THE FLAVONOID APIGENIN UPREGULATES CD26/DPPIV ON HUMAN COLORECTAL CARCINOMA CELLS

by

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The only limit to our realization of tomorrow will be our doubts of today –
Franklin D. Roosevelt

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ABSTRACT

CD26/dipeptidyl peptidase IV is a cell-surface glycoprotein expressed by various cell types, including epithelial cells of the normal human colon. CD26 levels increase during epithelial cell differentiation but become dysregulated during malignancies such as colorectal cancer (CRC). CD26 depletion appears to contribute to the aggressive and metastatic phenotype of solid tumours via enhanced concentrations of the nucleoside adenosine and of the chemokine CXCL12. Apigenin (4′,5,7-trihydroxyflavone) is a flavonoid present in various fruits, vegetables and herbs, but is most abundant in the leafy herb parsley and dried flowers of chamomile. Apigenin interferes with several molecular targets implicated in cancer progression, although its effect has never been investigated in the context of CD26. In the present study we aimed to determine the effect of apigenin and other flavonoids on the multiple functions of CD26 (dipeptidyl peptidase activity, capture of ecto-adenosine deaminase and binding to cellular fibronectin) in a variety of human CRC cells. The effect of apigenin on CD26 was also evaluated in combination with current chemotherapeutic agents employed in the management of metastatic CRC. We have demonstrated that apigenin, alone and in combination with chemotherapeutic agents, increases cell-surface CD26 and its multiple functions in a way that would oppose tumour progression. Our work has further revealed a novel and highly selective topoisomerase I-dependent pathway in CRC cells that is activated by apigenin and which elevates cell-surface CD26 in a way that shows a unique interaction with the topoisomerase I inhibitor irinotecan. We therefore suggest that apigenin may be able to act alone and in conjunction with irinotecan, to modulate the metastatic phenotype of CRC cells and alter colon cancer progression.

LIST OF ABBREVIATIONS AND SYMBOLS USED

ºC	Degree Celsius
µM	Micromolar
µg/ml	Micrograms/milliliter
nM	Nanomolar
5-FU	5-fluorouracil
ADA	Adenosine deaminase
A-7-O-N	Apigenin-7-O-neohesperidoside
ANOVA	Analysis of variance
ALP	Alkaline phosphatase
APN	Aminopeptidase N
APC	Adenomatous polyposis coli
AP2	Activator protein 2
Api	Apigenin
BSA	Bovine serum albumin
BCL-2	B-cell lymphoma 2
CAM	Cell adhesion molecule
cFN	Cellular fibronectin
CD	Cluster of differentiation antigen
CDK	Cyclin-dependent kinase
CK2	Casein kinase 2
COX-2	Cyclooxygenase-2
cpm	Counts per minute

CRC	Colorectal cancer
DCC	Deleted in colorectal carcinoma
DISC	Death-inducing signaling complex
DMEM	Dulbecco's modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DPP	Dipeptidyl peptidase
DR	Death Receptor
DRB	5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside
eADA	Ecto-ADA
ECM	Extracellular matrix
E-cadherin	Epithelial cadherin
EC_{50}	Concentration producing half-maximal effect
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	EGF receptor
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FAP-α	Fibroblast activation protein-alpha
FAP	Familial adenomatous polyposis
FGF	Fibroblast growth factor
FOLFIRI	[Drug regimen comprising] 5-FU, folinic acid, irinotecan

FOLFOX	[Drug regimen comprising] 5-FU, folinic acid, oxaliplatin
FdUMP	Fluorodeoxyuridine monophosphate
g	Gram
g	1 x gravity
GI	Gastrointestinal
GLP	Glucagon-like peptide
GLUT	Glucose transporter
GSK-3β	Glycogen synthase kinase 3β
GSPE	Grape seed-derived procyanidin extract
h	Hour
HDAC	Histone deacetylase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HIF-1	Hypoxia-inducible factor-1
HGF	Hepatocyte growth factor
HNF-1α	Hepatocyte nuclear factor-1α
HNPCC	Hereditary nonpolyposis colorectal cancer
HRE	Hypoxia-responsive element
HRP	Horseradish peroxidase
HtrA2	High temperature requirement protein A2
HUVEC	Human umbilical vein endothelial cells
IAP	Inhibitor of apoptosis
ICAM	Intercellular adhesion molecule
IC_{50}	Half maximal inhibitory concentration

Ig	Immunoglobulin
IL-8	Interleukin-8
JAK	Janus-activated kinase
JNK	C-Jun N-terminal kinase
kDa	Kilodaltons
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
MDRP	Multidrug resistance-associated protein
MEK	MAPK/ERK kinase
mRNA	Messenger RNA
MRP	Multidrug resistance protein
MSI	Microsatellite instability
MSP	Methylation-specific PCR
MT1	Membrane type 1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-dimethytetrazolium
Min	Minute
NAG-1	Non-steroidal anti-inflammatory drug-activated gene-1
NCS	Newborn calf serum
NFκB	Nuclear factor kappa-light chain enhancer of activated B cells
NS	Not significant
NSCLC	Non-small cell lung carcinoma
OX	Oxaliplatin

PCR	Polymerase chain reaction
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PIG3	P-53 inducible gene 3
PI3K	Phosphatidylinositide 3-kinase
PMA	Phorbol 12-myristate 13-acetate
PMF	3',4',5',7-pentamethoxyglavone
P-gp	P-glycoprotein
PSCA	Prostate stem cell antigen
Ras	Rat sarcoma
RNA	Ribonucleic acid
RT	Reverse transcriptase
SN-38	7-ethyl-10-hydroxy-camptothecin
ROS	Reactive oxygen species
sCD26	Soluble CD26
SCID	Severe combined immune deficiency
SDF-1	Stromal cell-derived factor-1
SDH	Succinate dehydrogenase
SDS	Sodium dodecyl sulfate
SE	Standard error
SI	Sucrase-isomaltase
siRNA	Small interfering RNA
Smac	Second mitochondria-derived activator of caspases

SOD	Superoxide dismutase
Sp1	Specificity protein 1
STAT	Signal transducers and activator of transcription
TBB	4,5,6,7-tetrabromobenzotriazole
TCF-4	T-cell transcription factor 4
TEMED	N,N,N,N-tetramethylethylenediamide
TF	Transcription factor
TGF	Transforming growth factor
TIMP	Tissue inhibitor of matrix metalloproteinases
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Topo	Topoisomerase
UGT	Uridine 5'-diphospho-glucuronosyltransferase
uPA	Urokinase-type plasminogen activator
uPAR	UPA receptor
USF-1	Upstream stimulatory factor 1
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor
XIAP	X-chromosome-linked inhibitor of apoptosis

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CHAPTER 1

INTRODUCTION

1.1 Colorectal cancer

1.1.1 Overview of colorectal cancer

Colorectal cancer (CRC) refers to cancers originating from the large intestine, which includes the colon and the rectum. CRC is one of the major causes of cancer morbidity and mortality in the Western world (Burz, Berindan-Neagoe et al. 2009). Every year in Canada, an estimated 23,300 patients will be diagnosed with CRC and about 9,200 deaths will be reported (Canadian Cancer Society 2012). The lifetime risk for developing CRC varies as much as 20-fold worldwide, a difference that can be explained by genetic predispositions, lifestyle choices and accessibility to screening programs (Parkin, Bray et al. 2005). The vast majority of CRC arises first as an adenoma from the epithelial surface of the colonic mucosa, to become malignant and invade the remaining mucosa, submucosa, muscularis propria and serosa (Levine and Ahnen 2006). Following local invasion, CRC spreads (metastasizes) to regional lymph nodes to subsequently affect the liver and other distant sites such as the lungs and the bone marrow (Weiss, Grundmann et al. 1986). Unfortunately, due to the late onset and non-specific nature of symptoms such as constipation or diarrhea, abdominal cramps, weight loss, bleeding and bloating, the majority of CRC patients are diagnosed at a late stage of their disease (Weekes, Lam et al. 2009). A later stage diagnosis implicates regional and/or distant metastasis, which often results in a poorer prognosis for CRC patients.

1.1.2 Colorectal cancer development and progression

The vast majority of CRC cases arise sporadically, although inherited forms such as Familial adenomatous polyposis (FAP) and Hereditary nonpolyposis colorectal cancer (HNPCC) also contribute to disease burden, accounting for approximately 5 to 10% of CRC cases (Soravia, Bapat et al. 1997). FAP individuals feature genetic mutations within the adenomatous polyposis coli (*APC*) gene (Groden, Thliveris et al. 1991). Pathologically, FAP is characterized by the appearance of numerous adenomas carpeting the colorectal mucosa, especially in the rectosigmoid area, thus significantly increasing the risk of developing CRC in these patients (Groden, Thliveris et al. 1991; Goss and Groden 2000). HNPCC, commonly known as the Lynch syndrome, is another condition associated with the early development of CRC (Markowitz and Bertagnolli 2009). HNPCC patients experience an elevated rate of base substitutions, a phenotype known as microsatellite instability (MSI) (Martin, Lord et al. 2010). Microsatellites are defined as multiple tandem repeats of base pairs, which are very prone to mismatch errors and are normally repaired by mismatch repair proteins such as hMSH2 and hMLH1 (Geiersbach and Samowitz 2011; Markowitz and Bertagnolli 2009; Liu, Nicolaides et al. 1995). Nevertheless, HNPCC patients feature germline mutations in one or several of the mismatch repair genes, which results in genomic instability and predisposes affected individuals to the development of CRC (Hampel, Frankel et al. 2005; Svrcek, El-Bchiri et al. 2007; Markowitz and Bertagnolli 2009).

The full route by which normal colonic epithelial cells progress to CRC has been well characterized, and involves the mutational inactivation of tumour suppressor genes coupled with a series of mutational activations of tumour oncogenes (Fearon and Vogelstein 1990). These mutational events typically occur in a predictable sequential manner as illustrated in (Fig. 1.1). The initial and rate-limiting event in the development of most colorectal adenomas involves the genetic mutation of the *APC* gene (Fearon 2011). Most *APC* mutations introduce a premature stop codon in the gene to produce a truncated, dysfunctional APC protein (Goss and Groden 2000; Luu, Zhang et al. 2004). As a result, APC can no longer exert its suppressive role within the Wnt signalling pathway (a pathway regulating cellular proliferation and differentiation). Dysfunctional APC permits β-catenin (a protein that links the cytoplasmic domain of E-cadherin to the actin cytoskeleton) translocation to the nucleus (Fearon 2011). Once there, β-catenin becomes part of the transcriptional complex with T-cell transcription factor 4 (TCF-4) and initiates the transcription of genes implicated in cellular proliferation, migration and adhesion, such as those for c-Myc, cyclin-D1 and matrix metalloproteinase-7 (MMP-7) (Fearon 2011). A mouse model commonly employed to study CRC progression and drug intervention studies includes the multiple intestinal neoplasia mouse. This particular model features a truncating mutation at codon 850 within the *APC* gene and its phenotype resembles that of human FAP patients (Su et al., 1992).

Other common genetic alterations following APC inactivation that promote further growth of the initiated adenomatous lesion and progression to the carcinoma stage, include KRAS, Deleted in Colorectal Carcinoma (DCC) and p53 genes (Fearon

and Vogelstein 1990; Calvert and Frucht 2002; Markowitz and Bertagnolli 2009). Despite the many efforts in determining molecules implicated in CRC initiation and expansion, attempts to identify molecules implicated in metastasis have been lacking. Molecules implicated in the overall prognosis of CRC patients have however been identified, and overexpression of the following genes: CD133 (Chen, Song et al. 2013), autophagy-related genes such as ATG10 (Jo, Kim et al. 2012), and MMP-9 (Li, Yuan et al. 2012) have been associated with a poorer survival.

The most important prognostic factor of patient outcome is the extent and severity of disease burden or the tumour stage. Tumour staging follows the Tumour-Node-Metastasis (TNM) system, describing tumour invasiveness, extent of lymph node involvement, and the presence and absence of metastasis as reviewed by (Compton and Greene, 2004). Altogether, the TNM system determines the stage of the affected cancer patient, which ranges from stage 0-IV. Staging allows medical experts such as oncologist and radiologist to determine the disease burden and consequently recommend optimal therapy (Compton and Greene, 2004; Newland, Chapuis et al., 1981).

1.1.3 Colorectal cancer therapy

Treatment strategies for CRC vary according to the disease stage as well as operational factors such as the geographical location of the health care organization. Suggested systemic therapy for metastatic CRC (in addition to surgery where warranted), typically involves a combination of 5-fluorouracil (5-FU) or its oral equivalent

capecitabine, oxaliplatin (OX) and/or irinotecan. These chemotherapeutic agents are often paired with cetuximab (a monoclonal antibody [mAb] targeting the epidermal growth factor receptor [EGFR]) or bevacizumab (a mAb targeting the vascular endothelial growth factor [VEGF] -A) (Javle and Hsueh 2009). Therapeutically, metastatic CRC patients treated with chemotherapeutic agents have a median survival of more than 20 months, compared to 6-8 months with supportive care alone (Goldberg, Rothenberg et al. 2007). In certain cases, patients undergoing therapy survive well beyond the median 20 months, although the disease eventually overcomes therapy due to the development of drug resistance and/or severe treatment-related toxicities, causing the cessation of treatment (Goldberg, Rothenberg et al. 2007).

1.1.3.1 The antimetabolite 5-fluorouracil

5-Fluorouracil is a pyrimidine antagonist, one of a class of antimetabolites that structurally resemble the naturally occurring RNA pyrimidine base uracil. The metabolism of 5-FU is complex, and following passive entry into cells, 5-FU becomes converted into various metabolites, including fluorodeoxyuridine monosphate (FdUMP) (Longley, Harkin et al. 2003). The primary mechanism of 5-FU cytotoxicity is exerted by the FdUMP metabolite, where FdUMP and the cofactor 5,10-methylene-tetrahydrofolate (CH_2FH_4) come together to form a stable ternary complex with thymidylate synthase (an enzyme implicated in DNA synthesis) to inhibit its activity (Myers, Young et al. 1975). Other toxicities exerted by 5-FU are mediated through the incorporation of other metabolites into RNA and DNA (Longley, Harkin et al. 2003). Since the mid-1980s, 5-FU has been administered in conjunction with leucovorin, a tetrahydrofolate precursor

that stabilizes the binding of 5-FU to thymidylate synthase as well as inhibits the enzyme itself, thus improving drug action and overall survival (Thirion, Michiels et al. 2004).

