (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml pepstatin A) containing 250 mM sucrose. Cells were placed on ice for 10 min and then lysed by passing 10-15 times through a 25 gauged needle. Cells were kept on ice for an additional 10 min. Homogenates were then centrifuged at 1,000 x g for 5 min at 4°C. The supernatent was removed and further centrifuged at 10,000 x g for 15 min at 4°C. The resulting mitochondrial pellets were resuspended in cold mitochondrial buffer.

For western blot analysis, equal amounts of mitochondrial and cytosolic proteins were resolved on 15% SDS-PAGE and followed the western blot analysis as outlined above. Anti cytochrome C mouse antibody was later used as the primary antibody.
2.2.7 Lipid analysis

Cells were plated in 6 well plates and grown to 80% confluency. Cells were then labeled with 2.5 μCi of [3H] palmitate/ml over-night. The radioactive medium was removed and washed twice with non-radioactive media containing 1% FBS. Cells were then starved in 1% FBS containing media for duration of 3 h, and were then incubated in various treatments. The duration of each treatment differed according to the experiment being performed.

Cells were then scraped in media and transferred to glass tubes. Cells were spun down at 12000 rpm for 1 min to remove media. Cells were then washed twice using ice cold PBS. 1 ml ice-cold methanol was added to the cell pellets and vortexed. An additional 1 ml of chloroform plus 0.9 ml of 2 M KCL + 0.2 M H₃PO₄ was added and mixed. Then samples were spun down at 18000 rpm in order to separate the phases. The lipids were extracted in the chloroform (bottom) layer, and the top aqueous layer was discarded. 400 μl of the extracted lipid was transferred into glass tubes and dried down for approximately 10 min with nitrogen and heat.

The lipids were re-dissolved in 50 μl of chloroform and ice-cold ceramide, were added. The lipids were then separated by thin layer chromatography (TLC) using Silica Gel 60 coated glass plates. For each ceramide and sphingomyelin, assay the appropriate standard was used (i.e. cold ceramide was used for ceramide assays). TLC plates were developed for 50% of their length in chloroform/methanol/acetic acid (9:1:1) and then air-dried. The plates were then redeveloped for their full length with petroleum ether/diethylether/acetic acid (60:40:1). The plates were then air-dried once again and stained with iodine vapor.

In case of sphingomyelin determination the lipids were developed using a TLC plate. The TLC plates were developed full length in chloroform/methanol/acetic acid/formic acid/distilled water (35:15:6:2:1). The plates were air-dried and stained with iodine vapor.

Radioactive ceramides were quantified after scraping the TLC plates at designated areas, followed by liquid scintillation counting.
2.2.8 Reactive oxygen species (ROS) assay

Cells were seeded on 12-well plates and grown to approximately 80% confluence. After 2 h incubation in 1% FBS containing media, 2 μM DCF-DA (2', 7'-dichlorofluorescein-diacetate) was added. After 1 h incubation with the dye the cells were exposed to various treatments (i.e. apigenin, curcumin and SSZ) for 4 h. Cells were then manually lifted by repeated pipetting, and spun down to remove the media at 12000 rpm for 1 min. The pellets were then re-suspended in 1 ml PBS and the fluorescence was then measured using flow cytometry (FL2).

In case of inhibitor studies, the ROS scavengers were added after cells had been labeled with 2 μM DCF-DA dye for 1 h. The scavengers were added 20 min prior to addition of drugs (i.e. apigenin, curcumin and SSZ).

To calculate the absolute fluorescence (ROS generation) the total number of cells, gated on the histogram obtained through flow cytometry, were multiplied by mean fluorescence. Experiments were conducted in triplicates and mean absolute fluorescence has been graphed.

2.2.9 Statistical analysis

Results were expressed as mean ± SEM. Statistical analysis was done with student's t-test. A p value of less than 0.05 was taken as significant.
Chapter 3. Sulfasalazine

3.1 Introduction

Sulfasalazine (SSZ) is a popular second line anti-inflammatory drug, which has been used over 50 years for treatment of different inflammatory bowel diseases (IBD) such as Ulcerative Colitis (UC) and Crohn's disease (Akahoshi et al., 1997). It was originally used for the treatment of rheumatoid arthritis (RA) and other inflammatory joint diseases (Rodenburg et al., 2000). In the 1940's, Svartz discovered that SSZ reduced colonic mucosal inflammation in arthritis patients (Svartz, 1968), thus giving birth to SSZ's modern use in treatment of IBD.

SSZ belongs to the sulfonamide drug family. Sulfonamide drugs were the first non-antibiotic, antibacterial agents. They are synthetic drugs that primarily act in a bacteriostatic fashion. SSZ interferes with folic acid metabolism by competing for dihydrofolate reductase, an enzyme involved in folic acid metabolism (Zimmerman, 1992; Baggot et al., 1993).

Chemically SSZ is comprised of a combination of 5'-aminosalicylic acid (5'-ASA) and sulfapyridine (SP). These two components are joined together via an azo bond (Figure 3.1), which can be cleaved by bacteria in the large intestine (Gaginella and Walsh, 1992).

In general, SSZ and SP are absorbed more easily as opposed to 5'-ASA (Gaginella and Walsh, 1992; Rodenburg et al., 2000). SSZ and SP are found in the range of $10^{-4}$M to $10^{-5}$M in serum. 5'-ASA is absorbed at much lower concentrations (Rodenburg et al., 2000). Typically, the concentration of SSZ in the gut reaches millimolar levels (Peppercorn and Goldman, 1973).

3.2 Rationale and Hypothesis

SSZ's role as an anti-inflammatory drug has been well established. Sulfasalazine and its constituents (5'-ASA and SP) inhibit: prostaglandin syntheses (Gaginella and Walsh, 1992), chemotaxis in inflammatory cells (Wandall, 1991), cytokine expression in mononuclear cells and angiogenesis (Smedegard and Bjork, 1995). Furthermore, SSZ sequesters nuclear factor $\kappa$B (NF$\kappa$B) in the cytoplasm (Munzert et al., 2002; Rodenburg et al., 2000).
In the case of cancer, SSZ is documented to reduce colorectal cancer prevalence (Suzuki et al., 2000). Moody et al. (1996) demonstrated long-term treatment with SSZ in UC patients, lead to reduction in the probability of development of colorectal cancer.

Currently there is abundant evidence that SSZ plays a role in apoptotic death in different cell lines. For example, SSZ treatment in Jurkat cells (a human leukemia cell line) leads to apoptosis via: accumulation of Bax, release of cytochrome C and thus activation of caspase-3. Furthermore, SSZ was found to stimulate translocation of apoptosis inducing factor (AIF) into mitochondria and thus triggering DNA fragmentation (Liptay et al., 2002). SSZ also causes apoptosis by inhibiting NFκB activity (Pentikainen et al., 2002). NFκB contains anti-apoptotic properties, which may lead to chemoresistance to cancer cells (Arlt et al., 2001).

It has been suggested that any augmentation of ceramide generation may enhance susceptibility of cancer cells to apoptosis (Hannun and Luberto, 2000). In this study, we wished to explore whether SSZ could effect apoptosis in CRC cell lines and if so, investigate the mechanisms involved in ceramide generation.
Figure 3.1  Structure of sulfasalazine.

A) The portion marked with the red box is similar to sulfapyridines structure.

B) The portion of the chemical structure outlined with blue is similar to structure of 5-ASA.

* Azo bond
3.3 Results

3.3.1 Sulfasalazine induces apoptotic death in colon cancer cell lines

To date, SSZ has been used in treatment of IBD, and its anti-carcinogenic properties have yet to be investigated. Thus, we initially looked at its effect on viability of colon cancer cells (HCT116, HT-29 and DLD-1).

We found that SSZ leads to reduction in cell viability in a concentration dependent manner. Three different colon cancer cell lines, HCT 116, DLD-1 and HT-29 were seeded in 96 well plates. Cells were starved overnight, and treated with various concentrations of SSZ over a 24 h period. Utilizing MTS assay, cell viability was assessed. Figure 3.2a, illustrates that 1 mM of SSZ over a 24 h period leads to reduction in cell survival by approximately 50% in all three selected cell lines. HCT116 cells were elected as a sample cell line used in most of the following studies.

Flow cytometry indicates a dose-dependent increase in sub-diploid compartment in HCT116 cells treated with different concentrations of SSZ (Figure 3.2b). HCT116 cells were seeded in 12 well plates, and starved for 1 h and treated with different concentrations of SSZ over a 4 h period. Cells were then removed and fixed with ethanol and stained with PI. Using a flow cytometer (Epics XL-MCL, Beckman Coulter, Fullerton, CA) the sub-diploid population was determined. In general the sub-diploid population is used as a marker for cell death (Vaculova et al., 2002). Figure 3.2b shows an approximate six-fold increase in sub-diploid population between non-treated cells and 2.5 mM SSZ treated cells.

Release of cytochrome C into the cytosolic fraction confirmed SSZ's role in apoptosis. After treatment of DLD-1 cells with 1 mM of SSZ over a 4 h period, the mitochondrial compartment was isolated and separated from the cytosolic fraction of each cell. Cytochrome C levels between the two cellular fractions were analyzed and compared. Western blot analysis of cytochrome C, illustrates leakage of cytochrome C from the mitochondrial compartment into the cytosol, a clear sign of apoptosis (Figure 3.3a). Similar results were obtained in HCT116 cells (data not shown).
Sulfasalazine leads to a decrease in cell survival in CRC cell lines

A) Viability in three colon cancer cell-lines (HCT116, DLD-1 and HT29) decreases in a dose dependent manner. The following graph depicts an MTS assay conducted over a 24 h period in HCT116, DLD-1 and HT29 CRC cell-lines. As SSZ concentrations increases, cell survival decreases.

B) Concentration course of SSZ depicts increase in sub-diploid population as analyzed by FACS in HCT116 colon cancer cell-line.
Likewise, HCT116 cells with varying concentrations of SSZ over 24 h were tested for PARP and pro-caspase-3 proteins. Western blot analysis of pro-caspase-3 and PARP (pro-apoptotic) proteins, demonstrates a gradual decrease in both pro-caspase-3 and PARP proteins (Figure 3.3b). All of the above-mentioned results point towards an apoptotic death.

To further support our findings, SSZ treated HT29 and HCT116 cells were stained with Hoechst 33285 (Figure 3.4a) and PI (Figure 3.4b) stains respectively. These stains are capable of detecting DNA fragmentation. Results illustrate intra-nuclear DNA fragmentation in cells treated with 1 mM SSZ in comparison to control cells (Figure 3.4).
Figure 3.3: Sulfasalazine treatment leads to cytochrome C release plus caspase-3 and PARP activation

A) Treatment of DLD-1 cells with 1 mM of SSZ leads to release of cytochrome C from the mitochondrial fraction into the cytosolic fraction. Cells were starved for 3 h in 1% FBS containing RPMI1640, and were then treated with 1 mM of SSZ for 4 h. Mitochondrial fractions and cytosolic fractions were separated. Western blot analysis indicated the movement of cytochrome C from mitochondria to cytosol.

B) Concentration course of SSZ depicts a decrease in PARP and Pro-caspase-3 proteins. HCT116 cells were treated with various concentrations of SSZ over a 24 h period. Western blot analysis of both PARP and Pro-caspase-3 indicates a decrease of both proteins in a concentration dependent manner.
A)

Cytochrome C

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B)

Parp

Pro-Caspase 3

[SSZ]

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Figure 3.4: Sulfasalazine treatment leads to DNA fragmentation

A) Pictorial representation of DNA fragmentation in HT29 colon carcinoma cell lines. Cells were stained with Hoechst 33285 after treatment with 1 mM of SSZ over a 4 h period. The panels on the left represent control cells and the right panels represent SSZ treated cells. The methodology has been outlined in chapter 2 (2.24).