1.1.3.2 The crosslinking agent oxaliplatin

Oxaliplatin (OX) is a third-generation platinum drug and unlike 5-FU and irinotecan, OX damages DNA in a cell-cycle non-specific manner. Compared to numerous anti-cancer agents, OX moves into the cells at a much slower rate. The slower uptake can be explained by the fact that OX relies on the copper transporter 1 for cellular accumulation (Holzer, Manorek et al. 2006; Howell, Safaei et al. 2010). The mechanism of action of OX is similar to that of alkylating agents whereby following cellular entry, OX forms intrastrand cross-links between two adjacent guanine-adenine bases or two adjacent guanine bases in the DNA, which results in the formation of bulky platinum:DNA adducts (Misset, Bleiberg et al. 2000). DNA adducts formed by OX interfere with the normal cellular machinery affecting DNA transcription and replication to subsequently trigger cellular death pathways (Burz, Berindan-Neagoe et al. 2009). Oxaliplatin is widely used in the therapy of CRC, and in combination with 5-FU and leucovorin (FOLFOX), represents the first-line treatment for metastatic CRC (Bokemeyer, Bondarenko et al. 2009).

1.1.3.3 The topoisomerase I inhibitor irinotecan

Irinotecan (also known as CPT-11) is a semisynthetic analogue of the natural alkaloid camptothecin, derived from the Chinese tree *Camptotheca acuminata*. Irinotecan

is activated through hydrolysis by carboxyesterases in the liver, to generate the active metabolite 7-ethyl-10-hydroxy-camptothecin (SN-38). In turn, SN-38 selectively inhibits the nuclear protein DNA topoisomerase I (topo I) (Zunino and Pratesi 2004; Sriram, Yogeeswari et al. 2005). Topo I functions to relieve supercoiling, in order to accommodate cellular processes such as DNA replication and transcription. To relieve DNA supercoiling, topo I cleaves a single-strand of DNA and forms a short-lived intermediate structure called the topo I covalent complex (also known as the cleavage complex) (Tentori, Leonetti et al. 2006). The formation of the covalent complex is necessary to allow the DNA strand to rotate and release torsional strain, to subsequently become religated (Tentori, Leonetti et al. 2006). The covalent complex generated by topo I is the cellular target of topo 1 inhibitors such as irinotecan. Binding of such drugs will stabilize the covalent complex and prevent religation of the single-strand break (Pommier, Pourquier et al. 1998). Toxicity exerted by topo inhibitors occurs once single-stranded breaks initially formed by topo I encounter a replication fork, to generate double-stranded breaks. The production of double-stranded breaks can result in a number of cellular consequences such as cell cycle arrest, DNA repair and/or activation of cellular death pathways (Gilbert, Chalmers et al. 2012). For the purpose of cancer therapy, cell cycle arrest and/or cell death are the preferred pathways activated by topo inhibitors, since activation of DNA repair pathways can interfere with drug toxicity. Irinotecan is often paired with 5-FU and leucovorin in a combination known as FOLFIRI, and is currently given as the second-line treatment for metastatic CRC (Rougier and Lepere 2005).

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1.2 Apigenin

1.2.1 Dietary factors influence the process of carcinogenesis

The risk factors for cancers of the gastrointestinal tract vary considerably across different geographical regions. Epidemiological and case-control studies have shown that some of these risk factors are dietary; for example the Japanese population is more susceptible to stomach cancer possibly caused by an elevated consumption of salty foods (Murata, Fujino et al. 2010), and African Americans are at a greater risk of developing CRC most likely due to an elevated consumption of processed meat (Norat, Lukanova et al. 2002; Ollberding, Nomura et al. 2011). Perhaps as much as 33% of all total neoplastic disease might be preventable with attention to lifestyle issues including diet (de Vere White, Hackman et al. 2010). The relationship between dietary factors and cancer has received great scientific and public attention. One area that has been of particular interest is the consumption of fruits and vegetables, reviewed in (Vainio and Weiderpass 2006; Gordaliza 2007; Key 2011).

Much of the interest relating to the roles of fruits and vegetables has focused on the early development of cancer, the process we know as carcinogenesis. Interfering with this process through natural dietary management or by the added ingestion of nutraceuticals has been termed 'chemoprevention' (Sporn, Dunlop et al. 1976). However, in the absence of effective population strategies for cancer prevention or risk avoidance there will always be significant disease incidence, and the role of the diet in influencing disease progression is also of importance. There is substantial current interest in the ability of natural products to dampen metastasis, in particular focusing on their abilities to interfere with oxidative stress and inflammatory processes (Khan and Mukhtar 2010; Reuter, Gupta et al. 2010; Gupta, Hevia et al. 2012).

1.2.2 The flavonoid apigenin

While fruits and vegetables are an abundant source of many cofactors, vitamins and minerals, the phytochemical constituents known as flavonoids have particular potential in acting against cancer (Liu 2004; Murthy, Mukherjee et al. 2009). Flavonoids are the most common type of phytochemical in the human diet (Ren, Qiao et al. 2003; Yao, Jiang et al. 2004). Thus far, over 6,000 different flavonoids have been characterized and are distinguished from other aromatic compounds by having a common phenylchromanone structure (C6-C3-C6) consisting of 2 benzene aromatic rings (A and B rings) linked by 3 carbons that are usually in an oxygenated central pyrone ring (C ring) (Harborne and Williams 2000; Birt, Hendrich et al. 2001; Yao, Jiang et al. 2004).

Based on the saturation level and opening of the central pyran ring, flavonoids can be classified into distinct sub-classes including flavanols, flavanones, flavanonols, flavonols, anthocyanidins, isoflavones and flavones (Lepiniec, Debeaujon et al. 2006; Patel, Shukla et al. 2007; Huang, Cai et al. 2010).

The compound 4′,5,7-trihydroxyflavone (IUPAC 5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one, molecular structure $C_{15}H_{10}O_5$, molecular mass 270.24 g/mol) is commonly referred to apigenin (Fig. 1.2). Apigenin exists in a wide range of plants, and is found at significant levels in many fruits, vegetables, herbs and spices. Major apigenin-containing food sources include thyme, cherries, tea, olives, broccoli, celery, and legumes; with the most abundant sources being the leafy herb parsley (*Petroselinum cripspum*) and dried flowers of chamomile (*Matricaria chamomilla*) (Manach, Scalbert et al. 2004; McKay and Blumberg 2006; Chung, Jiang et al. 2007).

Apigenin in fruits and vegetables occurs typically in a glycosidic form, where the tricyclic core structure is linked to a sugar moiety through hydroxyl groups (O-glycosides) or directly to carbon (C-glycosides). This modification appears to be critical for the storage and transport of apigenin in plants (Felgines, Texier et al. 2000; Ross and Kasum 2002; Braidot, Zancani et al. 2008). The common apigenin glycosides are apiin, apigenin-7-O-glucoside (apigetrin), apigenin-8-C-glucoside (vitexin), apigenin-6-C-glucoside (isovitexin) and apigenin 7-O-neohesperidoside (rhoifolin) (Nielsen, Young et al. 1999; Srivastava and Gupta 2007; Hanske, Loh et al. 2009; da Silveira, Trevisan et al.

2010). Apigenin glycosides may bind to proteins within plant sources, and be released as those proteins are broken down as part of the digestive process (Czubinski, Dwiecki et al. 2012).

1.2.3 Chemopreventive role of apigenin

For centuries, apigenin-containing plant preparations have been used as traditional medicines to treat diseases that have an inflammatory and/or degenerative component, such as asthma, insomnia, Parkinson's disease, neuralgia and shingles (Hamon 1989; Martens 1995; Awang-Dennis 2006). It was not until the 1960s that apigenin first attracted significant scientific interest, primarily due to its prevention of histamine release from basophils and its bronchodilating properties (see, (Spicak and Subrt 1958; Kawai, Hirano et al. 2007)). In the 1980s, apigenin was proposed to interfere with the process of carcinogenesis (Birt, Walker et al. 1986). Both initiation and promotion of carcinogenesis are suppressed by apigenin. Apigenin inhibits the mutagenicity of the carcinogens 2-aminoanthracene and benzo[a]pyrene (Birt, Walker et al. 1986). Apigenin pre-treatment also inhibits ornithine decarboxylase (Birt, Walker et al. 1986), the first enzyme in polyamine biosynthesis that is characteristically induced during carcinogenesis (Shantz and Levin 2007). Apigenin has been proposed as a cancer chemopreventative agent (Sarkar and Li 2004) and appears to confer protection against a wide array of cancers, as reviewed by (Patel, Shukla et al. 2007; Shukla and Gupta 2010).

1.2.4 Apigenin and the gastrointestinal tract

Interest in the physiological actions of apigenin and other flavonoids has driven research for several decades. Intraperitoneal administration of apigenin was found to inhibit small and large intestinal transit in mice, an effect that is mediated by alpha-2 adrenoceptors (Di Carlo, Autore et al. 1993). The Caco-2 system, which is a cell culture model of the intestinal epithelium, has also been used to investigate possible modulation of the P-glycoprotein (P-gp) transporter that mediates the efflux of various xenobiotics into the intestinal lumen. Flavonoids such as apigenin increase P-gp expression, which may be one mechanism that underlies their chemopreventive effect (Lohner, Schnabele et al. 2007).

Apigenin is less effective than tricin (4',5,7-trihydroxy-3',5'-dimethoxyflavone), and PMF (3',4',5',5,7-pentamethoxyflavone) in mouse models of CRC chemoprevention (Cai, Sale et al. 2009), suggesting that chemopreventive efficacy is favoured by O-methylation within the flavonoid core ring structure, and that apigenin itself may not be the premier flavonoid candidate for this purpose. However, substantially elevated apigenin levels may be attained with elective dosing, which may bring apigenin into the range for effective chemoprevention (Hoensch and Oertel 2011). Although apigenin has strong possibilities for chemoprevention, the focus here has been placed on the potential impact of apigenin on the progression and metastasis of existing gastrointestinal cancers (Fig. 1.3).

1.2.5 Actions of apigenin on cell growth and survival

1.2.5.1 Inhibition of the cell division cycle

Apigenin has been shown to inhibit proliferation and suppress cell cycle progression in a number of gastrointestinal cancer cell populations, including those of oral squamous carcinoma (Walle, Ta et al. 2007), esophageal (Zhang, Zhao et al. 2009), gastric (Wu, Yuan et al. 2005) and colorectal (Chung, Jiang et al. 2007) cancers, as well as cancers of organs associated with the gastrointestinal tract such as pancreatic carcinoma (Ujiki, Ding et al. 2006).

In human tongue squamous cell carcinoma SCC-9 cells, apigenin inhibited cellular proliferation with an IC_{50} of 40μM and this was associated with a decrease in cells found in the G1 phase of the cell cycle, which was in turn accompanied by an increase in the number of cells in G2/M, suggesting a block late in the cell cycle (Walle, Ta et al. 2007). Apigenin also inhibited cell growth in human esophageal cancer cells (both KYSE-510 cells, which harbor a p53 mutation, and OE33 cells), with a decrease in cells in the G1 phase and an increase in G2/M, suggesting cell cycle arrest at an apigenin concentration of 80μM (Zhang, Zhao et al. 2008; Zhang, Zhao et al. 2009). This was further associated with the down-regulation of the cell cycle regulator, cyclin B1, as well as an upregulation in p53-inducible gene 3 (PIG3) and downregulation of prostate stem cell antigen (PSCA) in both KYSE-510 and OE33 cells and an increase in p21/WAF1 in KYSE-510 cells (Zhang, Zhao et al. 2008; Zhang, Zhao et al. 2009). In human gastric cancer SGC-7901 cells, apigenin used at similar concentrations (up to 80μM) and with similar exposure times (24-48 h) as the above studies suppressed colony formation in a dose and time-dependent manner, but in this case the reduced proliferation was

associated with accumulation of cells in the S phase of the cell cycle (Wu, Yuan et al. 2005). There is therefore a consistent inhibition of proliferation at moderate (above ~20μM (Takagaki, Sowa et al. 2005)) concentrations of apigenin, but the specific nature of cell cycle interference may vary between different cellular targets.

Apigenin similarly inhibits proliferation in human CRC cells. Three of the most studied cell lines are HT-29, Caco-2 and SW480 human CRC cells, each of which show growth inhibition in response to apigenin using different assay systems (Wang, Heideman et al. 2000; Wang, VanAlstyne et al. 2004; Takagaki, Sowa et al. 2005; Chung, Jiang et al. 2007; Turktekin, Konac et al. 2011). The IC_{50} values with respect to cellular proliferation inhibition for SW480, HT-29 and Caco-2 cells treated with apigenin were all within the 40-70 μM range (Wang, Heideman et al. 2000), consistent with our own finding for HT-29 cells (Lefort and Blay 2011). The growth inhibition is accompanied primarily by a late cell cycle block with cells accumulating again at the more characteristic G2/M phase (Wang, Heideman et al. 2000; Wang, VanAlstyne et al. 2004; Takagaki, Sowa et al. 2005; Chung, Jiang et al. 2007), but with evidence also for a decrease in the number of cells in G1, while no changes noted in the S phase (Wang, Heideman et al. 2000; Wang, VanAlstyne et al. 2004; Chung, Jiang et al. 2007). Cellular accumulation in the G2/M phase between the different cell lines mirrors their relative sensitivities to growth inhibition (Wang, Heideman et al. 2000; Wang, VanAlstyne et al. 2004). The G2/M arrest is found to be reversible upon removal of the apigenin treatment (Wang, Heideman et al. 2000).

The mechanisms involved in apigenin-induced G2/M arrest and inhibition of growth have been studied. In HT-29 cells, apigenin at growth-inhibitory levels (≥ 30μM) enhanced the transcription of p21/WAF1 mRNA and production of p21/WAF1 protein, over a 6-24 h time course (Takagaki, Sowa et al. 2005). The cell cycle inhibitor protein p21/WAF1 is typically known for its involvement in G1 arrest, but also plays an important role in the G2/M checkpoint (Dulic, Stein et al. 1998; Foijer and te Riele 2006). The upregulation of p21/WAF1 by apigenin appeared to occur in a p53-independent manner, since (i) HT-29 cells are homozygous for the p53 gene, as reviewed in (Rodrigues, Rowan et al. 1990) and (ii) apigenin failed to trigger p53-responsive elements in a luciferase reporter assay in HT-29 cells unless the cells were co-transfected with a p53 expression plasmid (Takagaki, Sowa et al. 2005). However, the exact pathways are uncertain because p53 wild-type cells such as LoVo and HCT 116 also exhibited an elevation in p21/WAF1 following apigenin treatment (Zhong, Krisanapun et al. 2010). Apigenin also, over the same time course (24-48 h) and similar doses (30-80 μM) applied on CRC cell lines, reduces both the protein levels and activity of $p34^{cdc2}$, a key cyclin-dependent kinase involved in G2/M transition (Nurse 1990; Wang, Heideman et al. 2000) (Jackman, Firth et al. 1995). An interesting aspect of the cell-cycle arrest induced by apigenin relates to the APC gene mutation that is a particular feature of CRC. Normally, the APC protein plays a key role in the homeostasis of continuously proliferating tissues such as the intestinal epithelium, preventing uncontrolled growth and tumour formation. Defective APC is one of the critical elements in the pathway leading to colon cancer and more than 70% of CRC have inactivating APC gene mutations (Miyaki, Konishi et al. 1994). Each of the cell lines HT-29, Caco-2 and SW480 harbour

point or frameshift mutations in the APC gene (Rowan, Lamlum et al. 2000; Groden, Thliveris et al., 1991). When HT-29 cells were transfected with wild-type APC the resultant 'restored' cells (which were confirmed through western blotting to have normal levels of functional APC) no longer accumulated in the G2/M phase, as did the parental HT-29 cells (Chung, Jiang et al. 2007). This implicates APC in the pathway in which apigenin causes growth arrest.