B) Pictorial representation of DNA fragmentation in HCT116 cells treated with 2.5 mM SSZ over 4 h period. HCT116 cells were stained with PI. The methodology has been outlined in chapter 2 (2.24).
3.3.2 Sulfasalazine induces ceramide generation

Ceramides are known cellular second messangers, which lead to various events such as apoptosis. Previous studied have illustrated that SSZ exposure leads to ceramide generation in macrophages (Salh et al., 2002). Here we look at SSZ's effect on colon cancer cells.

HCT116 colon cancer cells were labeled overnight with $^{3}$H-palmitate. Cells were then starved for 3 h prior to treatment with various concentrations of SSZ over a 4 h time frame. Lipids were separated on a TLC plate and consequently relative ceramide generation was calculated. Analysis of ceramide generation illustrates a dose-dependent increase in HCT116 cells treated with SSZ. Maximum ceramide generation was observed when HCT116 cells were treated with 2.5 mM of SSZ (Figure 3.5a).

Furthermore, this increase in relative ceramide generation is also time-dependent. Our results depict a gradual increase in relative ceramide production in HCT116 cells over a 12 h period (Figure 3.5b) Thus, from these results, 1 mM of SSZ over a 4 h time frame are the treatment conditions used in the subsequent experiments.

3.3.3 Sulfasalazine induces ceramide generation through the de novo pathway

Ceramides generation occurs through two separate pathways: 1) de novo synthesis and 2) Sphingomyelin (SM) hydrolysis pathway (Gomez-Munoz, 1998; Mathias et al., 1998; Hannun and Luberto, 2000). To discriminate which pathway is involved in SSZ related elevation of ceramide generation, two de novo synthesis inhibitors, Myriocin (Wispriyono, 2002) and Fumonosin B1 (FB1) (Sweeney, 1996) were used.

Tritium palmitate treated HCT116 cells were pre-treated with the above inhibitors for half an hour before SSZ treatment. Over a 4 h incubation period cells were treated with 1 mM of SSZ plus either of the inhibitors.
Figure 3.5: Sulfasalazine increases ceramide generation time and dose-dependently.

A) The histogram indicates that over a 4 h period, SSZ (2.5 mM) leads to maximum increase in ceramide generation. HCT116 colon cancer cells were labelled overnight in H\textsuperscript{3}-Palmitate and starved for 3 h in 1% FBS containing McCoy's media. Cells were then treated with various concentrations of SSZ.

B) Illustrates a time course utilizing pre-labelled (H\textsuperscript{3}-Palmitate) HCT116 cells. Cells were starved for 3 h in 1% FBS containing media and treated for various time points (maximum of 12 h) with 1 mM of SSZ. The graph shows a time dependent increase in ceramide generation.
Results indicate that in the presence of both FB1 (10 μM) and SSZ (Figure 3.6a) or myriocin (25 nM) and SSZ (Figure 3.6b) there is an inhibition in ceramide generation. This indicates SSZ role in ceramide production is dependent on the de novo pathway.

3.3.4 Sulfasalazine induces ROS generation

Reactive oxygen species are generated as respiratory by-products. They have been shown to be involved in apoptotic death (Buttke and Sandstrom, 1994; Um et al., 1996; Salh et al., 2002). We have previously shown that SSZ leads to ROS production in macrophages, thus we wanted to see whether this was true for colon cancer cells.

Addition of 1 mM of SSZ leads to a dose-dependent increase in ROS generation in HCT116 colon cancer cells (Figure 3.7). HCT116 cells were starved for 2 h in 1% FBS containing McCoy's 5A media. Cells were then labelled with 2 μM DCHF-DA dyes an hour prior to treatment with SSZ.

To delineate the underlying mechanism leading to ROS generation, certain enzymatic and pharmacological inhibitors were used. Pharmacological inhibitors of ROS such as L-buthionine-[S,R]-sulfoximine (BSO), an agent depleting glutathione, butylated hydroxyanisol (BHA) (Sakurai et al., 2001), attenuates ROS post generation, N-acetylcysteine (NAC), and glutathione (GSH) precursor (Liu et al., 1998; Mansat-de Mas et al., 1999), cyclosporin A (CSA), inhibits the mitochondrial permeability along with some enzymatic ROS scavengers such as catalase (CAT) and superoxide dismutase (SOD) were utilized in an effort to identify the source of ROS generation. Results demonstrate that BSO (25 μM), CAT (1000 U), CSA (10 μM) and NAC (5 mM) were all able to reduce whereas BHA (25 μM) and SOD (1000 U) were unable to attenuate ROS generation in HCT116 colon cancer cells treated with SSZ (Figure 3.7c). These results are indicative of a mitochondrial mediated ROS generation.
Figure 3.6: Sulfasalazine elevates ceramide generation through the de novo pathway.

A) HCT116 cells were pre-labeled over night with H$^3$-Palmitate. Cells were treated with FB-1 (10 μM) for 0.5 h and then treated with 1 mM of SSZ for 4 h. The following shows that ceramide generation is significantly reduced in presence of SSZ plus FB-1.

B) HCT116 cells were pre-labeled over night with H$^3$-Palmitate. Cells were treated with Myriocin (25 nM) for 0.5 h and then treated with 1 mM of SSZ for 4 h. The following shows that ceramide generation is significantly reduced in presence of SSZ plus Myriocin.
A) 

![Graph of Relative Ceramide Generation](image)

B) 

![Graph of Relative Ceramide Generation](image)
Figure 3.7: Sulfasalazine treatment in CRC cells increases ROS generation.

A) HCT116 cells treated with various concentrations of SSZ (1-5 mM) exhibit an increase in ROS generation indicated by movement of cell masses on a log scale.

B) HCT116 cells treated with SSZ lead to a dose-dependent increase in ROS generation.

C) BSO, CAT, CSA and NAC reduce SSZ-induced ROS generation in HCT116 cells.
3.3.5 **Inhibition of Sulfasalazine mediated ROS generation leads to attenuation of cell death**

Role of SSZ mediated ROS generation in cell death was further examined. HCT116 cells were treated with various ROS inhibitors such as BSO (25 μM), CAT (1000 U), CSA (10 μM) and NAC (5 mM) half an hour prior to SSZ (2.5 mM) treatment. Flow cytometry was used in order to examine the sub-diploid population. Here we demonstrated that both NAC and CSA were able to most effectively reverse apoptosis. Catalase and BSO had less dramatic inhibitory effect on cell death (Figure 3.8). The data suggests a role for mitochondrial induced ROS generation in cell death in HCT116 colon cancer cells.

3.3.6 **Sulfasalazine mediated ROS generation is not upstream of ceramide pathway**

The relationship between SSZ mediated ROS and ceramide generation was investigated. Ceramide levels in ROS inhibited CRC cells were explored. Tritium palmitate labelled HCT116 cells were pre-treated with 5 mM of NAC for 0.5 h prior to stimulation with SSZ (1 mM). Cellular lipids were harvested and separated on a TLC plate. The level of ceramide generation was calculated.

Figure 3.9 exhibits no change in level of ceramide generation when ROS is inhibited. This indicates that ROS generation does not precede ceramide production in this cell model system.
Figure 3.8: ROS inhibition in SSZ treated cell leads to attenuation of cell death

HCT116 cells were starved for 3 h. Cells were then treated with various ROS inhibitors for approximately 1 h prior to stimulation with 1 mM SSZ over a 4 h period. ROS inhibited cells exhibited an increase in cell survival.
Figure 3.9: Inhibition of SSZ mediated induced ROS by NAC has no effect on ceramide generation.

HCT116 colon cancer cells were pre-labeled with $^3$H-Palmitate over night and subsequently starved for 3 h in 1% FBS containing McCoy’s 5A media. Cells were then first treated with 5 mM of NAC (0.5h) and then treated with 1 mM of SSZ over 4 h. No change in level of relative ceramide generation is observed.
3.4 Discussion

3.4.1 SSZ mediates apoptosis in colon cancer cells

For the past 60 years, Sulfasalazine has been one of the leading agents used for the treatment of IBDs such as ulcerative colitis (UC) (Gaginella and Walsh, 1992). Numerous studies have revealed that persistent UC may potentially lead to occurrence of colorectal cancer (Johnson et al., 1996).

Interestingly, it has been noted that in patients with UC that have been treated with SSZ over a long period tend to have lower probability of developing colorectal cancer (Moody et al., 1996; Suzuki et al., 2000).

Although SSZ's role in inducing apoptosis in CRC cell-lines has not been fully investigated, there is evidence of its apoptotic role in other cell lines such as Jurkat, Raw 264.7, germ line testicular cells and more (Liptay et al., 2002; Pentikainen et al., 2002; Salh et al., 2002).

Our studies illustrate for the first time the role of SSZ in induction of apoptosis in CRC cell lines. Initially we demonstrate that SSZ is capable of reducing cell survival dose-dependently in three different colon cancer cell-lines (HCT116, HT29, and DLD-1) (Figure 3.3a). This process was further analysed by use of flow cytometry. Flow cytometry is utilized in order to look at DNA integrity (Gomez-Lechon, 2002). Our results indicate that the sub-diploid population in HCT116 cells increases as the concentration of SSZ is increased (Figure 3.3b). Previous reports demonstrate a similar effect of SSZ on Raw 264.7 cells, where an increase in concentration of SSZ leads to elevation of the sub-diploid population (Salh et al., 2002).

Contradictory to our findings, Liptay et al. (2002) show that neither SW-620 colon carcinoma cells (a metastatic cell line unlike the CRC cell lines we have used), nor primary human synoviocytes were affected by SSZ (1 mM). Thus, suggesting that SSZ effects cell death in a cell line specific manner.

In order to provide evidence that cell death in SSZ treated cells is in fact apoptosis, we looked at cytochrome C release from mitochondria. Our findings demonstrate leakage of cytochrome C into the cytoplasm in SSZ treated cells (Figure 3.4a). The released cytochrome C from mitochondria leads to activation of caspase-3 and downstream substrates such as PARP (Figure 3.4b). Liptay et al. (2002) have demonstrated a similar response to SSZ stimulation in T-lymphocytes.
Moreover, to further substantiate SSZ role in apoptosis, nuclear DNA of CRC cells were stained with Hoechst 33285 and propidium iodine (HCT 116) stain. DNA fragments are observed in SSZ treated cells, indicative of apoptotic death.

3.4.2 SSZ elevates ceramide generation

Ceramides are cellular second messengers that are involved in variety of cellular events such as growth, viability and apoptosis (Kolesnick, 1991; Hannun, 1996; Quillet-Mary, et al., 1997). Our lab has previously studied SSZ's effect on ceramide generation in macrophages (Salh et al., 2002).

In this study we illustrate for the first time SSZ's capability to increase ceramide generation in CRC cell lines. This elevation in ceramide levels is accomplished both dose- and time-dependently (Figure 3.5). Obeid et al (1993) have previously demonstrated ceramides role in apoptosis by inducing DNA fragmentation. Thus, elevation of ceramide could be a possible route SSZ takes in order to induce apoptosis in the chosen CRC cell lines.

Since ceramides are generated via two different pathways; 1) the \textit{de novo} pathway and 2) SMase (sphingomyelinase) pathway (Gomez-Munoz, 1998; Mathias et al., 1998; Hannun and Luberto, 2000), we have conducted a few experiments to discover the origin of ceramide generation in CRC cell lines. Consequently, two \textit{de novo} pathway inhibitors were employed. Myriocin the \textit{de novo} sphingolipid synthesis inhibitor ISP-1 (Wispriyono, 2002) and Fumonosin B1 (FB1) a ceramide synthase inhibitor (Sweeney, 1996) were selected as \textit{de novo} pathway inhibitors. Both myriocin (Figure 3.6b) and FB1 (Figure 3.6a) in conjunction with SSZ treatment were capable of dramatically reducing ceramide generation. Therefore, our results for the first time ever illustrate SSZ’s involvement in the \textit{de novo} pathway in order to elevate ceramide generation. Thus, this ceramide generation may be involved in induction of apoptosis.