A similar growth inhibition and G2/M cell cycle block is found in human pancreatic cancer cell lines (AsPC-1, CD18, MiaPaCa2 and S2-013), which again harbour p53 mutations (Ujiki, Ding et al. 2006). A single exposure to apigenin (25-100 μM, for 24-72 h) substantially reduced DNA synthesis and cellular proliferation in each of these cell lines (Ujiki, Ding et al. 2006; Strouch, Milam et al. 2009). Furthermore, treatment of these pancreatic carcinoma cells with apigenin resulted in accumulation of the cells in the G2/M phase of the cell cycle, and a decrease in levels of the nuclear proteins cyclins A and B, which are involved in G2/M transition (Ujiki, Ding et al. 2006) and, as reviewed by (Gopinathan, Ratnacaram et al. 2011).

1.2.5.2 Sensitization to apoptosis

Apoptosis, commonly referred to as programmed cell death, is an essential process in all eukaryotic cells that ensures normal tissue turnover and prevents the accumulation of genetic mutations (Hengartner 2000; Elmore 2007). It occurs under physiological circumstances such as development or aging, but also under pathological circumstances like disease states or during exposure to noxious agents. It is distinct from

cell death by generalized cellular dissolution, termed necrosis. The two major routes of apoptosis are the intrinsic or mitochondrial pathway and the extrinsic or death receptor pathway, as reviewed by (Elmore 2007).

Apigenin has been shown to cause cell death in cells from the different cancers of the gastrointestinal tract, and this is presumed to occur principally through apoptosis, although the assays used to measure cell death do not always distinguish the actual mechanism. Typically cell death ensues with the higher doses that are used to inhibit proliferation, beginning at ~80μM, but the sensitivity to apigenin varies in cells from different origins, and the concentration ranges reported in the literature for inhibition of cell proliferation and induction of apoptosis overlap considerably. Unless cell death is specifically tracked using biochemical methods, it is difficult to distinguish growth inhibition from a reduction in cellular viability in most cell- or dye-based growth assays.

Cell death specifically due to apoptosis has been identified and confirmed in cancer cells of the gastrointestinal tract using many different assays and biochemical markers:

(i) DNA fragmentation (Zhang, Zhao et al. 2008; Kim, Jeon et al. 2011; Turktekin, Konac et al. 2011),

(ii) induction and enhanced activity of caspases (caspases-3, -7, -8, -9 and -10) (Izeradjene, Douglas et al. 2005; Khan and Sultana 2006; Zhang, Zhao et al. 2008; Zhang, Zhao et al. 2009; Masuelli, Marzocchella et al. 2011; Turktekin, Konac et al. 2011),

(iii) an accumulation of cells in the sub-G1 region of cell-cycle analysis (Horinaka, Yoshida et al. 2006),

(iv) induction of death receptor 5 expression (DR5) (Horinaka, Yoshida et al. 2006),

(v) induction of the pro-apoptotic proteins p53 and non-steroidal anti-inflammatory drug-activated gene (NAG-1) (a TGF-β protein that is involved in apoptosis and anti-tumorigenesis) (Zhong, Krisanapun et al. 2010),

(vi) recruitment of procaspase-8 to the death-inducing signalling complex (DISC) complex at the level of the tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptors (Izeradjene, Douglas et al. 2005),

(vii) release of cytochrome c from the mitochondria (Izeradjene, Douglas et al. 2005),

(viii) inhibition of function of anti-apoptotic proteins such as the family of inhibitor of apoptosis (IAP), such as X-chromosome-linked inhibitor of apoptosis (XIAP) (Izeradjene, Douglas et al. 2005), and

(ix) binding of annexin V to phosphatidylserine at the cell surface (Lee, Ryu et al. 2008).

The process of apoptosis involves a cascade of successive activations of a series of cysteine proteases termed caspases. The caspase family includes both upstream (initiator) caspases (e.g. caspases -2, -8, -9, -10 and -12) and downstream (executor) caspases (e.g. caspases -3, -6 and -7) (Kim 2005; Elmore 2007; Pereira and Amarante-Mendes 2011). The complex activation of the caspase network, which occurs by cleavage and dimerization is modulated by a series of regulators, including IAPs, B-cell lymphoma

2 (Bcl-2) family proteins and certain cell-surface receptors for extracellular ligands (Abraham and Shaham 2004; Olsson and Zhivotovsky 2011; Varfolomeev and Vucic 2011).

There are several major pathways of apoptosis in the cell. These include the intrinsic apoptotic pathway, which is activated upon a cellular stress signal such as DNA damage, reactive oxygen species (ROS), hypoxia, or loss of cell adhesion (Eskes, Desagher et al. 2000; Pereira and Amarante-Mendes 2011; Frish and Francis 1994) and the extrinsic pathway, which is triggered by activation of cell death receptors (DR), members of the TNF receptor gene superfamily (FAS) (Wajant 2002; Spencer and Sorger 2011). Activation of these apoptotic pathways ultimately results in the cleavage of essential structural proteins such as cytokeratins and nuclear laminins and eventual DNA fragmentation and degradation, culminating in cell death and disposal (Fuentes-Prior and Salvesen 2004; Pereira and Amarante-Mendes 2011).

Apigenin has been studied extensively for possible effects on apoptosis in cell populations of many different cancers and has been shown either to directly induce apoptosis or to sensitize cells to other pro-apoptotic stimuli in cancer cells of oral (Masuelli, Marzocchella et al. 2011), esophageal (Zhang, Zhao et al. 2008; Zhang, Zhao et al. 2009), colorectal (Farah, Parhar et al. 2003; Izeradjene, Douglas et al. 2005; Zhong, Krisanapun et al. 2010; Turktekin, Konac et al. 2011), liver (Khan and Sultana 2006; Kim, Jeon et al. 2011) and pancreatic (Lee, Ryu et al. 2008) origin.

In human CRC this has been shown for cells of the HCT 116, HT-29, SW480 and LoVo lines (Zhong, Krisanapun et al. 2010; Turktekin, Konac et al. 2011). Concentrations of apigenin used have typically been in the 1-90 μM range for 24-72 h. Apigenin treatment of these cells leads to the induction of the cell cycle inhibitor p21/WAF1, pro-apoptotic proteins p53 and NAG-1, enhanced caspase-3 activity as well as DNA fragmentation (Zhong, Krisanapun et al. 2010; Turktekin, Konac et al. 2011). Changes in phosphorylation of p53 are observed in response to apigenin and can also be observed *in vivo* with HCT 116 tumours (Zhong, Krisanapun et al. 2010).

Broadly similar findings for apigenin have been made in oral cancer cell lines derived from the tongue (CAL-27, SCC-15) and the pharynx (FaDu) (Masuelli, Marzocchella et al. 2011); esophageal carcinoma cells (OE33, KYSE-510) (Zhang, Zhao et al. 2008; Zhang, Zhao et al. 2009); liver cancer cells (Hep G2, Huh-7) (Khan and Sultana 2006; Kim, Jeon et al. 2011) and pancreatic cancer cells (MiaPaCa-2 and AsPC-1) (Lee, Ryu et al. 2008), although concentrations of apigenin used in these studies have ranged to as high as 240μM (Kim, Jeon et al. 2011).

Within the populations of CRC cells, a major proximal target of apigenin seems to be casein kinase II (CK2), a multifunctional serine-threonine kinase that is involved in cell growth and proliferation, whose levels are frequently dysregulated in various types of human cancers, as reviewed by (Tawfic, Yu et al. 2001). CK2 overexpression is associated with cellular growth promotion and apoptosis inhibition (Izeradjene, Douglas et al. 2005). Apigenin (40-60 μM) inhibits CK2 in HT-29 cells (Izeradjene, Douglas et al.

2005) and HCT 116 human CRC cells (Farah, Parhar et al. 2003). Further in HT-29 cells, inhibition of CK2 activity has been demonstrated to (i) sensitize cells to TRAIL-induced apoptosis by increasing the recruitment of procaspase-8 to the DISC at the level of the TRAIL receptors, (ii) enhance TRAIL-induced release of cytochrome *c*, second mitochondria-derived activator of caspases (Smac)/DIABLO and high temperature requirement protein A2 (HtrA2)/Omi, with concomitant inhibition of the function of members of the IAP family (c-IAPI and XIAP) and (iii) enhance caspase-8 cleavage (Izeradjene, Douglas et al. 2005).

1.2.6 Actions of apigenin on the tissue environment

1.2.6.1 Modulation of angiogenesis

The development of a supportive vascular network for a growing tumour, known as angiogenesis, requires the formation and growth of new blood vessels from the pre-existing vasculature. In mature normal tissue there is a homeostatic balance between pro- and anti-angiogenic factors, but this needs to be tipped towards an angiogenic phenotype during solid tumour growth (Ezekowitz, Mulliken et al. 1992; Harris 1997; Cao 2001). This 'angiogenic switch' is essential in the formation and progression of tumours in order to provide oxygen and nutrients, as without the trigger for expanded vascular support the tumours are unable to exceed 1-2 mm in diameter at either their primary or secondary sites (Folkman, Long et al. 1963; Abdelrahim, Konduri et al. 2010).

This switch involves turning on the production of signalling molecules involved in angiogenesis (pro-angiogenic molecules), including fibroblast growth factor (e.g. FGF-1 and FGF-2), transforming growth factor (TGF), interleukin 8 (IL-8), angiogenin, platelet-derived growth factors (PDGF-B and PDGF-C), epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) (Ferrara 2002). Of these factors, VEGF-A (typically known as VEGF), is a primary regulator and is rate limiting for pathological angiogenesis to occur (Ferrara 2009). VEGF is a 45kDa dimeric glycosylated peptide that is not stored within a cell but rather secreted, and it acts after binding primarily to the VEGF receptor 2 (VEGFR-2) on endothelial cells, although it can also bind to VEGFR-1 (Neufeld, Cohen et al. 1999; Ferrara, Gerber et al. 2003). Receptor activation stimulates the phosphatidylinositol 3-kinase (PI3K)/Akt and rat sarcoma (Ras)/ mitogen-activated protein kinase (MAPK) pathways, leading to integrin activation, altered cell adhesion, degradation of the extracellular matrix (ECM) and the migration of endothelial cells (Zhong, Chiles et al. 2000; Jiang, Jiang et al. 2001).

VEGF and its receptors are aberrantly expressed in consort with tumour growth and progression; VEGF receptor expression tends to increase as tumours become more invasive and aggressive (Dallas, Fan et al. 2007; Rigopoulos, Tsiambas et al. 2010). The gene for VEGF is transcriptionally upregulated through hypoxia-inducible-factor-1 (HIF-1), under conditions of hypoxia and whereas HIF-1α is rapidly degraded and practically undetectable under normal circumstances (Jaakkola, Mole et al. 2001). During hypoxic conditions, HIF-1α becomes stabilized such that HIF-1α is able to bind its constitutively expressed partner HIF-1β, to form a heterodimer that binds to hypoxia-responsive

elements (HREs). The stabilization of HIF-1 initiates the transcription of genes implicated in the hypoxic response including those for VEGF and other players in angiogenesis such as endothelin-1, (Kerbel, Viloria-Petit et al. 1998; Ryan, Lo et al. 1998; Semenza 1999; Jaakkola, Mole et al. 2001).

Various cancer cells have been tested for their responses to apigenin during hypoxia, which contributes to cancer progression. When HCT-8 CRC cells were exposed to hypoxia, they expressed higher levels of VEGF and HIF-1α, and a fairly modest exposure to apigenin (for 1 h at up to 20μM) inhibited the effect of hypoxia (Fang, Zhou et al. 2007). Similar effects of hypoxia and an opposing effect of apigenin have also been found for breast and prostate cancer cells as well as cells from hepatocellular carcinoma (Fang, Zhou et al. 2007; Kim, Jeon et al. 2011). The effect of apigenin on VEGF has been reported to occur through inhibition of VEGF promoter activity and this occurs in an HIF-1α dependent manner (Fang, Zhou et al. 2007). As a result of impaired VEGF signalling, apigenin compromises the angiogenic process and for cells to gain the capacity to migrate (Fang, Zhou et al. 2007; Kim, Jeon et al. 2011).

1.2.6.2 Alteration in glucose uptake

The cellular and molecular milieu around an expanding population of cancer cells is referred to as the microenvironment of a solid tumour (Brahimi-Horn, Chiche et al. 2007). The tumour microenvironment is known to facilitate tumour progression and is one factor that leads to resistance to drug and radiation therapies (Brown and Giaccia 1998). One key aspect of tumour physiology responsible for the creation of this

environment is the tissue hypoxia, that results from the evolution of an imperfect vasculature that is unable to properly serve the rapidly expanding cancer cell population (Vaupel, Kallinowski et al. 1989).

One consequence of hypoxia is the need for cells to adapt to a less oxygen-dependent form of energy metabolism, glycolysis. This is less efficient than the aerobic metabolism characteristic of normal tissues, and therefore yields less energy in the form of ATP per molecule of glucose (Opie 1990). As a result, cancer cells deplete glucose rapidly from the extracellular fluid and at the same time produce excess amounts of the metabolic waste compound lactic acid (Gatenby and Gillies 2004; Gatenby and Gillies 2007).

To compensate and adapt to low glucose conditions, cancer cells overexpress glucose transporters (GLUTs) in order to facilitate glucose transport into the cell. GLUT transporters such as GLUT-1 are expressed at low levels in benign epithelial tissues (Younes, Lechago et al. 1996), but are overexpressed in epithelial malignancies such as CRC (Younes, Lechago et al. 1996). The mechanism of GLUT-1 overexpression is poorly understood for gastrointestinal malignancies, but work with other cancers points to the involvement of HIF-1α, the prime mediator of the hypoxic response, in GLUT-3 transcriptional regulation (Weidemann and Johnson 2008; Anso, Zuazo et al. 2010) and the P13K/Akt pathway in translocating GLUT-1 from the cytosol to the plasma membrane (Clarke, Young et al. 1994).

In terms of possible effects of apigenin, there has been substantial work in this area using human pancreatic cancer cells, particularly the lines S2-013 and CD18 (Melstrom, Salabat et al. 2008; Melstrom, Salabat et al. 2011). When glucose influx was measured in terms of ^{14}C-2-deoxyglucose uptake, apigenin (25-100 μM) reduced cellular glucose uptake during a 24 h period (Melstrom, Salabat et al. 2008). This was at least partly due to a diminished expression of GLUT-1, since apigenin treatment of up to 50μM reduced GLUT-1 mRNA (measured using q-RT-PCR) by over 70% at 24 h in both cell lines (Melstrom, Salabat et al. 2008). The reduced expression of GLUT-1 was confirmed at the protein level through western blotting (Melstrom, Salabat et al. 2008).

The ability of apigenin to downregulate GLUT-1 and therefore reduce glucose uptake can also be seen to work in opposition to the cellular adaptation to hypoxia. In the same pancreatic cells (S2-013 and CD18 cell lines), apigenin was found to inhibit hypoxia-induced GLUT-1 upregulation as well as decreasing VEGF and HIF-1 at both gene expression and protein levels (Melstrom, Salabat et al. 2011). A similar normalization by apigenin after hypoxia has been noted for GLUT-3 mRNA levels in NCI-H157 lung cancer cells (Anso, Zuazo et al. 2010). Therefore, at least in principle, apigenin is able to oppose the process by which cancers can adapt to their tumour microenvironment to better utilize nutritional energy sources.

1.2.7 Actions of apigenin on cellular invasion, adhesion and migration

1.2.7.1 Inhibition of enzymes implicated in extracellular matrix degradation

The principal distinctive behaviour of a malignant tumour is its ability to invade into surrounding normal tissues (Chambers, Groom et al. 2002; Friedl and Wolf 2003). Although the invasive process involves a complex series of events, proteolytic enzymes play a major part. These enzymes are responsible for the digestion of components of the ECM, such as laminin, fibronectin, collagen, elastin and heparin sulfate proteoglycans (Mott and Werb 2004; Jinka, Kapoor et al. 2012). Such proteolytic enzymes include matrix metalloproteinases (MMPs) and urokinase-type plasminogen activator (uPA) (Mignatti and Rifkin 2000).

MMPs (a family of over 25 members) are zinc-dependent proteinases and are the main proteolytic enzymes involved in tumour invasion and progression (Sternlicht and Werb 2001; Yoon, Park et al. 2003). MMP activity increases in malignant tumours, allowing the cancer cells to degrade ECM, penetrate the local basement membrane and subsequently move distantly to other regions. MMPs also function to regulate cellular adhesion (modulating ECM attachment) to facilitate migration. They also interact with epithelial cadherin (E-cadherin) (which is involved in cell-cell adhesion) and β4 integrin (involved in cell-ECM adhesion) (Yoon, Park et al. 2003).

Particular groups of MMPs have been implicated in tumour invasion for certain types of cancer. For instance, oral carcinoma is associated with elevated expression of MMP-1, -2, -3, -9 and membrane type 1- (MT1)-MMP, esophageal cancer displays elevated MMP-7, -9 and MT1-MMP, and in gastric carcinoma invasive behaviour is associated with increased MT1-MMP expression, as reviewed in (Yoon, Park et al. 2003).

Tumour invasion can also be influenced by tissue inhibitors of matrix metalloproteinases (TIMPs), which are important negative regulators of MMPs (Goldberg, Marmer et al. 1989). The TIMP gene family consists of 4 members: TIMP -1, -2, -3 and -4. TIMPs interfere with the malignant progression of tumours, by regulating the expression and activities of MMPs (Kahari and Saarialho-Kere 1999). In this area of cell regulation, reports of the effect of apigenin on the MMP/uPA/TIMP family of proteins specifically in gastrointestinal cancers are sparse. Nevertheless, flavonoids such as apigenin have been shown to promote epithelial differentiation and alter cell migration in cells from the colon epithelium, actions that may involve MMP activity (Fenton and Hord 2004).

However, work done principally with breast cancer cells has identified a number of effects that may be important (Reddy, Krueger et al. 1999; Lindenmeyer, Li et al. 2001; Birchmeier, Birchmeier et al. 2003; Lee, Chen et al. 2008). Specifically apigenin at concentrations in the range of 40-90 μM and with exposures of 12-24 h has been found to (a) inhibit the mRNA production and protein secretion (assessed by enzyme zymography)

of uPA while not affecting levels of the uPA receptor (u-PAR) (Lindenmeyer, Li et al. 2001); and (b) inhibit both basal MMP-9 production and the increased MMP-9 expression elicited by phorbol 12-myristate 13-acetate (PMA) and EGF (Reddy, Krueger et al. 1999; Lindenmeyer, Li et al. 2001). Consistent with the expected result of these changes in ECM-degrading enzymes, apigenin reduced the ability of the cells to invade through Matrigel® (a component mimicking the ECM) (Reddy, Krueger et al. 1999; Lindenmeyer, Li et al. 2001). Inhibition of invasion was evident even if cells were first exposed to high levels of exogenous u-PA (which saturates u-PA binding sites at the cell surface) (Lindenmeyer, Li et al. 2001).

Apigenin also acts to combat the increased invasive ability of breast cancer cells that have been treated with hepatocyte growth factor (HGF), which enhances both uPA and MMP expression. HGF binds to its receptor 'c-Met' to activate multiple signalling pathways involved in cellular invasion, such as P13K/Akt, RAS/MAPK and Janus-activated kinase/ Signal Transducers and Activators of Transcription (JAK/STAT), as reviewed by (Eder, Vande Woude et al. 2009). HGF and c-Met are overexpressed in human tumours and this correlates with metastatic disease and poor prognosis (Birchmeier, Birchmeier et al. 2003; Lee, Chen et al. 2008). Apigenin (up to 40μM for 12 h) reduced the cellular migration and invasion in MDA-MB-231 cells induced by HGF and was more effective when combined with the P13K inhibitor wortmannin, suggesting that the effect of apigenin likely occurred through the P13K/Akt pathway (Lee, Chen et al. 2008). Consistent with this, apigenin also inhibited HGF-induced Akt

phosphorylation, while not affecting Met, extracellular-signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) protein levels (Lee, Chen et al. 2008).

1.2.7.2 Modulation of cell adhesion molecules

As tissue architecture is lost in a cancer, the cancer cells also lose their epithelial polarity, as reviewed by (Martin-Belmonte and Perez-Moreno 2012). Various cell adhesion molecules (CAMs) undergo progressive changes in expression, leading to changes in cell-ECM and cell-cell adhesion to facilitate cell movement through the less-structured ECM. These CAMs are divided into four major groups, the cadherins, integrins, selectins and members of the immunoglobulin superfamily such as intercellular adhesion molecules (ICAMs) and vascular cell adhesion molecules (VCAMs), as reviewed by (Makrilia, Kollias et al. 2009). Apigenin alters the expression and function of many of the CAMs.

E-cadherin is a transmembrane glycoprotein located at the adherens junction between epithelial cells and is responsible for maintaining the integrity and polarity of the epithelium, linking to the actin cytoskeleton through an association with β-catenin (Wang, Li et al. 2011). In epithelial cancers however, E-cadherin expression is frequently reduced or lost, and this results in enhanced cellular motility, invasion and a more aggressive cellular phenotype (Cavallaro and Christofori 2004). Apigenin is able to enhance E-cadherin levels and its interaction with β-catenin, and therefore prevent the

deregulation of β-catenin signalling that contributes to tumour formation (Shukla, MacLennan et al. 2007).

Apigenin also affects the functions of integrins, a complex family of heterodimeric cell-surface receptors that modulate cell-ECM and cell-cell interactions necessary for cell motility and invasion (Desgrosellier and Cheresh 2010; Jinka, Kapoor et al. 2012). For example, apigenin suppresses HGF-induced β4 integrin clustering at actin-rich membrane adhesive sites and cellular lamellipodia on cancer cells, through the P13K/Akt pathway (Lee, Chen et al. 2008). As well as affecting the CAM on cancer cells, apigenin can alter its partner on normal cells with which the cancer cell interfaces. For example, apigenin (10μM) treatment of human umbilical vein endothelial cells (HUVEC) in culture significantly reduces expression of VCAM-1 (Piantelli, Rossi et al. 2006). In vivo, intraperitoneal injections of apigenin (50 mg/kg) reduce VCAM-1 immunostaining on the endothelial cells of lung capillaries (Piantelli, Rossi et al. 2006). Since VCAM-1 is a potential adhesion target for cancer cells that are metastasizing, one would anticipate that apigenin would oppose that process. Indeed in an *in vivo* mouse model (C57BL/6N) of a highly metastatic murine B16-BL6 melanoma cell line, apigenin significantly reduced the extent of lung metastasis (Caltagirone, Rossi et al. 2000; Piantelli, Rossi et al. 2006). Accordingly, as well as altering the cellular environment within the primary tumour, apigenin may alter the environment of the lung such that the cancer cells were less likely to interact with the endothelium and colonize the lungs.

1.2.8 Apigenin Binding to Cellular Proteins

Apigenin may exert effects acutely by binding to enzymes involved in rapid cellular responses. For example, Maldonado-Rojas and Olivero-Verbel have examined the potential interaction of certain dietary compounds with cyclooxygenase-2 (COX-2), one of the major enzymes involved in eicosanoid production (Maldonado-Rojas and Olivero-Verbel 2011). Apigenin and other related compounds showed high binding affinity to COX-2, of the same order of magnitude as established synthetic COX-2 antagonists (Maldonado-Rojas and Olivero-Verbel 2011), raising the possibility that such compounds may act as competitive antagonists at COX-2 and modulate prostanoid production.

Apigenin may also inhibit the activity of nuclear enzymes, potentially impacting upon longer term events including the proliferation and survival of cancer cells (Johnson, Rupasinghe et al. 2011). In comparative studies of flavonoids and other compounds from citrus, Gonzalez de Mejia and colleagues found that flavonoids had the highest inhibitory activity on glycogen synthase kinase 3β (GSK-3β), a serine/threonine kinase whose levels are overexpressed in the nucleus of several cancer cell types and that controls many intracellular signaling pathways by phosphorylating substrates such as β-catenin (Johnson, Rupasinghe et al. 2011). Of the flavonoids tested, apigenin (IC_{50}, 1.9μM) and its metabolite luteolin (IC_{50}, 1.5μM) had the highest potencies for inhibition.

Apigenin (MW, 270.2) and luteolin (MW, 286.2) are small molecules with limited informational content to provide selective binding to discrete cellular targets and elicit specific response(s). However, there are structural correlates to its biological potency, and emerging knowledge of the domain interactions and thermodynamic factors

involved in modulation of enzyme activity. A study carried out by Chen and colleagues assaying for inhibition of 20S proteasomal activity found that potency was favoured by hydroxylation of the flavonoid B ring and/or unsaturation of the C ring, allowing apigenin and luteolin to function much more effectively than their saturated counterparts in proteasomal inhibition and therefore the induction of apoptosis in tumour cells (Chen, Chen et al. 2007). In a study investigating the interaction of flavonoids with the CDK6/cyclin D complex (Khuntawee, Rungrotmongkol et al. 2012), apigenin and other analogues were found to compete with ATP for binding into the ATP pocket of CDK6 and interfere with formation of the complex with cyclin D. Comparisons between apigenin, chrysin and fisetin showed a correlation between CDK6 binding affinity and the number of charge interactions through hydroxyl groups for the different flavonoids. As well, the less hydroxylated chrysin bound to CDK6 with a different orientation to the other two flavonoids, suggesting that the extent and topology of possible hydrogen bonds formed by these polyhydroxyflavones is important for the interaction with the target site (Khuntawee, Rungrotmongkol et al. 2012). Other studies (e.g. an investigation of the ability of flavonoids to suppress TNFα production *in vitro,* (Ueda, Yamazaki et al. 2004)) show a similar dependence of effect on the degree of hydroxylation. Most explorations of flavonoid interaction with enzymes support the view that extent of hydroxylation is important for action, and Johnson and colleagues have provided evidence that hydrogen bonding through hydroxyl residues lowers the energy of interaction and favours flavonoid interaction with the enzyme and inhibition of the (usually kinase) activity (Johnson, Rupasinghe et al. 2011). These workers also confirmed the lower interaction and lesser stability of resultant complexes when the flavonoid core was conjugated to

bulky sugar side groups, consistent with the observed poor biologic activity of the glycoside forms (Johnson, Rupasinghe et al. 2011).

1.3 The multifunctional CD26 molecule

1.3.1 CD26/ dipeptidyl peptidase IV

Cluster of differentiation antigen 26 (CD26) is another cell-surface glycoprotein implicated in cellular adhesion. Specifically, CD26 binds to fibronectin (Gonzalez-Gronow 1996) and to types I and III collagen (Loster, Zeilinger et al. 1995). The binding of CD26 to ECM proteins may be of importance in the metastatic phenotype of cancer cells, due to the effects on cell spreading and migration (Cheng, Abdel-Ghany et al. 1998; Ghersi, Dong et al. 2002; Boonacker and Van Noorden 2003). Other functions associated with the multifunctional CD26 molecule, as summarized in (Fig. 1.4) have also important implications in the metastatic process of cancer cells.

CD26 belongs to the serine protease post-prolyl peptidase family and is closely related to fibroblast activation protein-alpha (FAP-α) and distantly related to DPP8 and DPP9 (Busek, Stremenova et al. 2008). CD26 was originally studied in 1966 by Hopsu-Havu and Gleener and characterized from rat livers for its enzymatic function; it was initially termed glycylproline naphthylamidase (Hopsu-Havu and Glenner 1966). Since then, the same cell-surface protein became known as adenosine deaminase-binding protein and adenosine deaminase-complexing protein (Schrader and Stacy 1977). In 1984, Fox and colleagues identified the protein as a leucocyte antigen and in 1993, the

protein was finally termed CD26, independently by groups led by Houghton and Schlossman (Fox, Hussey et al. 1984; Kameoka, Tanaka et al. 1993; Morrison, Vijayasaradhi et al. 1993). Nowadays, this protein is interchangeably known as CD26 or DPPIV, in reference to its main enzymatic activity.

The gene for CD26 is located on the long arm of chromosome 2 in the region 2q24.3, and contains 26 exons (Abbott, Baker et al. 1994). The cDNA of CD26 contains 3465 base pairs and encodes 766 amino acids, with a monomer molecular weight of 110-kDa (Misumi, Hayashi et al. 1992; Abbott, Baker et al. 1994). Unlike most genes, the 5'-flanking region of CD26 does not contain a TATA or CAAT box, but rather contains a region of CpG islands, several Specificity protein 1 (Sp1) binding sites and a 300 bp sequence rich in C and G allowing for the binding of several transcription factors (Bohm, Gum et al. 1995). Such transcription factors include nuclear factor kappa-light chain-enhancer of activated B cells (NFκB), activator protein 2 (AP2), Sp-1 (Bohm, Gum et al. 1995), c-Myc (Abe, Havre et al. 2011), HIF-1α (Abe, Havre et al. 2011), upstream stimulatory factor 1 (USF-1) (Erickson, Lai et al. 2000) and HNF-1α (Erickson, Lai et al. 2000). Previous findings have reported that most of these transcription factors have the ability to enhance the transcription of CD26, with the exception of c-Myc, which in fact decreases CD26 transcription (Abe, Havre et al. 2011). The sequence for CD26 is well conserved between mammal species, with human CD26 showing 85% homology with that of rat, and 36% with yeast although the peptidase portion of the molecule differs significantly in lower vertebrates (Misumi, Hayashi et al. 1992; Mentlein 1999).