3.4.3 SSZ elevates reactive oxygen species

Reactive oxygen species are generated as by products of aerobic respiration. Mitochondria are one of the major sources of ROS production (Raha and Robinson, 2000). Other investigators have established ROS's role in induction of apoptosis (Buttke
and Sandstom 1994; Um et al., 1996; Salh et al., 2002). Additionally, Salh et al. (2002) have shown that SSZ triggers ROS generation in RAW 246.7 cells. Since we had shown effectively that SSZ lead to apoptosis, we wanted to analyse SSZ's role on ROS generation in colon cancer cells (HCT 116). Here we demonstrated that addition of 1 mM SSZ to CRC cells results in an increase in ROS generation (Figure 3.9). Our findings conflicts with studies conducted by Miyachi et al., (1987) which illustrated that addition of SSZ inhibited OH? (potent reactive oxygen species) production in leucocytes. This could be due to different cell lines and systems.

Utilizing pharmacological (BSO, CSA, and NAC) and enzymatic (CAT) ROS inhibitors we further investigated the possible source for SSZ induced ROS generation. We discovered that all of the inhibitors above were capable of inhibiting SSZ induced ROS generation. Since, most of the above inhibitors work at mitochondrial level (e.g. CSA); we believe that SSZ increases ROS through interactions with both mitochondrial chains plus interactions with glutathione pathway (GSH).

Subsequently, to see effects of SSZ mediated ROS on apoptosis, we took a closer look at the sub-diploid population in cells treated with both ROS inhibitors and SSZ. Figure 3.10 show that each of these inhibitors has fully or partially attenuated cell death. Salh et al. (2002) have shown similar attenuation in cell death upon treatment of RAW246.7 cells with NAC or CSA plus SSZ.

Various studies have established a link between ceramide elevation and ROS generation (Liu et al., 1998; Chan and Goldkorn, 2000; Andrieu-Abadie et al., 2001). Singh et al. (1998) by showing that inhibition of ROS by NAC could inhibit TNFα mediated conversion of SM to ceramide. Thus, we looked at effect of pre-treatment of HCT116 with NAC (5 mM) prior to stimulation with SSZ (1 mM) on ceramide generation. Figure 3.10 illustrates the lack of effect of NAC on ceramide generation in our cell lines. This data indicates that SSZ induced ROS generation and SSZ induced ceramide generation from the de novo pathway do not interact or overlap.

### 3.4 Summary and Conclusion

In this study we have demonstrated that SSZ is capable of inducing apoptosis in CRC cell lines. Apoptosis is accomplished by release of cytochrome C from the mitochondrial compartment into the cytosolic compartment. The release of cytochrome C leads to activation of caspase-3 and in turn PARP.
Furthermore, we have obtained results demonstrating SSZ's role in activation of ceramide generation. We have further analyzed the time- and dose-dependent ceramide elevation and have deduced that SSZ acts on the *de novo* pathway of ceramide synthesis, and thus leads to an increase in ceramide generation.

SSZ's role as a pro-oxidant has also been investigated. We found that addition of 1 mM of SSZ increases ROS generation. A closer look at the source of ROS generation indicates that mitochondria played a key role in free radical production.

Overall, administration of the anti-inflammatory drug SSZ to CRC cells leads to activation of various pathways which are all capable of inducing death. The results of this study are summarized in figure 3.10.
Figure 3.10: SSZ's mode of action in CRC cells.
Chapter 4. Apigenin

4.1 Introduction

Diet has been pegged as one of the major causes of variability in cancer incidence around the world. Epidemiological studies have made numerous observations concerning the importance of dietary factors in progression of colorectal malignancy (Zhao et al., 1991; Schatzkin, 1999). Factors such as fat, red meat, low fibre and low vegetable intake have been correlated with increased prevalence of colorectal cancers (Schatzkin, 1999). Therefore identification of dietary components which are involved in reduction of carcinogenesis are a prime area of interest in many laboratories worldwide.

There seems to be an inverse association between fruit and vegetable intake and cancer prevalence. This could be due to the presence of both micronutrient and non-nutrient components of fruits and vegetables. Previously, apigenin has been studied as a preventative agent for skin tumour promotion (Birt et al., 1996). Here we explore apigenin's role in colorectal cancer.

Apigenin is a plant flavonoid. Flavonoids are the largest class of polyphenols. There are more than 5000 different flavonoids described in the literature. Structurally, flavonoids consist of two aromatic rings linked through three carbons (Ross and Kasum, 2002). Figure 4.1 depicts the structure of apigenin. Originally, flavonoids were proposed as a required vitamin "P", but this was later dismissed (Rusznyak and Szent-Gyorgyi, 1936; Kuo, 1997). There are six major subclasses for flavonoids: Flavones, flavonols, flavanones, flavanols, isoflavones and anthocyanidins. Apigenin is part of the flavones subclass.

Apigenin is found in abundance in fruits and vegetables such as apples, beans, broccoli, celery, cherries, cloves, grapes, leeks, onions, barley, parsley and tomatoes. It is also found in plant-derived beverages such as tea and wine (Janssen et al., 1998).

Although originally it was thought that the absorption rate for flavonoids was not high, evidence to the contrary has been discovered. Studies on quercetin, a popular flavanoid, have demonstrated that approximately 52% of the quercetin available in onions are absorbed in humans (Hollman and Katan, 1999; Ross and Kasum, 2002).

The pharmacological safety of flavonoids is demonstrated through studies that estimate an average consumption of greater than 100 mg/day of all flavonoids. Studies
conducted in Netherlands indicate approximate consumption of 23 mg of flavones such as apigenin and luteolin on a daily basis (Hollman and Katan, 1999).

Figure 4.1 Structure of apigenin
4.2 Rationale and Hypothesis

Although there seems to be evidence for the anti-carcinogenic effects of apigenin in melanoma and prostate cancer, its mechanism of action in colon cancer is not yet fully understood.

Apigenin has been shown to inhibit growth, invasion and metastatic ability in melanoma (Caltagirone et al., 2000). It has also been shown to block tumour progression by inhibiting kinases, reducing expression of transcription factors and regulating cell cycle. It has been shown to inhibit enzymes such as topoisomerase I and II which are crucial for cell proliferation (Griffins et al., 1999). Apigenin is capable of inhibiting other major protein kinases such as Protein Kinase C (PKC) (Lin et al., 1997), mitogen-activated protein (MAP) kinase pathway. Apigenin has also been recorded as a potent inhibitor of Casein Kinase II (CK2), which is frequently unregulated in human cancers (Ford et al., 2000; Channavajhala and Seldin, 2002; Miro et al., 2002; Mead et al., 2003).

Ceramide has been indicated as an important mediator of various natural and pharmacological agents' actions in affecting cell growth, viability and differentiation (Kolesnick, 1991; Hannun, 1996; Quillet-Mary et al., 1997). Ceramide has also been shown to act on mitochondria, which is one of the major subcellular compartments contributing to apoptosis (Radin, 2001). Ceramides are known to effect the release of reactive oxygen species (ROS), which are generated by the respiratory chain, in mitochondria, thus leading to early apoptotic death (Davis, 2000; Radin, 2001).

Apigenin has been shown to cause ROS generation in leukemia cell lines. Apigenin induced apoptosis in HL-60 (leukemia cell line) has been shown to be due to release of cytochrome C and activation of caspase-3 and PARP. To date apigenin's role on the ceramide pathway has not been investigated.

Therefore in order to better understand the effect of apigenin as a dietary component in colon cancer, experiments were conducted to test the hypothesis that apigenin would induce cell death utilizing the ceramide and ROS pathways in colon cancer cells.
4.3 Results

4.3.1 Apigenin mediates apoptotic death in colon cancer cell lines

Apigenin's effect on cell survival in various cell lines has been observed (Wang et al., 1999). Here we demonstrate apigenin's effect on cell survival in three different CRC cell-lines.

Apigenin leads to apoptotic cell death in HCT116, DLD-1 and HT-29 colon cancer cell-lines. The mentioned cell-lines were seeded in 96 well plates and starved in 1% FBS containing media overnight. Subsequently, cells were then treated over a 24 h period with dose-escalating concentrations of apigenin. Utilizing the MTS assay, results demonstrate apigenin's capability to reduce cell viability in a concentration dependent manner (Figure 4.2a). As observed in figure 4.2a, concentrations 25 µM – 80 µM of apigenin show a marked reduction in cell survival in all three selected cell lines. Henceforth, HCT116 has been selected as a sample cell line used in most of the following studies.

Furthermore, flow cytometry analysis indicates a dose-dependent increase in sub-diploid population in HCT116 cells treated with varying concentrations of apigenin (Figure 4.2b). HCT116 cells were starved overnight in 1% FBS containing media. Cells were then treated with varying concentrations of apigenin over a 24 h period. Harvested cells were fixed using ethanol and nuclear DNA was stained with PI. Cells were separated and DNA integrity/ as measured by the quantity of sub-diploid DNA was analyzed on a flow cytometer. The results illustrate an increase in cell death in concentration dependent-manner (Figure 4.2b).

Apigenin's effect on apoptosis was investigated through its effect on release of cytochrome C. Western blot analysis of HCT116 cells treated with 80 µM of apigenin over a 24 h period demonstrates a leakage of cytochrome C from mitochondria into the cytosolic fraction (Figure 4.3a). This is a clear sign of apoptosis.

Similarly, HCT116 cells treated with varying concentrations of apigenin over a 24 h period were tested for PARP and pro-caspase-3 proteins. Western blot analysis of HCT116 cells treated with varying concentrations of apigenin demonstrate a gradual, dose-dependent decrease of both PARP and pro-caspase-3 proteins.
Figure 4.2: Apigenin reduces cell viability

A) Cell survival in HCT116, DLD-1 and HT29 colon carcinoma cells decreases dose-dependently upon their treatment with apigenin. Cells were seeded at 10,000/well density in 96-well plates. Cells were starved overnight in 1% FBS containing media and treated with various concentrations of apigenin over a 24 h time period. Cell viability was determined via (formazan) MTS method. Formazan was quantified by the amount of absorbance at 490 nm wave length, which is directly proportional to the number of viable cells in culture. Readings were taken in quintuplicate and results are representative of three independent experiments.

B) There is a direct correlation between sub-diploid population of HCT116 cells and apigenin concentration. With increase in apigenin concentrations (0-80 μM), there is a steady increase in sub-diploid population as analyzed from FACS data. The following results are representative of three independent experiments.
To further substantiation our data, HT29 and HCT116 cells treated with 80 μM of apigenin were stained with Hoechst 33285 (Figure 4.4a) and PI (Figure 4.4b) stains respectively. These stains are used to detect DNA fragmentation. The results demonstrate DNA fragmentation in HCT116 and HT29 cells.
Figure 4.3: Apigenin leads to apoptotic death in CRC cells

A) Treatment of HCT116 cells with 80 μM of apigenin leads to release of cytochrome C from the mitochondrial fraction into the cytosolic fraction. Cells were starved for 3 h in 1% FBS containing McCoy’s 5A media, and were then treated with 80 μM of apigenin for 24 h. Mitochondrial fractions and cytosolic fractions were separated. Western blot analysis indicated the movement of cytochrome C from mitochondria to cytosol. Results are representative of three independent experiments.

B) Concentration course of apigenin depicts a decrease in PARP and Pro-Caspase-3 proteins. HCT116 cells were treated with various concentrations of apigenin over a 24 h period. Western blot analysis of both PARP and Pro-caspase-3 indicates a decrease of both proteins at higher concentrations. Results are representative of three independent experiments.
A) 

Cytochrome C

<table>
<thead>
<tr>
<th>Apigenin 80μM</th>
<th>-</th>
<th>-</th>
<th>+</th>
<th>+</th>
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<tbody>
<tr>
<td>Cytosolic</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Mitochondrial</td>
<td>+</td>
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B) 

PARP

Pro-Caspase-3

<table>
<thead>
<tr>
<th>Apigenin [μM]</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>30</th>
<th>60</th>
<th>80</th>
</tr>
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Figure 4.4: Apigenin treatment in CRC cells leads to intra-nuclear DNA fragmentation

A) Pictorial representation of intra-nuclear DNA fragmentation HT29 colon carcinoma cell-lines. Cells were stained with Hoechst 33285-dye post treatment with 80 μM of apigenin over a 4 h period. The panels on the left represent control (untreated) cells while the panels on the right represents apigenin treated cells.