1.3.2 The distribution of CD26

CD26 is primarily expressed at the cell surface of epithelial cells (Kreisel, Hildebrandt et al. 1993), although CD26 can also be found in endothelial cells of capillaries, mesothelial cells (Kajiyama, Kikkawa et al. 2002), fibroblast-like synoviocytes (Riemann, Hansen et al. 2001) as well as cells of the immune system such as natural killer cells, and T and B lymphocytes (Heike, Mobius et al. 1988; Fleischer 1994; Gorrell, Gysbers et al. 2001; Boonacker and Van Noorden 2003). In tissues, CD26 has a broad distribution, being present in the kidney, lung, adrenal glands, liver, parotid gland, spleen, testis, skin, heart, pancreas, brain, spinal cord, appendix, prostate, exocrine glands, thymus, small intestine and the large intestine (Balis 1985; Houghton, Albino et al. 1988; Darmoul, Voisin et al. 1994; Morimoto and Schlossman 1998; Mentlein 1999). Furthermore, CD26 expression is not restricted to the cell surface of these cell types; a soluble, truncated version lacking the transmembrane domain can also be found in plasma, serum, saliva, tears, cerebrospinal fluid, semen and urine (Schrader and Stacy 1979). This soluble CD26 (sCD26) is thought to originate predominantly from the proteolytic cleavage of the cell-surface CD26 present on lymphocytes, endothelial cells and liver epithelial cells (Cordero, Salgado et al. 2009).

1.3.3 CD26 levels in cancer

CD26 has been of particular interest in cancer, since levels of this protein are frequently dysregulated. CD26 downregulation has been reported in numerous malignancies including prostate (Bogenrieder, Finstad et al. 1997), ovarian (Kajiyama,

Shibata et al. 2006), liver (Stecca, Nardo et al. 1997), neuroblastoma (Arscott, LaBauve et al. 2009), non-small cell lung carcinoma (NSCLC) (Wesley, Tiwari et al. 2004), melanoma (Morrison, Vijayasaradhi et al. 1993) and CRC (Sakamoto, Watanabe et al. 1993). The role of CD26 in cancer is perhaps not as straightforward as we might hope, since the converse of upregulated CD26 expression has been associated with proliferation, disease progression and resistance to therapy in thyroid carcinoma (Kotani, Aratake et al. 1991), esophageal carcinoma (Goscinski, Suo et al. 2008), mesothelioma (Amatya, Takeshima et al. 2011), B-chronic lymphoblastic leukemia (Bauvois, De Meester et al. 1999) and Non-Hodgkin's lymphoma (Carbone, Gloghini et al. 1995).

Studies of CD26 expression in CRC suggest variable levels depending on the situation. Two groups have quantified CD26 changes during the malignant development of CRC. While Trotta and Balis observed CD26 to be increased in human CRC, a contrasting decrease in rat tumours was found (Trotta and Balis 1978). Ten Kate et al subsequently reported CRC human tumours to express variable levels of CD26 (Ten Kate, Wijnen et al. 1984). A later study performed by Ten Kate et al., confirmed the expression pattern of CD26 in adenocarcinomas of the colon to be variable, and also found the cellular staining pattern to differ between CRC as compared to the normal colonic mucosa. Specifically, the staining pattern of CD26 in the normal intestinal mucosa was primarily localized at the epithelial brush border, although also present in cytoplasmic granules and at the basal portion of some cells. In carcinoma tissues however, three different staining patterns were noted: membranous, diffuse cytoplasmic and granular cytoplasmic (Ten Kate, Wijnen et al. 1985). Ten Kate et al. concluded that

the variable cellular distribution of CD26 could indicate tumour heterogeneity, which would also explain why variable levels of CD26 have been reported for CRC (Ten Kate, Wijnen et al. 1984).

The inconsistency of reports on the distribution and levels of CD26 at different stages in the malignant transformation of CRC could also be explained by several other factors: (i) the use of different methods of quantification (western blot analysis, radioimmunoassay, enzymatic function of DPPIV); (ii) different sensitivities of detection depending on the location of CD26 (nuclear, cytoplasmic, cell-surface and soluble); and (iii) the particular anatomical location of the tumour within the colon (levels of CD26 expression, as reflected by DPPIV enzyme activity, were found to be two-fold higher in tumours found in the right side (ascending) as opposed to the left side (descending) of the colon (Fric, Sovova et al. 2000)).

The general view that CD26 reflects a more differentiated state of the epithelium has been further challenged by a recent study conducted by Pang and colleagues, which suggests that the combination of CD26 along with CD44 and CD133 could identify a colon cancer population with stem cell properties. CD44 and CD133 have been previously identified as stem cell markers and are therefore associated with poorly differentiated cells (Haraguchi, Ohkuma et al. 2008). Pang and colleagues suggest that the additional presence of CD26 in colorectal cells is a correlate of subsequent distant metastasis and poor differentiation. CD26-expressing cells from human colorectal tumour biopsies were found to be more tumorigenic as compared to CD26-negative cells, as

measured by tumour formation in SCID mice. Lastly, the presence of CD26 was associated with invasion, metastasis and chemoresistance to 5-FU and OX (Pang, Law et al. 2010).

This study contradicts the broader literature on the role and implications of CD26 in metastasis and chemoresistance. Furthermore, although cells within CRC metastases in the liver show substantial levels of CD26, this may be the result of local expression as the epithelial cells of the liver are known to express this cell-surface protein at levels significantly greater than that of the colon (Balis 1985). With respect to chemoresistance, the study of Pang et al measured cellular viability and apoptosis as well as tumour volume in SCID mice bearing $CD133^{+}CD26^{+}$ or $CD133^{+}CD26^{-}CD44^{+}$ tumours. Treatment with chemotherapeutic agents caused a substantial decline in cellular viability, enhanced apoptosis and reduced tumour burden, while enhancing CD26 expression (Pang, Law et al. 2010). Pang et al therefore suggest that these tumours are 'chemoresistant' since the tumour is being enriched by a $CD26^{+}$ population, although this does not directly correlate CD26 with chemoresistance.

1.3.4 CD26 expression reverses the malignant phenotype

The first study to report that malignant tissue was associated with altered CD26 expression was performed in 1978, on human and rat tissues (Trotta and Balis 1978). The importance of CD26 in cancer was first firmly established in melanoma studies conducted during the late 1980's. Houghton and colleagues examined 51 cultures of

melanocytes and 102 cultures of melanoma cell lines, which were derived from either primary or metastatic lesions (Houghton, Albino et al. 1988). They reported elevated CD26 levels in melanocytes, while CD26 was absent in melanoma cells (regardless of disease and proliferative stage). The loss of CD26 was then mechanistically implicated in the malignant process of melanoma, since re-introducing CD26 (to levels comparable to melanocytes), drastically reversed the malignant phenotype (Wesley, Albino et al. 1999).

Wesley and colleagues went on to demonstrate the importance of CD26 in NSCLC and prostate carcinoma. In the NSCLC cell line SK-LUC-8, CD26 protein and mRNA levels were found to be markedly reduced or absent, compared to normal human bronchial lung epithelial cells (Wesley, Tiwari et al. 2004). As in the earlier melanoma study, the re-expression of CD26 in SK-LUC-8 led to a drastic change in cellular morphology, induced cellular apoptosis, reduced migration and inhibited cellular proliferation. Wesley and colleagues also determined that CD26 re-expression suppressed tumorigenicity in nude mice (Wesley, Tiwari et al. 2004). Similar findings were reported in cancer cells of the prostate and ovarian that featured restored CD26 levels (Wesley, McGroarty et al. 2005; Kajiyama, Shibata et al. 2006).

Changes in CD26 expression have been identified in a number of malignancies. Considering the basal expression of the tissue, the microenvironment of a tumour and the multi-functional nature of CD26, it is not surprising that the function of CD26 may vary according to tumour stage and type. Overall, these studies suggest that for many epithelial cell types (although not universally), CD26 expression is lost late during

malignant progression. Once re-introduced into these various cancerous cells, CD26 restores cellular morphology, markers of differentiation such as E-cadherin make their appearance, and the invasive and metastatic phenotype becomes suppressed. It is therefore not surprising that this molecule is often referred to as a tumor suppressor gene (Wesley, McGroarty et al. 2005; McGuinness and Wesley 2008).

1.3.5 Regulation of chemokine pathways

For metastasis to occur, cancer cells first intravasate into and extravasate from the vasculature and/or the lymphatics, to reach a secondary site(s). Although this to some extent depends upon physical events it is also a directed process, which is why cancers have a characteristic profile of common metastatic sites (Balkwill 2004; Dittmar, Heyder et al. 2008). Chemotaxis, the phenomenon by which the movement of cells is directed in response to an extracellular gradient of a specific stimulus, is thought to be implicated in intravasation, extravasation and colonization (Roussos, Condeelis et al. 2011).

An important molecule involved in chemotaxis in CRC and other cancers is the CXCR4 chemokine receptor. CXCR4 was initially described for its involvement in stem cell homing, but is now recognized for its additional involvement in tumour survival, angiogenesis and metastasis (Raman, Baugher et al. 2007). Overexpression of this chemokine receptor is considered advantageous for the metastatic journey of these cells, as CXCR4 expressing cancer cells ultimately settle in regions that are abundant in its chemokine ligand CXCL12 (Muller, Homey et al. 2001; Liu, Ji et al. 2010).

The CXCR4/CXCL12 axis has been implicated in the metastasis of colon cancer as well as pancreatic and other carcinomas (Koshiba, Hosotani et al. 2000; Scotton, Milliken et al. 2001; Matsusue, Kubo et al. 2009; Liu, Ji et al. 2010). Activation of the CXCR4 G-protein-coupled receptor by CXCL12 initiates the activation of signalling molecules implicated in chemotaxis (phospholipase C), growth (MAPK), survival (P13K/Akt) and gene transcription (JAK/STAT) (Lataillade, Domenech et al. 2004; Teicher and Fricker 2010).

Depending on the cancer, elevated CXCR4 expression correlates with metastasis, tumour grade, poor prognosis and disease reoccurrence (Russell, Hicks et al. 2004; Ding, Fu et al. 2009; Matsusue, Kubo et al. 2009; Mongan, Fadul et al. 2009; Liu, Ji et al. 2010). In CRC, CXCR4 expression is associated with cellular proliferation, migration, VEGF release and enhanced ICAM-1 expression (Ottaiano, Franco et al. 2006). An alternate receptor that responds to CXCL12, with greater binding affinity than CXCR4, is the chemokine receptor CXCR7 (Burns, Summers et al. 2006; Luker, Lewin et al. 2012). Like CXCR4, CXCR7 has been associated with the malignant progression of various tumour types, including those associated with a gastrointestinal origin such as hepatocellular carcinoma (Salmaggi, Maderna et al. 2009; Zheng, Li et al. 2010).

The availability of CXCL12 to provoke the CXCR4 and CXCR7 pathways depends in large part upon CD26. The intrinsic dipeptidyl peptidase enzyme activity of CD26, DPPIV, allows the N-terminal cleavage and loss of bioactivity of CXCL12 and

other peptides involved in cell regulation, such as RANTES (CCL5) and glucagon-like peptide-1 (GLP-1) (Christopherson, Hangoc et al. 2002; Liu, Christensson et al. 2009; Matteucci and Giampietro 2009). DPPIV therefore interferes with the directional migration of CXCR4 and CXCR7-expressing cancer cells, to locations that are abundant in the CXCL12 chemokine.

Although there are conflicting data, cancer cells frequently exhibit reduced levels of CD26 expression (Moehrle, Schlagenhauff et al. 1995; Kikkawa, Kajiyama et al. 2005), which removes a restraint on the ability of CXCL12 to facilitate tumour progression. This escape process would be blocked if levels of DPPIV activity could somehow be upregulated. The possibility that some intervention would increase CD26 (and therefore favour inactivation of CXCL12 and CXCR4 function) is appealing. Our work points to the possibility that apigenin may provide such an opportunity.

1.4 Rationale

Dietary flavonoids are used as complementary and alternative medicine in conjunction with current chemotherapeutic agents, almost entirely on the initiative of the patients themselves as possible preventative measures, but they may also have value in assisting to improve the drug response and limit toxicities of modern therapy. In this research we chose to examine the effects of flavonoids, with special emphasis on apigenin, on pre-existing cancer cells either alone or in combination with chemotherapeutic drugs. CD26 is a potential suppressor of the CXCL12-CXCR4 axis implicated in the metastasis of various cancers, including CRC. CD26 expression by

cancer cells is frequently lost late during the malignant transformation. Rather than investigating cytotoxicity due to apigenin, we have instead focused on its action on the multifunctional protein CD26 (because of its role in tumour development and spread), with the following hypothesis and objectives:

1.5 Hypothesis

The flavonoid apigenin, alone or in combination with chemotherapeutic agents, will upregulate the level and biological activities of the multifunctional protein CD26 on human colorectal carcinoma cells, which would be predicted to oppose the process of cancer progression.

1.6 Objectives

1. To determine if dietary flavonoids such as apigenin alter the levels and functions of CD26 on human colorectal carcinoma cells (Chapter 2).

2. To identify if the flavonoid apigenin retains its anti-tumour properties in conditions found within the tumour environment and examine if apigenin can be successfully combined with chemotherapeutic agents employed in human colorectal carcinoma therapy, to modulate CD26 (Chapter 2).

3. To examine the molecular mechanisms by which apigenin enhances CD26 and increases the potency of irinotecan to do so: implications for the involvement of MAPK, CK2 and topoisomerase I (Chapter 3).

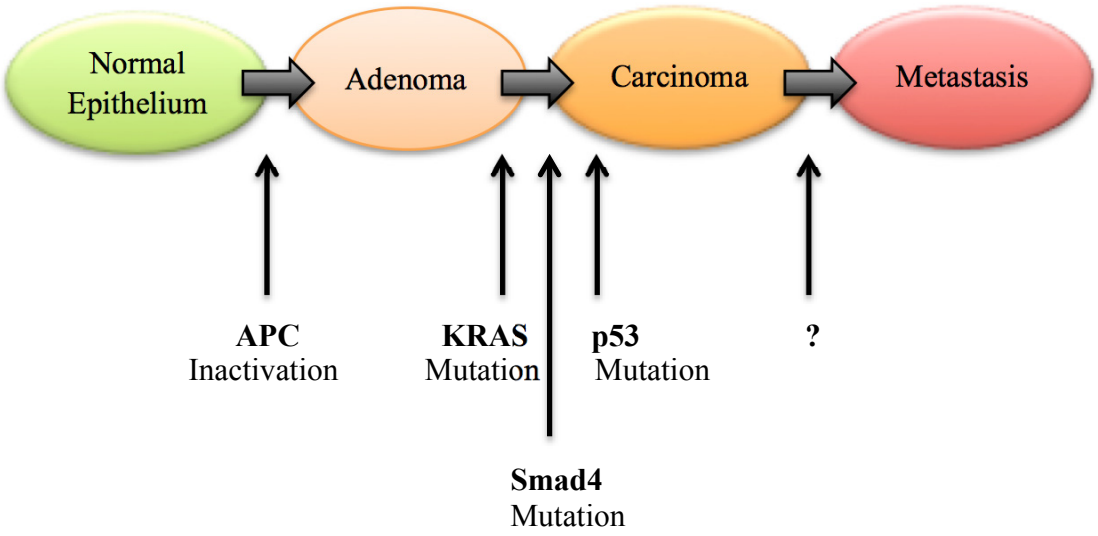
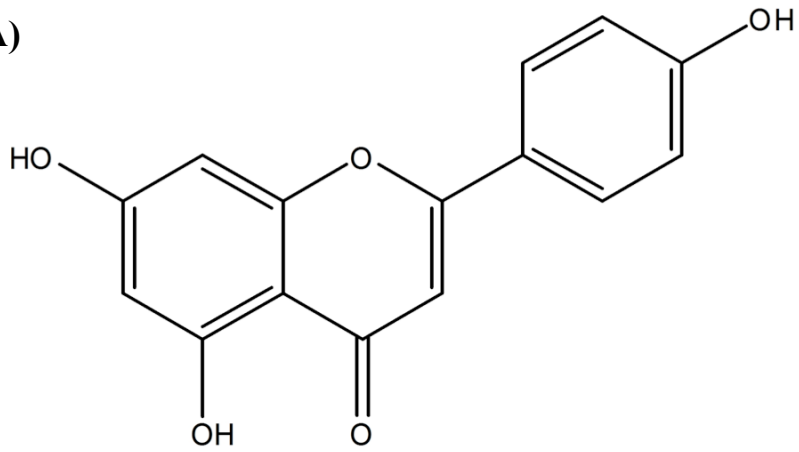


Figure 1.1 Colorectal cancer progresses in a multi-step process, as proposed by Fearon and Vogelstein.

Adapted from (Fearon and Vogelstein 1990).

(A)



(B)

Molecular structure	$C_{15}H_{10}O_5$
Molecular mass	270.24 g/mol
CAS Number	520-36-5
Appearance	yellow crystalline powder
Melting point	315 °C
Aqueous solubility	insoluble
Solubility	DMSO, hot ethanol

Figure 1. 2 Structure and Basic Properties of Apigenin.

(A) The structure of apigenin (5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one). **(B)** Major properties of apigenin.

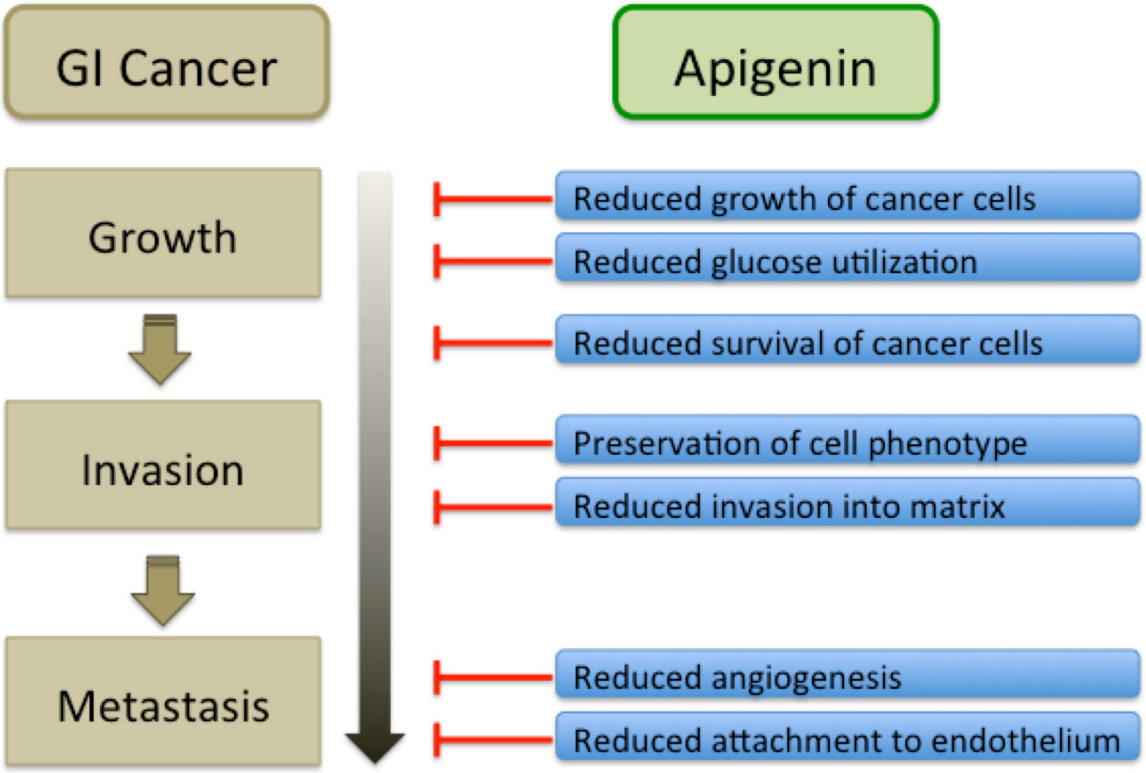


Figure 1.3 The various effects of apigenin and their potential to oppose the progression of gastrointestinal cancers.

Some of the major demonstrated actions of apigenin are shown relative to successive stages in the development of gastrointestinal (GI) cancers that might be inhibited.

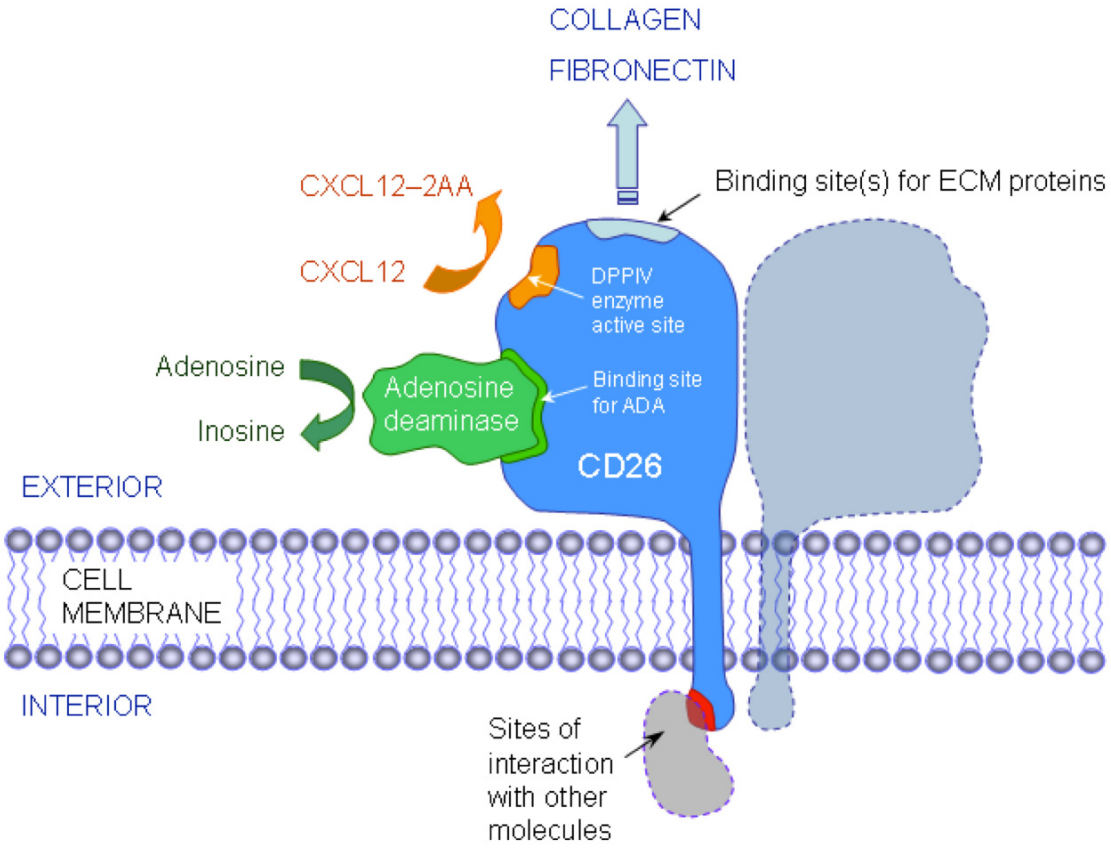


Figure 1.4 CD26 is a multifunctional protein.

The dipeptidyl peptidase (DPPIV) enzyme activity, binding of adenosine deaminase and proteins of the extracellular matrix (ECM) along with interacting intracellularly with other molecules, are shown in this diagram from (Blay 2008) with permission.

CHAPTER 2

THE DIETARY FLAVONOID APIGENIN ENHANCES THE ACTIVITIES OF THE ANTI-METASTATIC PROTEIN CD26 ON HUMAN COLON CARCINOMA CELLS

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(É.C. Lefort did all of the experimental work in this publication, was principally responsible for the experimental design, and had the major role in preparation and submission of the manuscript.)

2.1 Introduction

Due to the late onset of symptoms, CRC patients are often diagnosed at a late stage of the disease (Duke's stages C or D) (Weekes, Lam et al. 2009). As a result, these patients have a median survival of 8.5 months, although if a chemotherapeutic regimen is undertaken this can be prolonged to 20 months (Simmonds 2000). Despite recent advances, the long-term prognosis of metastatic CRC remains poor, with less than 10% of patients surviving 5 years (Kohne, van Cutsem et al. 2005). Understanding the molecular mechanisms by which colon cancer cells develop and maintain this metastatic behavior would allow us to further slow disease progression and improve survival.

CD26 is a 110-kDa, cell-surface, type II membrane glycoprotein that plays an important role in tumour progression (Pro and Dang 2004). CD26 is normally expressed on epithelial cells of the human colon as well as at other epithelial sites (Balis 1985; Kotackova, Balaziova et al. 2009). A notably multifunctional molecule, CD26 (i) has an intrinsic hydrolase activity that cleaves N-terminal dipeptides from polypeptides with either L-proline or L-alanine at the penultimate position (De Meester, Korom et al. 1999; Havre, Abe et al. 2008); (ii) acts as the major cellular binding protein for eADA (Dinjens, van der Boon et al. 1986; Schrader, West et al. 1990; Dong, Tachibana et al. 1997); (iii) binds proteins of the ECM such an fibronectin (FN) (Bauvois 1988; Hanski, Huhle et al. 1988; Loster, Zeilinger et al. 1995; Cheng, Abdel-Ghany et al. 1998; Cheng, Abdel-Ghany et al. 2003); and (iv) participates in complex signalling mechanisms by associating with the serine protease FAP-α, the protein tyrosine phosphatase CD45 and the chemokine receptor CXCR4 (Thompson, Ohnuma et al. 2007).

The intrinsic enzyme activity of CD26, DPPIV, allows the cleavage of a number of chemokines and other peptides involved in cell regulation, of which the major substrates are CXCL12, glucagon-like-peptides and RANTES (Christopherson, Hangoc et al. 2002; Liu, Christensson et al. 2009; Matteucci and Giampietro 2009). This creates a key regulatory node for processes involved in immune disorders, diabetes, HIV and cancer (Ohtsuki, Tsuda et al. 2000; Rosenstock and Zinman 2007; Thompson, Ohnuma et al. 2007; Arscott, LaBauve et al. 2009).

Cancer is frequently accompanied by a decrease in CD26 expression (Moehrle, Schlagenhauff et al. 1995; Tsuji, Sugahara et al. 2004; Kikkawa, Kajiyama et al. 2005; Wesley, McGroarty et al. 2005), which reduces both the intrinsic DPPIV and the associated eADA activities. This in turn reduces the local degradation of CXCL12 and the main substrate for eADA, the purine nucleoside adenosine (6-amino-9-β-D-ribofuranosyl-9-H-purine). CXCL12, through its receptor CXCR4, acts to promote metastatic spread and reduces ultimate survival (Ottaiano, Franco et al. 2006; Ding, Fu et al. 2009). Adenosine is produced at increasing levels within the tumour microenvironment of solid tumours (Blay, White et al. 1997; Tan, Mujoomdar et al. 2004). Adenosine has been shown to promote angiogenesis, stimulate cell motility and enhance tumour cell growth (Woodhouse, Amanatullah et al. 1998; Montesinos, Desai et al. 2002; Mujoomdar, Hoskin et al. 2003; Mujoomdar, Bennett et al. 2004). Both of these soluble agents therefore facilitate metastasis. The loss of CD26 in tumours removes a restraint on the ability of CXCL12 and adenosine to promote tumour progression and spread. Indeed, if CD26 is reintroduced to cells that express low levels of this protein the

malignant phenotype can be reverted, a phenomenon that has now been demonstrated in melanoma, ovarian cancer, non-small cell lung carcinoma and prostatic carcinoma (Wesley, Albino et al. 1999; Kajiyama, Kikkawa et al. 2002; Wesley, Tiwari et al. 2004; Kikkawa, Kajiyama et al. 2005; Wesley, McGroarty et al. 2005). The level of expression of CD26 and its corresponding enzyme activities (DPPIV and eADA) therefore play a major part in determining the cancer cell phenotype and disease outcome in CRC, particularly in terms of cell motility and metastasis. Agents that enhance CD26 levels would be predicted to have anti-metastatic potential.

The consumption of fruit and vegetables is of wide interest in the context of cancer (Vainio and Weiderpass 2006; Gordaliza 2007). Most of our current knowledge of such dietary components relates to cancer risk rather than any impact upon survival. Ingestion of phytochemicals by cancer patients undergoing treatment requires careful study, since it is theoretically possible that such agents might also interfere with the action of current chemotherapeutic drugs (Block, Koch et al. 2008). Nevertheless, evaluation of possible anti-metastatic actions is an area of high interest. Apigenin is a flavonoid, belonging to the class of secondary plant metabolites that have a common phenylchromanone structure (C6-C3-C6) with one or more hydroxyl constituents, and is ubiquitously present in many fruits, vegetables, herbs and spices (Birt, Hendrich et al. 2001; Yang, Landau et al. 2001). Apigenin has a range of activities that may be beneficial in the context of cancer (Patel, Shukla et al. 2007). In initial studies of flavonoids, we found apigenin to have the greatest promise of several phytochemicals for useful regulation of CD26. We therefore examined whether apigenin might act to oppose the deficits in CD26 functions, which could be one

underlying mechanism for its reported activity against tumour progression in experimental cancers. We further examined what would be the outcome if it were combined with chemotherapeutic drugs.