B) Pictorial representation of intra-nuclear DNA fragmentation in HCT116 cells treated with 80 μM of apigenin over a 4 h period. HCT116 cells were stained with PI.
A) Control  HT29  Apigenin 80 μM

B) Control  HCT116  Apigenin 80 μM
4.3.2 Apigenin elevates ceramide generation in CRC cells

Ceramides are known second messengers, which lead to various cellular events such as apoptosis. Therefore, apigenin's ability to cause apoptotic death was analysed by looking at ceramide generation. HCT116 colon cancer cells were labelled overnight with tritium palmitate. Cells were than starved over a 3 h period prior to treatment with various concentration of apigenin. Figure 4.5a shows that apigenin is capable of increasing relative ceramide generation over a 24 h period. Maximum increase in relative ceramide generation was seen with 10 μM of apigenin. From inspection of the time course data it is evident that apigenin (10 μM) leads to maximum ceramide generation at 24 h, and thus its effect is time dependent (Figure 4.5b).

However, since treatment of cells with 80 μM of apigenin lead to apoptotic death, we looked at earlier time points of this concentration in regards to ceramide generation. Figure 4.5c illustrates that around 4 h, 80 μM apigenin leads to maximal generation of relative ceramide generation.

From these results, 80 μM of apigenin over 4 h period, are the treatment conditions used in the upcoming experiments.

4.3.3 Apigenin mediated ceramide generation is independent of the de novo pathway

Ceramides are generated through either the de novo pathway or through sphingomyelin hydrolysis (SMase pathway) (Gomez-Munoz, 1998; Mathias et al., 1998; Hannun and Luberto, 2000). In order to decipher which pathway leads to increase in ceramide generation, two inhibitors were employed.

Myriocin, which blocks sphingolipid synthesis (Wispriyono, 2002) and fumonosin B1 (FB1), which blocks ceramide synthase (Sweeney, 1996), were used as de novo pathway inhibitors.

Tritium labelled HCT116 cells were pre-treated with either FB-1 (10 μM) (Figure 4.6a) or myriocin (25 nM) (Figure 4.6b) for 0.5 h prior to treatment with apigenin (80 μM). Over a 4 h incubation period, the relative ceramide generation was calculated and compared with apigenin control. Figure 4.6 demonstrates that there is no significant difference in level of ceramide generated. Thus apigenin induced ceramide generation is independent of the de novo pathway.
Figure 4.5: Apigenin elevates ceramide generation time and dose-dependently

A) Apigenin (10 μM) leads to maximum increase in ceramide generation over a 24 h period. HCT116 colon cancer cells were labelled overnight in H\textsuperscript{3}-palmitate and starved for 3 h in 1% FBS containing McCoy's 5A media. Cells were than treated with various concentrations of apigenin over a 24 h period. Ceramide generation was calculated and graphed. The following results are representative of three independent experiments.

B) Apigenin (10 μM) treatment causes increase in ceramide generation in a time-dependently. HCT116 cells were starved for 3 h in 1% FBS containing media and treated for various time points (up to 48 h) with 10 μM of apigenin. The graph bellow shows a time-dependent increase in ceramide generation. The results are examples from three independent experiments.

C) Apigenin (80 μM) leads to maximum increase in ceramide generation over a 4 h time period. This figure demonstrates a time course utilizing pre-labelled HCT116 cells. Cells were starved for 3 h in 1% FBS containing media and treated for various time points (up to 6 h) with 80 μM of apigenin. The graph bellow shows a maximum increase in ceramide generation at 4 h time point.
A) [Graph showing relative ceramide generation for different concentrations of Apigenin (0uM, 5uM, 10uM, 80uM).]

B) [Graph showing relative ceramide generation over time (0h, 1h, 2h, 4h, 8h, 10h, 24h, 48h).]
C)
Figure 4.6: Apigenin induced ceramide generation is independent of the de novo pathway

A) HCT116 cells were pre-labelled over night with H\textsuperscript{3}-palmitate. Cells were treated with FB-1 (10 µM) for 0.5 h and then treated with 10 µM of apigenin for 4 h. The following shows that ceramide generation is not effected in presence of apigenin plus FB-1.

B) HCT116 cells were pre-labelled over night with H\textsuperscript{3}-palmitate. Cells were treated with myriocin (25 nM) for 0.5 h and then treated with 80 µM of apigenin for 4 h. The following shows that ceramide generation is not effected in presence of apigenin plus myriocin.
A)

![Graph showing Relative Ceramide generation for different treatments.]

- Ctl
- FB 1
- 10 uM FB 1 + Apigenin

B)

![Graph showing Relative Ceramide production for different treatments.]

- Control
- Myriocin 25 nM
- Apigenin 80 uM
- Apigenin 80 uM + Myriocin 25 nM
4.3.4 Apigenin mediates ROS generation

Reactive oxygen species are typically generated as respiratory by-products. ROS is involved in early and late apoptotic event (Buttke and Sandstrom, 1994; Um et al., 1996; Salh, 2002).

Here we illustrate that treatment of HCT116 cells with apigenin at two different concentrations of 10 µM and 80 µM leads to ROS generation. HCT116 cells were starved for 2 h in 1% FBS containing McCoy's 5A media and then pre-labelled with free radical recognizing dye, DCFH-DA (2 µM) for 1 h prior to treatment with apigenin. Cell fluorescence was measured using flow cytometry (FL3). Figure 4.7(a-b) illustrate an increase in production of ROS levels in HCT116 cells treated with either 10 µM or 80 µM of apigenin.

In hopes of better understanding the source of this ROS generation, certain enzymatic and pharmacological inhibitors were used. Pharmacological inhibitors of ROS such as L-buthionine-[S,R]-sulfoximine (BSO), butylated hydroxyanisol (BHA) (Sakurai et al., 2001), and N-acetylcysteine (NAC) (Liu et al., 1998; Mansat-de Mas et al., 1999), along with catalase (CAT) an enzymatic ROS scavenger were utilized in an effort to identify the source of ROS generation.

Figure 4.8 demonstrates that BSO (2.5 µM), BHA (2.5 µM), CAT (1000 U), and NAC (5 mM) were all capable of dramatically reducing ROS generation in HCT116 colon cancer cells treated with apigenin (80 µM).

4.3.5 Inhibition of ROS leads to attenuation of cell death

To test whether apigenin induced ROS was involved in apoptosis; we used some of these ROS inhibitors and looked at cell death. Using flow-cytometry to examine the sub-diploid population, we treated our HCT116 cells with BSO (2.5 µM), CAT (1000 U), CSA (10 µM), BHA (2.5 µM) and caspase 1 inhibitor (DVD-FMR) for half an hour prior to Apigenin (80 µM) treatment.

BSO most effectively reversed apoptosis; BHA and CSA, also decreased cell death, but in a less dramatic fashion (Figure 4.8). Additionally, the combination of caspase-1 inhibitor plus apigenin, also lead to attenuation of cell death. Thus, these
results demonstrate that apigenin can affect glutathione transferase by inhibiting it and thus increasing ROS generation. This ROS generated due to treatment of apigenin in HCT116 cells, is involved in cell death. This is evident because inhibition of ROS generation lead to attenuation of cell death in HCT116 cells treated with apigenin.

4.3.6 Apigenin mediated ROS production is not upstream of the ceramide pathway

The relationship between apigenin mediated ROS and ceramide generations were further analyzed. HCT 116 cells were pre-labelled over night with H³-palmitate. Cells were starved for 3 h in 1% FBS containing McCoy's 5A media. HCT116 cells were then treated with 5 mM NAC for 0.5 h prior to treatment with 80 μM of apigenin. Cellular lipids were harvested, and separated using TLC. Thus, the level of relative ceramide generation was calculated. The results from this experiment demonstrated that inhibition of ROS generation has no effect on relative ceramide generation. This indicates that ROS generation does not precede ceramide production in this cell model system (results not shown)
Figure 4.7: Apigenin elevates ROS generation.

A) HCT116 cells treated with varying concentrations of apigenin (0-80 μM) exhibit an increase in ROS generation indicated by movement of cell masses on log scale.

B) HCT116 cell treated with apigenin lead to a dose-dependent increase in ROS generation. The following is a graphical representation of ROS generation in HCT116 treated with 10 μM and 80 μM of apigenin. Methodology is outlined in chapter 2 (section 2.2.8).

C) BHA, BSO, CAT and NAC were all able to reduce apigenin induced ROS generation in HCT116 cells. Methodology is outlined in chapter 2 (section 2.2.8).
A)

Apigenin 0µM

Apigenin 10µM

Apigenin 80µM
B) Absolute Fluorescence

C) Absolute Fluorescence

Treatment
Figure 4.8: Reduction of apigenin induced ROS generation leads to attenuation of cell death.

HCT116 cells were starved for 3 h and then treated with various ROS inhibitors and in one case caspase inhibitor, for approximately 1 h before treatment with 80 μM of apigenin for 4 h period. The results indicate a reduction in cell death and increase in cell viability upon inhibition of ROS and caspases 1 in HCT116 cells.
4.4 Discussion

4.4.1 Apigenin mediates apoptosis in CRC cells

The dietary flavonoid apigenin, found in abundance in fruits and vegetables, leads to apoptotic death in various cell types such as HL60 cells (Wang et al., 1999). However, to date there is limited research conducted on apigenin’s role in colorectal cancers; thus, in this study we have looked at the effect of apigenin on CRC cell-lines.

Initially, effect of various concentrations of apigenin on three different CRC cell-lines (HCT116, DLD-1 and HT29), were examined. Reduction in cell viability in all three cell lines was observed. This observation parallels studies conducted by Lee et al. (2002) in HL60 (human pro-myeloleukemic cells). Other investigators have also looked at effects of apigenin in other cell lines such as LNCaP cells (prostate cancer cells) (Gupta et al., 2002; Kobayashi et al., 2002).

Further confirmation of apigenin’s effect on viability is evident in PI stained cells which have been sorted using a flow cytometer. FACS analysis indicates an increase in sub-diploid population, which contains dead or damaged cells, with increased concentration of apigenin, in HCT116 cells (Figure 4.2b).

Therefore, to confirm apoptosis, apoptotic markers such as release of cytochrome C into the cytosol, cleavage of PARP and activation of caspase-3 were monitored (Abu-Qare and Abou-Donia 2001). The release of Cytochrome C into the cytosol initiates a cascade of cellular events, which ultimately lead to ‘programmed cell death’. Cytochrome C has been shown to cause caspase-3 activation, which eventually leads to cleavage of PARP (Figure 4.3 b, c). Our results corroborate with those found by Wang et al. (1999) who also show release of cytochrome C and activation of caspase-3 and thus PARP cleavage upon treatment with apigenin in HL60 cells.

Interestingly, here we show that there is a reduction in cell death in HCT116 cells pre-treated with a general caspase inhibitor and subsequently treated with apigenin (80 μM). This confirms apigenin’s role in apoptotic CRC cell death via activation of caspases. Using two different DNA binding dyes (PI and Hoechst 33285) we were able to demonstrate DNA fragmentation in apigenin treated cells.

Therefore, there seems to be overwhelming results in support of apigenin’s role in induction of apoptotic death in CRC cells.
4.4.2 Apigenin elevates ceramide generation

Ceramides act as second messengers, which lead to diverse cellular events such as growth, differentiation and apoptosis (Quillet-Mary, et al., 1997; Kolesnick, 1991; Hannun, 1996). Ceramides pro-apoptotic role has been illustrated through work done by Obeid, et al. (1993), who has shown its involvement in production of DNA fragmentation. To date there are no studies investigating the role of flavonoids on ceramide generation.

In this study for the first time we demonstrate apigenin’s capability to induce ceramide generation in a concentration and time-dependent manner (Figure 4.5). To ensure that higher concentration of apigenin (80 μM) also lead to induction of ceramides generation, earlier time points were considered. Figure 4.6 shows that 80 μM of apigenin is capable of increasing ceramide generation best over 4 h stimulation period. This is merely to illustrate the legitimacy of the following experiments using these conditions.

Ceramides are generally generated via two separate pathways, 1) de novo synthesis and 2) sphingomyelin hydrolysis (SMase pathway) (Gomez-Munoz, 1998; Mathias et al., 1998; Hannun and Luberto, 2000). To distinguish which pathway apigenin interferes with in order to boost relative ceramide generation different inhibitors were utilized.

Two inhibitors, 1) Myriocin, the sphingolipid synthesis inhibitor ISP-1, (Wispryono, 2002) and 2) Fumonosin B1 (FB1) a ceramide synthase inhibitor (Sweeney, 1996) were selected as de novo pathway inhibitors. Neither Myriocin (Figure 4.7a) nor FB1 (Figure 4.7b) in conjunction with apigenin treatment were capable of changing levels of ceramide generation. Thus, indicating that apigenin leads to elevation of ceramides independent of the de novo pathway. Further investigation is needed to look at SMase activity in presence of apigenin.

4.4.3 Apigenin elevates ROS

Previously, investigators have established a link in reactive oxygen species and its role in induction of apoptosis (Buttke and Sandstrom 1994; Um et al., 1996; Salh et al., 2002). In general, reactive oxygen species are generated as by products of aerobic respiration. Mitochondria are one of the major sources of ROS production (Raha and
Robinson, 2000). Apigenin has lead to apoptotic death utilizing the mitochondrial apoptotic pathway (i.e. cytochrome C release).

Thus, next the effects of apigenin on ROS generation in our colon cancer cells were analyzed.

Treatment of HCT116 cells with 80 μM of apigenin lead to elevation of ROS generation (Figure 4.8). Our results are in agreement with previous studies conducted in HL60 leukemia cells, where in they have shown an increase in ROS production upon stimulation of the cells with apigenin (Wang et al., 1999; Lee et al., 2002).

Next, utilizing pharmacological (BHA, BSO and NAC) and enzymatic (catalase) inhibitors of the ROS pathway, we took a closer look at source of ROS generation by apigenin. We discovered that all of the mentioned inhibitors in conjunction with apigenin stimulation were capable of reducing and in some cases attenuating ROS generation in HCT116 cells (Figure 4.9).

Subsequently, to see what effect ROS inhibition has on viability and apoptosis, the sub-diploid population of HCT116 cells were taken into account. Figure 4.8b illustrates a reversal in cell death in HCT116 cells treated with both inhibitor (CSA, BHA and BSO) and apigenin. Additionally, we have shown attenuation of cell death in presence of a generic caspase inhibitor in conjunction with apigenin. This serves as a positive control indicating a link between ROS produced by apigenin and apoptosis.

Since BSO interacts at the level of glutathione (GSH), we believe that apigenin leads to production of ROS via manipulation of this pathway. Breinholt et al. (1999) have illustrated that in colon of female rats, administration of dietary apigenin did not induce glutathione transferase. Since activation of glutathione transferase is associated with reduction in ROS, this corroborates with our findings that apigenin is capable of inducing ROS by disabling glutathione transferases regular antioxidant activity.

4.5 Summary and Conclusion

This study illustrates the role of a dietary flavanoid in induction of cellular death. Here we have demonstrated apigenin's capability to induce apoptosis in CRC cell lines. Addition of apigenin to CRC cells leads to release of cytochrome C and thus activation of caspase 3 and PARP, hallmarks of apoptosis.
Moreover, we have demonstrated for the first time Apigenin's role in inducing ceramide generation in both dose- and time-dependent manner. Further enzymatic studies are needed to analyze apigenin's effect on SMases activity.

We have also demonstrated that apigenin plays a role as a pro-oxidant in CRC cell lines. It seems that apigenin is capable of increasing ROS generation by inhibiting the glutathione transferase enzyme.

Overall, administration of apigenin to CRC cell lines lead to increase in apoptotic death. The mode of action of this flavanoid has been demonstrated in figure 4.9.
Figure 4.9: Apigenin's probable mode of action in CRC cells.
Chapter 5. Curcumin

5.1 Introduction

Colorectal cancer is one of the leading causes of cancer deaths in western society. Dietary manipulations have been shown to have a concrete impact on the prevention of many human cancers (Chauhan, 2002). One such dietary component is curcumin.

Curcumin (diferuloylmethane) is a yellow pigment that has been isolated from the ground rhizome of Curcuma species. Figure 5.1 shows the structure of curcumin. Several different species of Curcuma, indicated in table 5.1 (adapted from Lin and Lin-Shiau, 2001), have been analyzed for their curcuminoids (curcumin, demethoxycurcumin and bisdemethoxycurcumin) content. C. longa L. (turmeric) has been shown to have the highest concentration of curcumin as compared with other species (Lin and Lin-Shiau, 2001).

In general, curcumin is used for its colouring and flavour in various foods such as curry, mustard, bean cake, cassava paste and potato chips. It has also been implemented in drugs and cosmetics (Lin and Lin-Shiau, 2001).

Curcumin has been used and administered for centuries in Asian countries without any toxic effects. Some cultures are known to have consumed in excess of 100 mg of curcumin as a dietary spice on a daily basis (Ammon and Wahl, 1991).

Currently there is abundant support for curcumin's role as an anti-carcinogenic and anti-proliferative constituent in overall cancer treatment (Nagabhusahn and Bhide, 1992; Huang et. al., 1997). Epidemiological data indicates that curcumin plays a role in reducing rates of colorectal cancer in Asian countries (Chauhan, 2002). This is exciting since the development of a non-toxic natural agent, which contains chemo-preventive properties against colon cancer, has been a focus of investigation in many laboratories around the world.
Figure 5.1  Structure of Curcumin.
(adapted from: http://216.239.51.100/search?q=cache: Sr63amWDmMsC: www.hbcse.tifr.res.in/icho/ Theory/PDF/Org. pdf+curcumin+and+structure&hl=en&ie=UTF-8)

Table 5.1  Contents of curcuminoid the Rhizome of Curcuma Species
(adapted from Lin and Lin-Shiau, 2001).

<table>
<thead>
<tr>
<th>Curcuma Species</th>
<th>Total</th>
<th>Curcumin</th>
<th>Demethoxycurcumin</th>
<th>Bisdemethoxycurcumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcuma longa Linn.</td>
<td>3.97</td>
<td>1.84</td>
<td>1.09</td>
<td>1.01</td>
</tr>
<tr>
<td>Curcuma xanthorrhiza Roxb.</td>
<td>2.10</td>
<td>1.43</td>
<td>0.86</td>
<td>0.12</td>
</tr>
<tr>
<td>Curcuma wenyujin</td>
<td>0.20</td>
<td>0.13</td>
<td>0.07</td>
<td>0.02</td>
</tr>
<tr>
<td>Curcuma sichuanensis</td>
<td>0.04</td>
<td>0.01</td>
<td>0.01</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Curcuma Kwangsineasis</td>
<td>1.54</td>
<td>0.89</td>
<td>0.57</td>
<td>0.23</td>
</tr>
<tr>
<td>Curcuma aeruginosa Roxb.</td>
<td>0.04</td>
<td>0.01</td>
<td>0.01</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Curcuma elata Roxb.</td>
<td>0.01</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>
5.2 Rationale and Hypothesis

Even though curcumin has been demonstrated to be a potent inhibitor of chemical carcinogenesis, its mechanism of action is still not fully understood. At cellular level curcumin has been linked to several different events. Curcumin leads to inhibition of NFκB and thus acting in an anti-inflammatory fashion (Singh and Aggarwal, 1995), suppresses cell proliferation by inhibiting protein kinase PKC plus epidermal growth factor receptor (EGFR). It has also been demonstrated to inhibit arachidonic acid (Conney et al., 1991) metabolism and COX-2 activity (Huang et al., 1991; Lin and Lin-Shiau, 2001).

Curcumin appears to exert its anti-carcinogenic properties by inhibiting proliferation and inducing apoptosis in certain gastric and colon cancer cells (Moragoda et al., 2001). Curcumin leads to activation of various apoptotic pathways.

Ceramide has been indicated as an important mediator of various natural and/or chemical agents that are capable of influencing cell growth, viability and differentiation (Kolesnick et al., 1991; Hannun, 1996; Quillet-Mary et al., 1997). Ceramides have previously been described to initiate stress activated protein kinases such as SAPK / c-Jun kinase, and thus lead to apoptotic cell death (Verheij et al., 1996). Ceramides have also been shown to act on mitochondria, which is one of the major subcellular compartments contributing to apoptosis (Radin, 2001). It has been shown that ceramide leads to the release of ROS, which are generated by the respiratory chain in mitochondria, thus leading to early apoptotic death (Davis et al., 2000; Radin, 2001).

We hypothesized that curcumin would effect cell death utilizing the ceramide, JNK and ROS pathways. Therefore, the following experiments were conducted in order to better understand the underlying mechanism involved in induction of cell death by curcumin in colorectal cancer cells.
5.3 Results

5.3.1 Curcumin induces apoptotic death in colon cancer cell lines

In this study we demonstrate that curcumin leads to reduction in cell viability in a concentration dependent manner. Three different cell lines, HCT116, DLD-1 and HT-29 colon cancer cells were starved overnight and treated with dose-escalating concentrations of curcumin over a 24 h period. Utilizing MTS assay, cell survival was analyzed. Results indicate that exposure of all three CRC cell-lines to 50 μM curcumin over a 24 h period leads to a drastic reduction in cell survival in all three selected cell lines (Figure 5.2a).

Flow cytometry indicates a dose-dependent increase in sub-diploid population in HCT116 cells treated with various concentrations of curcumin over a 4 h period (Figure 5.2b). HCT116 cells had been seeded in 12-well plates and starved for 1 h in 1% FBS containing McCoy's 5A media prior to treatment with various concentrations of curcumin. Cells were then removed mechanically, fixed in ethanol and stained with propidium iodide (PI). Using flow cytometry HCT116 cells were then sorted and the percent sub-diploid population was determined as a marker for cell death. Results illustrate an increase in cell death, presented as a percentage of PI positive cells (sub-diploid population), in a dose-dependent fashion (Figure 5.2b).

We confirmed that this curcumin induced cell death is apoptotic by looking at cytochrome C release into the cytosol. Both HCT116 and DLD-1 cells were treated with 100 μM of curcumin over a 4 h period. Western blot analysis indicates a leakage of cytochrome C from the mitochondria into the cytosolic fraction (Figure 5.3a).

Furthermore, treatment of HCT116 colon cancer cells with different concentrations of curcumin over 24 h leads to a decrease in pro-caspase-3 and cleavage of PARP protein as determined via western blot analysis (Figure 5.3b).

To further corroborate our findings, curcumin treated cells were stained with either PI stain (Figure 5.4a) or Hoechst 33285 stain (Figure 5.4b). Intra-nuclear DNA fragmentation in cells treated with 100 μM of curcumin is observed in comparison to control cells (Figure 5.4).
Figure 5.2: Curcumin leads to reduction in cell death

A) Viability in HCT116, DLD-1 and HT29 colon carcinoma cells decreases dose dependently upon their treatment with curcumin (0-500 µM). Cells were seeded at 10,000/well density in 96-well plates. Cells were treated overnight in 1% FBS containing media and treated with various concentrations of curcumin over 24 h time period. Cell viability was determined via (formazan) MTS method. Formazan was quantified by the amount of absorbance at 490 nm absorbance, which is directly proportional to the number of viable cells in culture. Readings were taken in quintuplicate and results are representative of three independent experiments.