2.2 Materials and Methods

2.2.1 Materials

HT-29 and HRT-18 human CRC cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). Irinotecan and 5-FU were purchased from Mayne Pharma (Montreal, Quebec, Canada). Calf spleen ADA was obtained from Worthington (Lakewood, NJ). Oxaliplatin, bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), FN derived from human foreskin fibroblasts, apigenin (4′,5,7-trihydroxyflavone), apigenin-7-O-neohesperidoside (A-7-O-N, rhoifolin), kaempferol (3,4′,5,7-tetrahydroxyflavone), genistein (4′,5,7-trihydroxyisoflavone), thymidine and MTT (3-(4,5-dimethylthiazol-2yl)2,5-dophenyltetrazolium bromide) were purchased from Sigma-Aldrich (St. Louis, MO). [Methyl-3H]-thymidine (TRK-300) was obtained from GE Healthcare Life Sciences). Flavonoids were dissolved in DMSO to make stock solutions and then further diluted in medium to give a final DMSO concentration of less than 0.02 % (v/v), a concentration that does not affect CD26 levels (Tan, Mujoomdar et al. 2004). Mouse anti-human CD26 mAb (clone M-A261), mouse IgG isotype-matched control mAb (clone W3/25), rabbit anti-human ADA polyclonal Ab and mouse IgG anti-human fibronectin (clone 2B7-D4) were from BD Pharmingen (San Diego, CA). Secondary ^{125}I-labeled goat anti-mouse IgG fragment and secondary ^{125}I-labeled goat

anti-rabbit mouse IgG fragment were obtained from PerkinElmer Life Sciences (NEN, Boston, MA).

2.2.2 Cell culture

HT-29 and HRT-18 cells were cultured in 80-cm^2 flasks containing Dulbecco's modified Eagle medium (DMEM) without antibiotics, with 10% (v/v) heat-inactivated newborn calf serum (NCS). We routinely perform all of our cell culture without the addition of antibiotics to limit accidental infection, as antibiotics may mask the low level growth of certain bacteria that can affect experimental results (personal observations). Sufficiently rigorous sterile technique makes the routine use of antibiotics unnecessary. Cultures were maintained at 37ºC in a humidified atmosphere of 90% air/10% CO_2. Once a sub-confluent monolayer was reached, cells were detached with brief exposure to 0.05% (w/v) trypsin and 0.53 mM ethylenediamine tetraacetic acid (EDTA). For experimental purposes, cells were seeded at 90,000 cells/ml into 48-well plates. Once cultures reached 60% of confluent density, they were treated with compounds of interest or with control vehicle as specified in figure legends.

2.2.3 Assays for cell viability

Following 8- and 48-h exposure to selected flavonoids, three different viability assays were performed. The first consisted of an MTT assay, as fully described in (Blay and Poon 1995). Briefly, following 2-3 h incubation with 5mg/ml MTT at 37°C, cells

were solubilized in DMSO and the dissolved purple formazan dye was then quantitated by spectrophotometer according to its absorbance at the 492nm wavelength. The second viability assay consisted of the [^{3}H]-thymidine incorporation assay, as previously described (Mujoomdar, Hoskin et al. 2003). Briefly, cells were incubated with 1µCi/ml [*methyl*-^{3}H]-thymidine at an overall concentration of 1µM for a 19 h period. The precipitated macromolecules were then dissolved and the radioactivity that had become incorporated into DNA was measured using a Beckman LS 5000TA liquid scintillation counter (Beckman Coulter, Mississauga, ON). Lastly, cell number was tracked directly as a measurement of cell viability, and cells were dissociated from culture with trypsin/EDTA before being counted with a Coulter® Model ZM151183 particle counter (Beckman Coulter, Mississauga, ON).

2.2.4 Radioimmunoassay for cell-surface CD26

Following a typical 48 h treatment, cell-surface CD26 was quantified with a radioimmunoassay as previously described (Tan, Mujoomdar et al. 2004). The culture plates were placed on ice and all subsequent washes and incubations were performed at 4˚C. First, the original medium was aspirated and wells were washed once with 250µl of ice-cold phosphate-buffered saline (PBS; 137mM NaCl, 24.8mM Tris-HCL, 5mM KCL, 0.7mM Na_2HPO_4, 0.5mM $MgSO_4$ and 1mM $CaCl_2$; pH 7.2) containing 0.2% (w/v) BSA. Cells were then incubated for 1 h with 125µl PBS containing 1% (w/v) BSA and 1µg/ml anti-CD26. Following the incubation period, cells were washed twice with 500µl PBS containing 0.2% (w/v) BSA and were incubated for 1 h with 125µl PBS containing 1%

(w/v) BSA and 1μCi/ml ^{125}I-labeled goat anti-mouse IgG Ab. Cells received two final washes with 500μl PBS containing 0.2% (w/v) BSA. Finally, 500μl of 0.5M NaOH was added to each well in order to solubilize the cells, and radioactivity was assessed using a gamma counter. Radioactive counts were corrected for both non-specific binding with an isotype control and any difference in cell number.

2.2.5 Assay for dipeptidyl peptidase IV enzymatic activity

The enzymatic function of the CD26 protein was measured through a colorimetric DPPIV enzymatic assay, as previously described (Tan, Mujoomdar et al. 2004). Briefly, culture plates were placed on ice and were washed twice with ice-cold PBS. Cells were then incubated for 3 h at 37ºC with 200μl of a 2mM concentration of the substrate for DPPIV; gly-pro-ρ-nitroanilide in 100mM HEPES buffer, containing 0.12M NaCl, 5mM KCl, 1.2mM $MgSO_4$, 8mM glucose and 1% (w/v) BSA. Following the incubation period, 100μl of the product was transferred into a 96-well plate and absorbance measured at a wavelength of 405nm. Readings were corrected by subtracting background absorbance and adjusting for final cell number.

2.2.6 Radioimmunoassay for ecto-adenosine deaminase binding

In order to measure CD26's ability to bind eADA, a modified radioimmunoassay was performed with the addition of one simple step. Prior to incubation with primary anti-ADA Ab, cells were incubated with saturating levels of calf spleen ADA (10μg/ml

of ADA for 60 min at 4˚C). Then cells were washed twice with ice-cold PBS and the assay proceeded using rabbit anti-bovine ADA primary Ab at a concentration of 1µg/ml. The secondary Ab was ^{125}I-labeled goat anti-rabbit secondary Ab, and radioactive counts were corrected for non-specific background as well as cell number.

2.2.7 Radioimmunoassay for fibronectin binding

In order to measure CD26's ability to bind FN, a similar method to the measurement of eADA binding capacity was utilized. Cells were first treated with FN at a 50µg/ml concentration for 60 min at 4˚C, then assayed using 1µg/ml mouse anti-human fibronectin primary Ab and ^{125}I-labeled goat anti-mouse secondary Ab, with corrections as before.

2.2.8 Statistical analysis

Unless otherwise noted, figures show data that are representative of independent experiments done on at least three separate occasions. Statistical analyses were performed using Prism 3.0 software (GraphPad, San Diego, CA). Comparisons of data were performed using one- or two-way analysis of variance (ANOVA) followed by Dunnett's or Bonferroni's comparison tests, or t-tests, as indicated in the figure legends. For all analyses a P value < 0.05 was considered as the minimum for statistical significance.

2.3 Results

2.3.1 Apigenin and genistein increase the cell-surface levels of functional CD26 on human colorectal cancer cells

We first examined whether the plant flavonoids apigenin, kaempferol or genistein could affect the levels of CD26 on CRC cells. We focused on the human CRC cell line HT-29, which has been used for many of our mechanistic studies on CD26/DPPIV (Tan, Mujoomdar et al. 2004; Tan, Richard et al. 2006).

We initially treated HT-29 cells with a single 50μM dose of flavonoid for 48 h. Using a radioimmunoassay that selectively detects cell-surface CD26 protein (Tan, Mujoomdar et al. 2004), CD26 was consistently found to be upregulated by both apigenin and genistein but not by kaempferol (Fig. 2.1). We confirmed these results using a functional assay that measures the ability to bind eADA (Schrader and Stacy 1979). The upregulation of CD26 as its eADA binding capacity by genistein and apigenin paralleled that measured in the CD26 immunoassay (Fig. 2.1). Kaempferol was again without effect, showing that the upregulation of CD26 is not common to all flavonoids. We also tested the CD26 response to flavonoids in another CRC cell line, HRT-18. Neither kaempferol nor genistein had an effect on CD26 in HRT-18 cells (Appendix, Figure A2.1, A and B). However, apigenin again increased the levels of cell-surface CD26 (Fig. 2.1). The CD26 response to apigenin is therefore not restricted solely to HT-29 cells.

In HT-29 cells the effect of apigenin on CD26 cell-surface protein (an increase of 56.3 ± 0.03%, mean ± SEM, 10 independent experiments) and on eADA binding (an increase of 55.0 ± 0.06%, mean ± SEM, 7 independent experiments) was consistently higher than that for genistein. For that reason, because of the insensitivity of HRT-18 cells to genistein and due to the diverse pharmacology of genistein (Banerjee, Li et al. 2008) we restricted our focus to the actions of apigenin.

Figure 2.2 shows the dose-dependence effects of apigenin on CD26 protein (Fig. 2.2, A) and its three known functional properties; (i) the binding of eADA ((Schrader and Stacy 1979; Dinjens, van der Boon et al. 1986; Schrader, West et al. 1990; Dong, Tachibana et al. 1997)), as indicated above (Fig. 2.2, B), (ii) its DPPIV activity ((De Meester, Korom et al. 1999; Havre, Abe et al. 2008), Fig. 2.2, C), and (iii) its ability to bind FN ((Cheng, Abdel-Ghany et al. 1998; Cheng, Abdel-Ghany et al. 2003), Fig. 2.2, D). Apigenin consistently increased all four measures of CD26 cell-surface abundance and activity, with the effect on FN binding being very similar to those for protein abundance and eADA binding (53.0 ± 0.04%, mean ± SEM, 5 independent experiments), but elevation of DPPIV enzymatic activity being somewhat lower (43.0 ± 0.01%, mean ± SEM, 4 independent experiments).

The threshold concentration of apigenin required to produce a statistically significant increase in CD26 or its functions at 48 h after exposure was from 1-10 μM in all experiments, and the EC_{50}, although variable, was typically 3-30 μM. We compared the effect of apigenin with that of its 7-ortho-neohesperidoside derivative (A-7-O-N),

which has been shown to be essentially devoid of bioactivity in several studies of apigenin (Srivastava and Gupta 2007; Benavente-Garcia and Castillo 2008). A-7-O-N is commonly known as rhoifolin and is structurally similar to apigenin apart from a pentose sugar ring appended to the molecule. In order to mediate its full range of biological actions, the glycosidic bond present within the molecule needs to be hydrolyzed (Srivastava and Gupta 2007). We found that A-7-O-N had no effect on CD26 or its DPPIV activity (Figs 2.2 A, C) and used it as a control in further studies.

Measurement of CD26 at different time points (Fig. 2.3) showed that there was no interference of apigenin with the measurement of immunoreactive CD26 (Fig. 2.3, A, left triplet of bars), but that there was a progressive elevation of immunoreactive cell-surface CD26 that was first apparent 3h following treatment and subsequently reached a maximum at 24-48 h (Fig. 2.3, A and B).

2.3.2 The effect of apigenin is not cytotoxic but coincides with a decline in cell proliferation

All of our data in Figs 2.1-2.3 were corrected for any change in cell number, thus we were confident that the effect on CD26 was not due to a cytotoxic action of apigenin. We confirmed that apigenin was not cytotoxic by measuring cell viability. To do this we used cell counting or MTT assays following short term (8h) treatment with agents. Such acute treatment with apigenin had no effect on cell number (Fig. 2.4, A), nor was any change seen with the control compound (Fig. 2.5, A). Interestingly, when we used the

MTT dye reduction assay to measure viability, we consistently observed (4 independent experiments) a dose-dependent increase in conversion of MTT to its formazan derivative (Fig. 2.4, B). This presumably reflects an apigenin-induced increase in cellular metabolism, specifically seen as an elevation in the activity of succinate dehydrogenase (SDH), which is the enzyme that produces the formazan product in the MTT assay. This increase in the activity of mitochondrial SDH was not seen with the control compound (Fig. 2.5, B).

Longer term assays measuring cell number, SDH activity or DNA synthesis are reflective of cell growth. Over the 48 h (typical) period of our assays, 100μM apigenin inhibited the growth of HT-29 cells. This was seen comparably whether counting cells or using the MTT assay (Fig 2.6, A and B). The viability of cells at the end of the culture period measured using a trypan blue dye exclusion assay was not statistically different with a 30μM dose of apigenin, and decreased less than 9 % (from 98.3 ± 0.5 to 89.6 ± 0.7 $P<0.01$) at the highest dose of 100μM after 48 h. However, measurement of the rate of DNA synthesis (Fig. 2.6, C) showed that the cells were almost completely growth-inhibited at 100μM apigenin. It is significant though, that elevation of CD26 begins at apigenin concentrations approximately 10-fold less than growth inhibition.

2.3.3 The elevation of CD26 levels by apigenin occurs under conditions present in the tumour microenvironment

The assumption for many plant flavonoids is that the activity of the agents is due in large part to their anti-oxidant and cellular protective properties (Patel, Shukla et al. 2007; Butt and Sultan 2009; Vasquez-Garzon, Arellanes-Robledo et al. 2009). This raises the question of whether or not agents such as apigenin would act in a similarly in the more hostile tumour microenvironment as they do in standard cell culture. We therefore tested whether apigenin would have the same effect on CD26 and its functions under conditions prevalent in the tumour microenvironment.

Solid tumours such as CRC are typically hypoxic due to a compromised neovasculature (Vaupel, Kallinowski et al. 1989; Brown and Giaccia 1998). We therefore assessed whether apigenin would be able to enhance CD26 levels and activities in reduced oxygen partial pressure (1.0% v/v) in culture. In five independent experiments, apigenin was able to increase cell-surface CD26 protein levels on HT-29 cells under hypoxic conditions, and in 3 of those 5 experiments hypoxia enhanced the potency (caused a leftward shift in the dose-response curve) of the action of apigenin (Fig. 2.7, A). A hypoxic environment is therefore not an impediment to this phenomenon and may in fact enhance the effect of apigenin.