B) There is a direct correlation between sub-diploid population of HCT116 cells and curcumin concentration. With increase in curcumin concentrations (0-1 mM), there is a steady increase in sub-diploid population as analyzed from FACS data. The results depicted are representative of three independent experiments.
5.3.2 Curcumin elevates ceramide production

Ceramides are known second messengers that lead to activation of numerous pathways such as apoptosis (Gomez-Munoz, 1998; Mathias et al., 1998; Hannun and Luberto, 2000). Currently, the relationship between curcumin and ceramide generation in colon cancer cells is unknown.

HCT116 colon cancer carcinoma cells were labelled overnight with tritium palmitate. Cells were than staved for 3 h prior to treatment with various concentrations of curcumin over a 4 h time frame. Lipids were extracted and separated on a TLC plate, and consequently relative ceramide generation was analyzed. Results illustrate a dose-dependent increase in relative ceramide generation upon treatment of HCT116 cell with Curcumin (Figure 5.5a).

Moreover, this increase in relative ceramide generation is also time dependent. According to the illustrated results (Figure 5.5b), 50 μM of curcumin over 24 h, leads to a gradual increase in relative ceramide production. Ceramide generation due to treatment of HCT116 cells with 50 μM of curcumin peaks at 12 h, and seems to level off after this time point. From these results, we elected to use 50 μM of curcumin over a 4 h period, as the treatment condition in the ensuing studies.
Figure 5.3: Curcumin leads to apoptotic death

A) Cytochrome C leakage is observed in both HCT116 and DLD-1 cells treated with 100 μM of curcumin. Cells were starved for 3 h in 1% FBS containing media, and were then treated with 100 μM of curcumin for 4 h. Mitochondrial fractions and cytosolic fractions were separated. Western blot analysis indicates the movement of cytochrome C from mitochondria to cytosol. Results are representative of three independent experiments.

B) Concentration course of curcumin depicts a decrease in PARP and Pro-Caspase 3 proteins. HCT116 cells were treated with various concentrations of curcumin over a 24 h period. Western blot analysis of both PARP and Pro-Caspase 3 indicates a decrease of both proteins in the higher concentrations. Results are representative of three independent experiments.
### A)

**Cytochrome C**

<table>
<thead>
<tr>
<th>Condition</th>
<th>HCT116</th>
<th>DLD-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin 100μM</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cytosolic</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

### B)

**Parp and Caspase 3**

<table>
<thead>
<tr>
<th>Condition</th>
<th>1 μM</th>
<th>10 μM</th>
<th>100 μM</th>
<th>1 mM</th>
<th>2.5 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.4: Curcumin treatment leads to intra-nuclear DNA fragmentation

A) Pictorial representation of intra-nuclear DNA fragmentation in HCT116 a colon carcinoma cell line. Cells were stained with Hoechst 33285-dye post treatment with 100 μM of curcumin over a 4 h period. The panels on the left represent control (untreated) cells while the panels on the right represents curcumin treated cells.

B) Pictorial representation of intra-nuclear DNA fragmentation in HCT116 cells treated with 50 μM of curcumin over a 4 h period. HCT116 cells were stained with PI. The panels on the left represent control (untreated) cells while the panels on the right represents curcumin treated cells.
5.3.3 Curcumin mediated ceramide generation is independent of the de novo pathway

Generally, ceramides are generated via two different pathways, the de novo pathway and the sphingomyelin hydrolysis pathway (Gomez-Munoz, 1998; Mathias et al., 1998; Hannun and Luberto, 2000). In order to delineate which pathway curcumin utilizes to boost relative ceramide generation, two de novo pathway inhibitors, myriocin (Wispriyono, 2002) and fumonosin B1 (FB1) (Sweeney, 1996), were used.

Tritium palmitate labelled HCT116 cells were pre-treated with the above inhibitors half an hour prior to curcumin (50 μM) treatment over a 4 h incubation period. Lipids were isolated, separated on TLC plates and relative ceramide generation was calculated. Figure 5.6 illustrates that there is no change in relative ceramide levels in FB1 (10 nM) (Figure 5.6a) plus curcumin or Myriocin (25 nM) (Figure 5.6b) and curcumin treated cells. These results indicate that ceramide generation due to curcumin exposure is independent of the de novo synthesis pathway in CRC cells.

5.3.4 Curcumin mediated ceramide generation is desipramine insensitive

To further analyze pathways involved in curcumin induced ceramide generation, the sphingomyelinase (SMase) inhibitor desipramine was utilized. Desipramine is an inhibitor of acidic sphingomyelinase (Zhang et al., 2002).

HCT116 and DLD-1 CRC cells were pre-labelled with H³-palmitate overnight. Cells were then starved for 3 h in 1% FBS containing media. Both cell lines were then treated for 0.5 h with desipramine, prior to a 4 h treatment with curcumin (50 μM). Results illustrate that desipramine had no effect on curcumin dependent ceramide synthesis in two different colon cancer cell lines (HCT116 and DLD-1) (Figure 5.7). Hence, these results indicated that even though there is an increase in ceramide generation upon curcumin treatment in these cell lines, pre-treatment with desipramine lead to no change in the level of ceramide generation. This lends support to the notion that ceramide generation is unlikely to be due to acidic SMase activity.
Figure 5.5: Curcumin elevates ceramide generation time and dose-dependently

A) Curcumin (500 μM) leads to maximum increase in ceramide generation over a 4 h period. HCT116 colon cancer cells were labelled overnight in H$_3$-palmitate and starved for 3 h in 1% FBS containing McCoy's 5A media. Cells were then treated with various concentrations of curcumin over a 4 h period. Ceramide generation was calculated and graphed. The following results are representative of three independent experiments.

B) Illustration of a time course utilizing pre-labelled HCT116 cells. Cells were starved for 3 h in 1% FBS containing media and treated for various time points (up to 24 h) with 50 μM of curcumin. The graph shows a time-dependent increase in ceramide generation. The results are collated from three independent experiments.
Figure 5.6: Curcumin induced ceramide generation is independent of the *de novo* pathway.

A) HCT116 cells were pre-labelled over night with H\(^3\)-palmitate. Cells were treated with FB-1 (10 \(\mu\)M) for 0.5 h and then treated with 50 \(\mu\)M of curcumin for 4 h. The following shows that ceramide generation is not affected in the presence of curcumin plus FB-1.

B) HCT116 cells were pre-labelled over night with H\(^3\)-palmitate. Cells were treated with myriocin (25 nM) for 0.5 h and then treated with 50 \(\mu\)M of curcumin for 4 h. The following shows that ceramide generation is not effected in presence of curcumin plus myriocin.
A) 

![Graph A](image)

Relative Ceramide Generation

![Graph Legend A](image)

B) 

![Graph B](image)

Relative Ceramide Generation

![Graph Legend B](image)
5.3.5 Curcumin induces ROS generation

Reactive oxygen species are generated as by products of aerobic respiration. ROS have been shown to contribute to apoptotic cell death (Buttke and Sandstorm 1994; Um *et al.*, 1996; Salh *et al.*, 2002).

Curcumin leads to ROS generation in HCT116 colon cancer cells, dose-dependently (Figure 5.8 a, b). HCT116 cells were starved for 2 h in 1% FBS containing McCoy’s 5A media. Cells were then labelled with 2 μM of DCHF-DA, an ROS recognizing dye, for an hour prior to 4 h treatment with curcumin. Cells were later sorted using a flow-cytometer and absolute florescence, ROS generation, was calculated.

To further understand the underlying mechanisms involved in ROS generation, certain enzymatic and pharmacological inhibitors were used. Figure 5.8c illustrates that pharmaceutical ROS inhibitors, BSO (2.5 μM) and NAC (5 mM) plus enzymatic ROS inhibitor, extracellular catalase (1000 U), were all able to quench curcumin related ROS generation. Since the above inhibitors interact with the glutathione transferase, curcumin mediated ROS generation is associated with this biochemical reaction. Therefore, curcumin induces ROS generation through modulating specific inhibitors working at mitochondrial permeability transition.

5.3.6 Inhibition of curcumin mediated ROS generation leads to attenuation of cell death

ROS generation has been linked with early and late apoptosis. Therefore, the role of curcumin mediated ROS in cell death was further analyzed. Utilizing flow cytometry plus the previously mentioned ROS inhibitors, sub-diploid population in HCT116 colon cancer cells were determined.

HCT 116 cells were pre-treated with ROS inhibitors such as BSO (2.5 μM) and NAC (5 mM) for 0.5 h. Cells were then treated for 4 h with 100 μM of curcumin. Nuclear DNA was marked with PI stain and cells were sorted using flow cytometry.

Results illustrate that pre-treatment of HCT116 cells with BSO and NAC lead to an impressive attenuation of apoptotic death (Figure 5.9). This data demonstrates that curcumin mediated ROS generation leads to cell death in HCT116 colon cancer cells.
Figure 5.7: Curcumin induced ceramide generation is desipramine insensitive

Pre-labelled (H\(^3\)-Palmitate) HCT116 and DLD-1 cells were starved for 3 h in 1% FBS containing media. Cells were then treated for 0.5 h with desipramine (5 and 10 \(\mu\)M) prior to treatment with curcumin (50 \(\mu\)M). Relative ceramide generation was analyzed. Cells treated with both desipramine and curcumin show no inhibition of ceramide synthesis, thus indicating insensitivity of these cells to this inhibitor.

![Graph showing relative ceramide generation](image)

<table>
<thead>
<tr>
<th>Desipramine ((\mu)M)</th>
<th>0</th>
<th>0</th>
<th>10</th>
<th>10</th>
<th>5</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin ((\mu)M)</td>
<td>0</td>
<td>50</td>
<td>0</td>
<td>50</td>
<td>0</td>
<td>50</td>
</tr>
</tbody>
</table>
Figure 5.8: Curcumin elevates ROS generation.

A) HCT116 cells treated with varying concentrations of curcumin (0-1 mM) exhibit an increase in ROS generation dose-dependently. HCT116 were starved in 1% FBS containing McCoy's 5A media. Cells were pre-labelled with DCHF-DA, ROS recognizing dye, for 0.5 h prior to incubation with curcumin for 4 h. Cells were sorted using flow cytometry and fluorescence emitted by the dye was calculated.

B) HCT116 cell treated with curcumin lead to a dose-dependent increase in ROS generation.

C) BSO (2.5 μM), CAT (1000 U) and NAC (5 mM) (ROS inhibitors) were all able to reduce curcumin induced ROS generation in HCT116 cells.
Control

Curcumin 5 μM

Curcumin 10 μM

Curcumin 50 μM

Curcumin 100 μM
B)

![Graph showing ROS generation with varying curcumin concentrations for HCT116.](image)

C)

![Graph showing absolute fluorescence for different treatments.](image)
Figure 5.9: **ROS inhibition leads to reduction in cell death in HCT116 CRC cells treated with curcumin**

Inhibition of ROS leads to a decrease in cell death. HCT116 cells were starved for 2 h in 1% FBS containing McCoy's 5A media. Cells were then labelled via DCHF-DA 1 h prior to treatment with ROS inhibitors. Subsequently, HCT116 cells were stimulated with curcumin (100 μM) for 4 h. In ROS inhibited cells, cell survival dramatically increased.
5.3.7 Curcumin induced ROS generation is not upstream of ceramide pathway

Based upon previous studies illustrating a link between ROS and ceramide pathway we hypothesized that curcumin mediated ROS generation effects the ceramide pathways (Liu et al., 1998; Singh et al. 1998; Chan and Goldkorn, 2000; Andrieu-Abadie et al., 2001). Thus, the role of curcumin mediated ROS on ceramide generation was analyzed. HCT116 cells pre-labelled with tritium palmitate over-night. Cells were starved for 3 h and treated with NAC (5 mM) for 0.5 h prior to curcumin (50 μM) treatment. Cellular lipids were isolated and separated on a TLC plate. Using a scintillation counter the amount of ceramide generation was calculated.

Our results demonstrated no change in ceramide generation upon treatment with both NAC and curcumin (Figure 5.10). Thus the data illustrates that ROS generated via curcumin does not precede ceramide generation in HCT116 colon cancer cells.

5.3.8 Curcumin activates JNK

Based on previous work illustrating curcumin’s inhibitory effect on AP-1 DNA binding, a JNK pathway transcription factor we hypothesized that curcumin will inhibit JNK activation in our cell lines (Park et al., 1998; Chen et al., 1999; Chen et al., 2003).

HCT116 cells were seeded in 12 well plates and starved for 1 h in 1% FBS containing McCoy’s 5A media. Cells were then treated with 100 μM curcumin over a 2 h period. Cell lysates were collected at various time intervals, and the proteins were separated on SDS PAGE gel. Western blot analysis demonstrates an increase in JNK activity 60 min after curcumin stimulation (Figure 5.11a) These results were compared to levels of JNK protein (Figure 5.11b), which did not change over 2 h period. Thus we can conclude that curcumin stimulation lead to activation of JNK protein kinase in HCT116 cell lines.
Inhibition of curcumin induced ROS by NAC has no effect on ceramide generation

HCT116 colon cancer cells were pre-labelled with H3-palmitate over night and subsequently starved for 3 h in 1% FBS containing McCoy's 5A media. Cells were then treated for 0.5 h with NAC (5 mM) and then treated with 50 μM of curcumin over a 4 h period. No change in ceramide generation is observed.
Figure 5.11: Curcumin activates JNK pathway

A) Western blot analysis indicates an increase in activation of JNK via phosphorylation of this protein in a time dependent manner. HCT116 cells were plated in 6-well plates and starved in 1% FBS containing McCoy’s 5A media for 1 h. Cells were then stimulated with 100 μM curcumin for various (0-120 min) time points.

B) The following western blot is of the same experiment showing no change in the protein levels of JNK. Thus this result shows JNK activation while the protein level remains constant.

A) Phospho-JNK

B) JNK

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>30</th>
<th>60</th>
<th>120</th>
</tr>
</thead>
</table>
5.3.9 Inhibition of JNK attenuates cell death in curcumin treated cells

Role of curcumin induced JNK in cell survival was subsequently analysed. Two different cell lines, HCT116 (Figure 5.13b) and HT-29 (Figure 5.12c) were seeded in 12 well plates. These cells were pre-treated with JNK inhibitor; Anthra [1,9-cd] pyrazol-6 (2H)-one1, 9-pyrazoloanthrone (SP600125), for half hour prior to treatment with curcumin. This JNK inhibitor (Figure 5.13a) inhibits JNK by competing with ATP for binding to its ATP binding site. The concentrations of SP600125 were used as directed in Bennett et al. (2001) and Han et al. (2001). Cells were treated with curcumin for 4 h and cells were mechanically lifted and stained with PI.

Flow cytometry was utilized in order to examine sub-diploid population in the two cell lines. We found that in both cell lines cell death was reversed upon inhibition of JNK, indicating that curcumin induced JNK activation is involved in cell death (Figure 5.12b).

5.3.10 Inhibition of JNK has no effect on curcumin induced ROS generation

Having demonstrated a protective effect of JNK inhibition on curcumin induced cell death, we hypothesised that generation of ROS and JNK activation were connected. Therefore the effect of inhibition of JNK on ROS was investigated. Primarily, we looked at the level of ROS generation in CRC cells that had been pre-treated with the JNK inhibitor. After 1 h starvation in 1% FBS containing McCoy's media, HCT 116 cells were treated with SP600125 (JNK inhibitor) for 0.5 h prior to stimulation with Curcumin (100 μM) for 1h. The cells were collected and separated using FACS. The results indicate no change in ROS generation in JNK inhibited cells. Figure 5.13, illustrates that in our cell system, JNK activity does not precede ROS generation.

5.3.11 Inhibition of JNK has no effect on ceramide levels

The role of curcumin mediated JNK activation on the ceramide pathway was studied. HCT116 cells were seeded in 6 well plates and pre-labelled overnight with tritium palmitate. Cells were then starved for 1 h in 1% FBS containing McCoy's 5A
media. HCT 116 cells were then treated with SP600125 (JNK inhibitor) for 0.5 h prior to stimulation with 100 μM Curcumin (1 h).

Lipids were isolated and separated on a TLC plate, and thus ceramide levels were calculated. The results demonstrate that in the presence of JNK inhibitors there is no change in ceramide generation, this excludes a link between JNK activity and ceramide levels (Figure 5.14).

Therefore, there seems to be no evidence for curcumin induced JNK to be upstream of ceramide pathways.

5.3.12 Inhibition of ROS has no effect on JNK activation via curcumin

The relationship between ROS generation and JNK pathway activation were analyzed. HCT116 cells were seeded in 6-well plates and grown to 80% confluence. Cells were then starved in 1% FBS containing McCoy’s 5A media over a 1h period. ROS was inhibited by NAC (5 mM), 0.5 h prior to stimulation of cells with curcumin (100 μM). Cellular proteins were isolated and separated on a SDS-PAGE gel.

Western blot analysis demonstrates no change in JNK activation in ROS inhibited cells (Figure 5.15). Therefore, activation of ROS by curcumin is not linked to JNK activation by the same compound.
Inhibition of curcumin induced JNK activation instigates attenuation of cell death in two CRC cell lines

A) Structure of JNK inhibitor, SP600125.

B) Flow cytometry analysis of HCT116 cells treated with both JNK inhibitor and curcumin shows a reduction in sub-diploid population in comparison to curcumin treatment alone. HCT116 cells were plated in 12-well plates and starved for 1 h. Cells were then treated with JNK inhibitor (20 μM) prior to treatment with Curcumin (100 μM) for 1 h in 1% FBS containing McCoy’s 5A media. The treated cells were mechanically removed and stained with PI. Utilizing a flowcytometer, sub-diploid population was determined.

C) Flow cytometry analysis of HT-29 cells treated with both JNK inhibitor and curcumin shows a reduction in sub-diploid population in comparison to curcumin treatment alone. HT-29 cells were plated in 12-well plates and starved for 1 h in 1% FBS containing M199. Cells were then treated with JNK inhibitor (20 μM) prior to treatment with Curcumin (100 μM) for 1 h. The treated cells were mechanically removed and stained with PI. Utilizing a flowcytometer, sub-diploid population was determined.
Figure 5.13: Inhibition of JNK does not interfere with curcumin induced ROS generation.

HCT116 cells were seeded on 12-well plates, upon reaching 80% confluency cells were starved for 1 h in 1% FBS containing media and incubated with DCDA-FA, ROS recognizing dye for 1 h. HCT116 cells were then pre-incubated with 20 μM of JNK inhibitor for 0.5 h prior to stimulation with curcumin (100 μM). Utilizing flow cytometry, ROS generation via emission of the dye were analyzed and absolute fluorescence calculated. The data indicates that JNK does not precede ROS pathway.
Figure 5.14: Inhibition of JNK has no influence on relative ceramide generation.

Ceramide generation is not affected in HCT116 and DLD-1 cells that have been pre-treated with SP600125 (JNK inhibitor). Cells were plated on 6-well plates and labelled with $H^3$-palmitate overnight. Cells were then starved for 1h and treated with 20 μM of JNK inhibitor for 0.5 h. Cells were then stimulated with 50 μM curcumin and relative ceramide generation was calculated.
Figure 5.15: Effect of ROS on the activation of JNK in curcumin treated cells.

HCT116 cells were seeded in 6-well plates. Cells were starved for 1 h in 1% FBS containing McCoy's 5A media. 5 mM of NAC was administered 0.5 h prior to curcumin (100 μM) stimulation. Results are indicative of two separate pathways of activation in case of JNK and ROS by curcumin.
5.4 Discussion

5.4.1 Curcumin administration leads to apoptotic death in CRC cells

Utilizing the MTS assay, we have effectively shown that curcumin leads to a dose-dependent reduction in cell viability in different colon carcinoma cells (HCT116, DLD-1 and HT-29) (Figure 5.2a).

Next, we wanted to see if this decline in cell survival was due to apoptotic death, thus we looked at apoptotic markers. Flow cytometry was employed as a tool in order to analyse: size of the nuclei, chromatin complexity, and DNA integrity (Gomez-Lechon, 2002). In general, sub-diploid population depicts dead and damaged cells, where the cellular DNA is degraded. Thus, this is an effective method of looking for cell death. Sub-diploid population in HCT116 cells, treated with different concentrations of curcumin, was determined (Figure 5.2b). Results indicated a dose-dependent increase in sub-diploid population, with exception of 1 mM of curcumin. The observed recline in sub-diploid population in 1 mM curcumin treated HCT116 cells was largely due to increase in cell death, and thus fewer cells to analyze. Mori et al. (2001) report similar reduction in viability in their cell line of choice Colo-320 cell upon curcumin treatment.

Subsequently, we looked at the release of cytochrome C from mitochondria. Figure 5.3a depicts release of cytochrome C into the cytosolic fraction in two different cell lines (DLD-1 and HCT116). The release of cytochrome C triggers a cascade of cellular events that leads to apoptosis. Curcumin's effect on release of cytochrome C in different cell lines has been previously observed. Bhaumik et al. (1999) demonstrated that treatment of AK-5 tumour cells with curcumin lead to elevation of cytochrome C in the cytoplasm. There are numerous studies done on other cell lines (i.e. LNCaP, human prostate cancer cells; HL60, leukemia cells) showing elevation of cytoplasmic cytochrome C in curcumin treated cells (Pan et al., 2001; Deeb et al., 2003). Recently, Rashmi et al. (2003) have reported that some heat shock proteins such as hsp70, hsp90 and hsp27 have a protective effect against curcumin induced apoptosis in SW480 (derived from primary tumour) and SW620 (derived from the secondary lymph node tissue) colon cancer cells.

Release of cytochrome C leads to activation of series of caspases, such as caspase-3. PARP or poly (ADP-ribose) polymerase is also involved in DNA repair. Upon activation of caspase-3, PARP is cleaved and thus unable to repair DNA damage.
Figure 5.3b shows that as curcumin concentration increases both PARP and pro-caspase-3 protein levels diminish, thus indicating initiation of apoptosis. This data correlates with Anto et al.'s (2002) data showing that curcumin causes apoptosis in HL-60 cells by activation of caspase-3 and cleavage of PARP.

Additionally, as direct evidence of apoptosis, pictures of HCT116 cells were acquired. Figure 4 illustrates nuclear DNA fragmentation, which is the hallmark of apoptotic death. Propidium iodide and Hoechst stain was used to stain the DNA. DNA fragments are observed in curcumin treated cells, indicative of apoptotic death.

5.4.2 Curcumin elevates ceramide generation

Ceramides are second messengers involved in numerous cellular events, such as growth, viability and differentiation (Kolesnick, 1991; Hannun, 1996; Quillet-Mary et al., 1997). Figure 5.5 demonstrates curcumin's ability to elevate ceramides, both dose (Figure 5.5a) and time (Figure 5.5b) dependently. Ceramides have previously been shown to act in a pro-apoptotic manner leading to DNA fragmentation (Obeid, et al., 1993). Thus, elevation of ceramide levels due to curcumin treatment could be a possible mechanism leading to apoptosis. To date, the relationship between curcumin and ceramides in colon cancer cells has not been fully explored.

Ceramides are generally generated via two separate pathways, 1) de novo synthesis and 2) sphingomyelin hydrolysis (SMase pathway) (Gomez-Munoz, 1998; Mathias et al., 1998; Hannun and Luberto, 2000). To determine which pathway curcumin interferes with in order to boost relative ceramide generation, inhibitors of each pathway were employed.

Two inhibitors of the de novo synthesis were chosen to exclude this pathways intervention in curcumin induced ceramide generation. Myriocin the de novo sphingolipid synthesis inhibitor ISP-1 (Wispryono, 2002) and fumonosin B1 (FB1) a ceramide synthase inhibitor (Sweeney, 1996) were selected as de novo pathway inhibitors. The result of use of these yeast derived inhibitors indicated that curcumin elevates ceramide generation independent of the de novo pathway (Figure 5.6).

Activation of Sphingomyelinase has been thought to be the main pathway leading to generation of ceramides participating in apoptosis, following stress stimuli (Obeid and Hannun, 1995; Verheij et al., 1996; Mathias et al., 1998). Using desipramine, a non-specific sphingomyelinase inhibitor, (Erdreich-Epstein et al., 2002)
levels of ceramide generation in HCT116 cells were determined. Figure 5.7 showed no significant difference in curcumin induced ceramide generation in the presence of desipramine. These results are indicative that acidic SMases are not involved in curcumin dependent ceramide generation in HCT116 cells.

5.4.3 Curcumin elevates ROS

Typically, reactive oxygen species are generated as by products of aerobic respiration. Mitochondria are one of the major sources of ROS production (Raha and Robinson, 2000). Other investigators have established ROS's role in induction of apoptosis (Buttke and Sandstrom 1994; Um et al., 1996; Salh et al., 2002). Curcumin lead to apoptotic death utilizing the mitochondrial apoptotic pathway (i.e. cytochrome C release). Thus, we next wanted to see curcumin's effect on ROS generation within CRC cells.

Upon stimulation of HCT116 colon cancer cells, we observed an increase in ROS generation (Figure 5.8a). Similarly, Bhaumik et al. (1999) have shown an increase in ROS generation in curcumin treated AK-5 cancer cells. In contrary to our result, many have shown curcumin to be an anti-oxidant (Somasundaram et al., 2002). This contrast maybe due to:

1) Concentrations of curcumin used (we used (50-100 µM) concentrations as oppose to (1 µM) )
2) The inherent differences in cell lines involved

Next utilizing pharmacological (BSO and NAC) and enzymatic (catalase) inhibitors of the ROS pathway, we took a closer look at source of ROS generation by curcumin. We discovered that all of the above-mentioned inhibitors lead to attenuation of curcumin induced ROS generation. Since both NAC and BSO interact at the level of glutathione (GSH), we believe that curcumin leads to production of ROS via manipulation of this pathway.

Subsequently, to see what effect ROS inhibition has on viability and apoptosis, the sub-diploid population was observed. Upon treatment of HCT116 cells with both inhibitor and curcumin, cell death is reversed (Figure 5.8b). Kim et al. (2001) illustrated that pre-treatment with NAC also led to prevention of apoptosis in MCF10A human breast epithelial cells.
Various studies have established a link between ceramide elevation and ROS generation (Liu et al., 1998; Chan and Goldkorn, 2000; Andrieu-Abadie et al., 2001). Singh et al. (1998) have shown that inhibition of ROS by NAC could inhibit TNFα mediated conversion of SM to ceramide. Thus, we looked at the effect of pre-treatment of HCT116 with NAC prior to treatment with curcumin on ceramide generation. Figure 5.9 illustrates the lack of effect of NAC on ceramide generation in our cell lines. This data indicates that ROS generated here via curcumin does not affect ceramide generation.

5.4.4 Curcumin activates JNK

Jun N-terminal kinase (JNK) is a serine threonine protein kinase. The activity of this stress-activated protein kinase is facilitated by inflammatory cytokines, bacterial endotoxin, osmotic shock, UV radiation, and hypoxia (Derijard et al. 1994; Kallunki, et al., 1994; Bennett et al., 2001). Once JNK is phosphorylated (activated) it leads to activation of series of genes, such as activator protein-1 (AP-1), which precedes apoptosis (Verheij, 1996; Brenner et al., 1997; Salh et al., 2000; Chen et al., 2003).

Previously, curcumin has been used as a JNK inhibitor (Chen et al., 2003; Sowa et al., 2002). We wanted to see curcumin’s effect on JNK. Contrary to popular belief, we found that curcumin in fact activated JNK in HCT116 colon cancer cells. Figure 5.10 depicts activation of JNK by curcumin over a two-hour period.

Recently Mansouri et al. (2003) demonstrated that inhibition of JNK lead to attenuation of apoptosis. Since we have shown curcumin activated JNK, we wanted to see if inhibition of this JNK activation leads to apoptosis. Therefore, using the JNK specific inhibitor SP600125 (Han et al., 2001; Bennett et al., 2001), we looked at sub-diploid population in two different colon cancer cells, HCT116 and HT-29. We discovered that inhibition of curcumin-induced JNK activation, lead to attenuation of cell death and subsequently apoptosis (Figure 5.11).

Previously, Lo et al. (1996) have shown that ROS induces JNK activation. We next wanted to see whether this curcumin induced JNK activity, caused death utilizing either ceramides or ROS generating pathways.

First, we looked at level of ROS production while inhibiting JNK activity. Figure 5.12 demonstrates no difference in ROS level in HCT116 cells treated with SP600125
and curcumin. Our observations suggests that curcumin induced JNK does not commit cell to death via ROS generation.

We next wanted to see whether this curcumin induced JNK activity, causes death utilizing either ceramides or ROS generating pathways. First, we looked at level of ROS production while inhibiting JNK activity. Upon inhibition of JNK, no difference in ROS generation was observed (Figure 5.12). Thus, indicating in our cells JNK is not stimulating ROS generation in order to cause cell death.

Similarly, we looked at relative ceramide generation in presence of JNK inhibitor. Ceramides have been shown to trigger apoptosis through activation of the JNK pathway (Mathias et al., 1998). Looking at two different colon cancer cell-lines we demonstrated no significant change in ceramide production in presence of JNK inhibitor. This indicates that JNK is not upstream of ceramide generation. Therefore, a potential explanation for our results could be that curcumin leads to apoptosis via increasing ceramide generation, which in turn increases JNK activity. Thus, this activation of JNK probably leads our cells to their demise.

5.5 Summary and Conclusion

In the above study we investigated the role of curcumin on cell death in CRC cell lines. Administration of curcumin to CRC cells leads to activation of various apoptotic mechanisms. Here we have illustrated release of cytochrome C from the mitochondria into the cytosol, a trademark of apoptosis signalling. Further confirmation is available by looking at caspase-3 activation and PARP cleavage. DNA fragmentation is also evident in PI and Hoechst stained cells.

Here we demonstrated for the first time curcumin's capability for elevation of ceramide generation in CRC cell lines. Curcumin induces ceramide generation in a dose- and time-dependent manner. Although the potential route of ceramide elevation is still not clear, here we have demonstrated that inhibitors of de novo pathway did not have an effect on ceramide generation. Thus indicating that most probable route of action is through the SMase pathway. Further enzymatic studies on various SMases need to be conducted in order to better understand the mechanism involved in curcumin induced ceramide generation.
ROS generation is known to be involved in induction of cell death. Here we demonstrated an increase in ROS generation in curcumin treated CRC cells. Upon further analysis, we determined that curcumin activated GSH and thus induced ROS generation. Although there are various studies indicating a link between ROS pathway and ceramide pathway we were unable to establish a link.

In this study we have demonstrated for the first time curcumin’s ability to activate the SAPK/JNK stress pathway. Further analysis indicated that upon inhibition of JNK in curcumin treated cells, cell survival was decreased. This establishes role of JNK in induction of cell death by curcumin. The effect of JNK on ROS generation was also studied. We found that in the presence of JNK inhibitor, curcumin treated cells level of ROS generation did not change. Conversely, no change was seen in ceramide generation in JNK inhibited and curcumin treated CRC cells. This lead us to believe that curcumin is capable of inducing apoptotic by activation of various pathways which there is no necessary cross talk between them. Figure 5.16 illustrates a pictorial summary of curcumin’s activity.
Figure 5.16: Curcumin mode of action in CRC cells.
Chapter 6: Summary

6.1 Summary

In this study we have looked at the effect of an anti-inflammatory drug (SSZ) and two dietary components apigenin and curcumin on CRC cell lines. Here we have demonstrated that treatment with each of these three agents leads to apoptotic cell death. Apoptosis is characterized by: release of cytochrome C into the cytosol, activation of caspase-3, cleavage of PARP, and nuclear DNA fragmentation.

Furthermore, we have established that all three agents are capable of increasing ceramide generation, a lipid second messenger that is known to be involved in apoptosis. We have demonstrated that ceramide generation by apigenin and curcumin (dietary components) were independent of de novo synthesis. Conversely, we have demonstrated that SSZ is capable of increasing ceramide generation through the de novo synthesis pathway in our CRC cell lines.

Additionally, ROS generation was monitored in CRC cell lines treated with apigenin, or curcumin or SSZ. We have shown that ROS generation in our cell lines elevates upon treatment with of each of the mentioned agents. Further analysis revealed that in apigenin treated cells ROS was mainly produced via the mitochondria.

6.2 Future Direction

This thesis represents a mere stepping stone in the "big picture" of colon cancer. Further analysis of each agent is needed in order to better understand their mode of action. This thesis may be divided into three reports on the effect of various dietary and chemo-preventative agents on colon cancer cells. Thus to improve this research I will talk about each section separately.

6.2.1 Sulfasalazine

In this thesis, we were able to definitively demonstrate SSZ's effect on cell death, as well as illustrating its effect on ceramide generation and ROS generation. Although we have demonstrated SSZ route of ceramide generation, looking at its potential effect
on the SMases would be an interesting addition. Also, blocking of de novo synthesis and looking at death pathways such as caspase-3 activation, in order to decipher if ceramides generated through the de novo pathway could potentially lead to apoptosis.

Further analysis on the ROS pathway will be conducted. For example transfection of CRC cells with catalase containing plasmids would enhance our knowledge on the effect of SSZ on this enzyme. Also, effect of catalase transfection on ceramide pathways will be conducted in order to further analyze a possible link between ceramide generation and ROS generation in CRC cells.

6.2.2 Apigenin

In our studies we demonstrated that apigenin was capable of causing cell death in CRC cells. We also demonstrated that treatment of CRC cells with apigenin lead to elevation in ROS and ceramide generation.

In terms of apoptotic signals, further analysis of mitochondrial pathways will be analyzed in the future. Thus, the effect of apigenin on BCI-2 protein family members will be further studied.

Further studies will be conducted in order to decipher source of ceramide generation in apigenin treated cells. We have definitively demonstrated that apigenin does not increase ceramides through the de novo pathway. Next we will look at the SMases enzymatic activity to determine the level which apigenin affects this pathway.

In apigenin treated cells, further analysis could be conducted in order to decipher whether there is any shunting to other metabolites such as ceramide 1-phospahate. This is especially crucial for apigenin since we observed ceramide generation earlier in higher concentrations (80 μM) in comparison to lower concentrations (10 μM).

6.2.3 Curcumin

This thesis demonstrated that, administration of the dietary constituent curcumin, to CRC cells lead to decrease in cell viability, increase in ceramide and ROS generation and activation of SAPK/JNK stress pathways.

Further analysis of curcumin on BCL-2 family members will be conducted on CRC cells, in order to see curcumin’s effect on this anti/pro apoptotic family of proteins.
Similar to apigenin, curcumin treatment of CRC cells was not through the de novo pathway. Thus, further enzymatic studies of the SMases will be conducted to decipher the pathway from which ceramides are generated.

Additionally, over expression of JNK will be conducted in order to analyze its effect on ceramides and ROS generative pathways. Also, through addition of exogenous ceramide ROS and ceramide generation and JNK activity will be observed.

**OVERALL:** The above studies of each three chemical agent could be further analyzed by conducting an *in vivo* study. Administration of each agent (apigenin, curcumin and SSZ) into the feed of *min* mice models with colon cancer could be conducted; their colons excised and further analysis for ceramide generation and death pathways (i.e. presence of activated caspase-3, cleavage of PARP, Tunnel staining), and activation of ROS pathways may be conducted.


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