In addition to hypoxia, cells within a solid tumour are subject to drastic alterations in energy metabolism, which are accompanied by reduced levels of extracellular glucose and enhanced lactate production (Gatenby and Gillies 2004; Gatenby and Gillies 2007). We examined the response to apigenin either with lactate increased to 20mM or with a reduction in glucose concentration (from 4.5g/l in our standard DMEM medium to

0.5g/l). Neither high lactate concentration nor reduced glucose levels in any way impeded the response to apigenin (Fig. 2.7, B). Apigenin was also able to elevate CD26 in the presence of high (up to 100μM) concentrations of adenosine, which is a significant by-product of hypoxic stress (Headrick and Willis 1989; Olsson and Pearson 1990; Gutierrez 1991) (Appendix Figure A2.2).

We combined these stressors, making the hypoxic conditions even more stringent (48h in 0.2% v/v oxygen) and combining with high lactate and low glucose. As is evident from the data shown in Fig. 2.7, C, apigenin is fully able to increase CD26 under these conditions, which parallel the situation in the tumour microenvironment. Apigenin is therefore not likely to lose its ability to upregulate CD26 in the context of a tumour. Interestingly, lowering the glucose concentration itself enhanced CD26 levels ($P<0.001$).

2.3.4 Apigenin enhances the effect of chemotherapeutic agents in upregulating CD26

Chemotherapeutic agents utilized for the treatment/management of CRC include irinotecan, 5-FU and OX (Javle and Hsueh 2009). We have previously reported that these chemotherapeutic agents upregulate the cell-surface expression of CD26 (Blay, Lowthers et al. 2004). We investigated whether apigenin was able to further enhance the effects of chemotherapeutic agents in upregulating CD26. Irinotecan, 5-FU and OX each significantly increased the cell-surface expression of CD26 and its DPPIV enzyme activity (Fig. 2.8, A and B). This increase was further enhanced by the addition of

apigenin (30μM, Fig. 2.8). The ability of apigenin to enhance CD26 was consistently most robust when paired with irinotecan, a topoisomerase I (topo I) inhibitor (Iyer and Ratain 1998).

While the effects of apigenin and chemotherapeutic drugs in enhancing CD26 and its functions were simply additive at optimal doses (Fig. 2.8), further examination revealed a particular interaction between the effects of apigenin and the drug irinotecan (Fig. 2.9). When we normalized and scaled data from multiple experiments to distinguish the dose-dependency of the CD26 response for 5-FU, there was no effect of apigenin on the potency of 5-FU (Fig. 2.9, A). The EC_{50} values for 5-FU action in the absence and presence of 30μM apigenin were 0.16 ± 0.04 and 0.17 ± 0.07 μg/ml respectively (mean ± SEM, 7 independent experiments, not statistically different with P=0.90). We also detected no sign of interaction if apigenin were combined with OX (Appendix Figure A2.3). However, combining apigenin with irinotecan caused a leftward shift in the dose-response curve for irinotecan (Fig. 2.9, B) and led to a 4.2-fold enhancement of its potency, with a reduction in the EC_{50} for irinotecan from 4.68 ± 1.17 μg/ml to 1.11 ± 1.26 μg/ml in the presence of 30μM apigenin (mean ± SEM, 6 independent experiments, statistically different with P=0.009). We also observed an interaction if the experimental design was reversed, adding a fixed dose of irinotecan to a range of apigenin concentrations (Fig. 2.9, C). In this situation the addition of 2μg/ml of irinotecan led to almost a 30-fold enhancement of the potency of apigenin for increasing CD26, lowering the EC_{50} from 32.8 ± 1.11 μM to 1.10 ± 1.35 μM (statistically different, P=0.004).

2.4 Discussion

Since malignant progression has been characterized by a decrease in CD26 (Moehrle, Schlagenhauff et al. 1995; Tsuji, Sugahara et al. 2004; Kikkawa, Kajiyama et al. 2005; Wesley, McGroarty et al. 2005) and a reversal in this downward trend reduces disease impact (Wesley, Albino et al. 1999; Kajiyama, Kikkawa et al. 2002; Wesley, Tiwari et al. 2004; Kikkawa, Kajiyama et al. 2005; Wesley, McGroarty et al. 2005) we wished to establish if dietary flavonoids could restore the levels of this anti-metastatic protein.

Both apigenin and genistein significantly increased CD26 and each of its functions on HT-29 cells, but kaempferol did not. Kaempferol differs in having a hydroxyl group on the second ring (Benavente-Garcia and Castillo 2008), which may be a structural correlate of the activity relevant to this response. It is clear that CD26 upregulation is not a feature of all flavonoids. As well, the response to genistein is not likely to be evident for all cancers, as we saw no upregulation of CD26 on HRT-18 cells. However, apigenin had substantial effects on CD26 in a sensitive cancer cell population (HT-29 cells) and the elevation of CD26 was reproducible in another cell line (HRT-18 cells). The slow time course of the upregulation of CD26 by apigenin (maximum at 24-48 h) suggests that there is a series of cellular events that must be completed after exposure to apigenin, since the flavonoid itself has a half-life of only several hours (Meyer, Bolarinwa et al. 2006).

We also used apigenin-7-O-neohesperidoside (A-7-O-N) in this study, since apigenin is usually found in nature in a glycosylated form (Avallone, Zanoli et al. 2000). However, A-7-O-N failed to demonstrate an upregulation on CD26 and its functions, and was considerably less active on cellular metabolism, cell proliferation or cellular viability, consistent with other work carried out using prostate cancer cells (Srivastava and Gupta 2007). We found A-7-O-N to be a useful control substance for apigenin in this study.

The glycosylation of apigenin would not be a hindrance if such a form were used therapeutically in human patients by the oral route, or in response to dietary sources. Flavonoid glycosides are typically cleaved to release the active aglycone through the activity of β-glucosidases. Conversion to apigenin can be observed after incubating apigenin glycosides with small intestinal tissue (Srivastava and Gupta 2007) or cell-free extracts (Day, DuPont et al. 1998), or with purified β-glucosidase enzyme (Lambert, Kroon et al. 1999). In humans the metabolism of dietary flavonoid glycosides occurs primarily in the small intestine, where deglycosylation occurs via β-glucosidase activities both at the epithelial cell apical membrane and through cytosolic β-glucosidases after epithelial uptake (Nemeth, Plumb et al. 2003). Bacterial glucosidases may play a part further along the intestine where there is more abundant microflora (Liu and Hu 2002). Most apigenin would therefore be released and taken up in the proximal intestine. However, enterohepatic recycling typically leads to the resecretion of glucuronidated forms, which should be reconverted to their aglycones by the intestinal microflora in the

lumen of the large intestine (Liu and Hu 2002). Oral apigenin glycosides will therefore be active at the level of the colon.

Consistent with its effects on CD26 protein, apigenin upregulated DPPIV in a dose-dependent fashion. This would increase cleavage of CXCL12, which is the chemokine most susceptible to DPPIV action (Shioda, Kato et al. 1998; Lambeir, Proost et al. 2001; Sun, Pedersen et al. 2008). The subsequent CXCL12 inactivation will reduce activation of its receptor CXCR4 on cancer cells, and lower the stimulation of downstream effector pathways that are involved in cell survival, proliferation and directional migration (Raman, Baugher et al. 2007; Ding, Fu et al. 2009; Teicher and Fricker 2010). The CXCR4 chemokine receptor is highly expressed in metastatic CRC (Matsusue, Kubo et al. 2009) and has been linked to metastatic spread and lowered ultimate survival (Ottaiano, Franco et al. 2006). The upregulation of DPPIV activity by apigenin will therefore tend to reduce the impetus for metastasis, and this may be one of the mechanisms that contributes to an ability of apigenin to reduce tumour progression and spread.

Similarly, CD26 cell-surface upregulation led to an increase in binding of eADA and therefore the ability to deaminate adenosine at the cell surface. CD26 is the major binding protein for eADA, inactivating the tumorigenic compound adenosine into inosine (Gorrell, Gysbers et al. 2001). Increased adenosine hydrolysis, occurring as a result of the apigenin-driven increase in eADA, will therefore be a further event that opposes tumour progression. Interestingly we have shown that adenosine itself exerts a self-favouring

down-regulation of CD26, which leads to a 'cycle of self-exacerbation' that accentuates the damaging effects of adenosine (Meininger, Schelling et al. 1988; Mujoomdar, Hoskin et al. 2003; Tan, Mujoomdar et al. 2004). Apigenin therefore acts to interrupt this negative cycle and reduce effects of adenosine that will favour tumour spread.

Finally, we have confirmed the prediction that apigenin will enhance the ability of CRC cells to bind fibronectin through CD26. While the interactions of cells with FN and other ECM components are complex, we speculate that increased anchorage to FN through CD26 (independently of integrin-dependent adhesion) will tend to restrain cells in association with local basement membrane and reduce the likelihood of migration to other locations. This suggestion is consistent with other observations where apigenin-treated cells were found to have greater adherence to the extracellular matrix and exhibit reduced motility (Lindenmeyer, Li et al. 2001; Franzen, Amargo et al. 2009). This may further limit tumour dissemination.

The elevation of CD26 and its associated functions by apigenin should proceed under the harsh conditions of the tumour microenvironment. We found no diminution of the effect of apigenin with hypoxia, elevated lactate concentration, or reduced glucose levels. Indeed, we found evidence that the apigenin effect might be accentuated under the hypoxic conditions characteristic of the tumour. The enhancement of potency of apigenin by hypoxia was substantial (~20-fold in the experiment shown in Fig. 2.7, A). Under normoxic conditions, the elevation in CD26 due to apigenin is still increasing at a concentration of 100μM (Figs 2.2, A and 2.7, A), the highest dose tested due to concerns

over toxicity. However, a maximum effect was seen at only 10μM apigenin when the oxygen tension was reduced (Fig. 2.7, A). The decline in the CD26 response to apigenin at higher concentrations in (Fig. 2.7, A) we interpret as the expected upper end of the dose-response curve when the cells are sensitized to apigenin in this way.

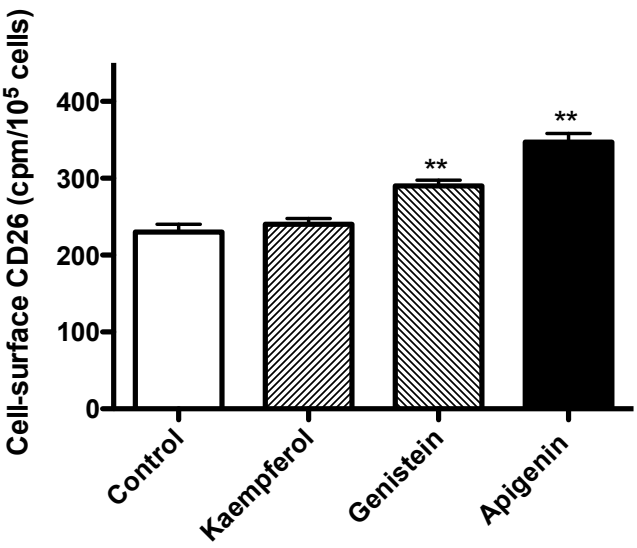
As a result of these multiple actions of apigenin through its upregulation of CD26, we predict that the phenotype of the cell will be less migratory, less proliferative and more static. We have shown that the apigenin sensitivity of CD26 upregulation is maintained under conditions that prevail within the harsh tumour environment: hypoxia, high levels of lactate (and therefore lowered pH) and a reduction in concentrations of glucose. We have also demonstrated that the effects of apigenin continue in the presence of each of the three common chemotherapeutic drugs used for this cancer: 5-FU, OX and irinotecan; and that the activity of each of these drugs (as assessed by their own effects on CD26) are not compromised by the presence of apigenin.

It is exciting that the effects of chemotherapeutic agents and apigenin can be combined to give a much greater effect on CD26 – the increase over control levels approached 4-fold in some experiments. This was found to apply to all functional activities of CD26/DPPIV. In the case of irinotecan (but not 5-FU or OX) there was a specific interaction with the action of apigenin. It may be that this overlapping activity reflects some cross-talk in the mechanism of action of apigenin with this chemotherapeutic agent. It has been shown that flavones (which include apigenin) are able to stabilize and inhibit the topo I-DNA complex (Boege, Straub et al. 1996), which

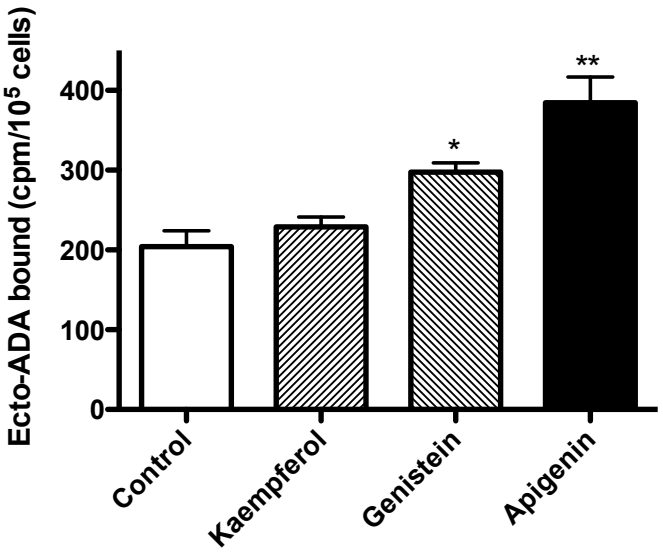
overlaps with the primary mechanism of irinotecan (Iyer and Ratain 1998). At least in the context of CD26 upregulation, part of apigenin's action may involve topoisomerase(s).

In summary, we have shown that apigenin enhances cell-surface levels of CD26 and its three functions that are normally suppressed in cancer and whose absence contributes to the aggressive phenotype. Apigenin increases cellular DPPIV activity and therefore the degradation of CXCL12 which is known to promote metastasis. It enhances the binding of eADA and therefore interrupts the pro-tumour 'self-exacerbating' cycle of adenosine. Finally, apigenin increases association with FN in the ECM. These cellular actions may point to an anti-metastatic potential for apigenin. Furthermore, we suggest that it may be useful as an adjunct intervention alongside conventional chemotherapy to further restrain the cancer cell phenotype in late-stage CRC.

(A)


Cell-surface CD26 (cpm/10^5 cells)
400
300
200
100
0
**
**
Control
Kaempferol
Genistein
Apigenin

(B)


Ecto-ADA bound (cpm/10^5 cells)
400
300
200
100
0
*
**
Control
Kaempferol
Genistein
Apigenin

(C)

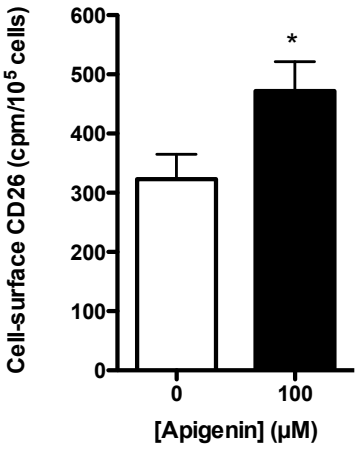
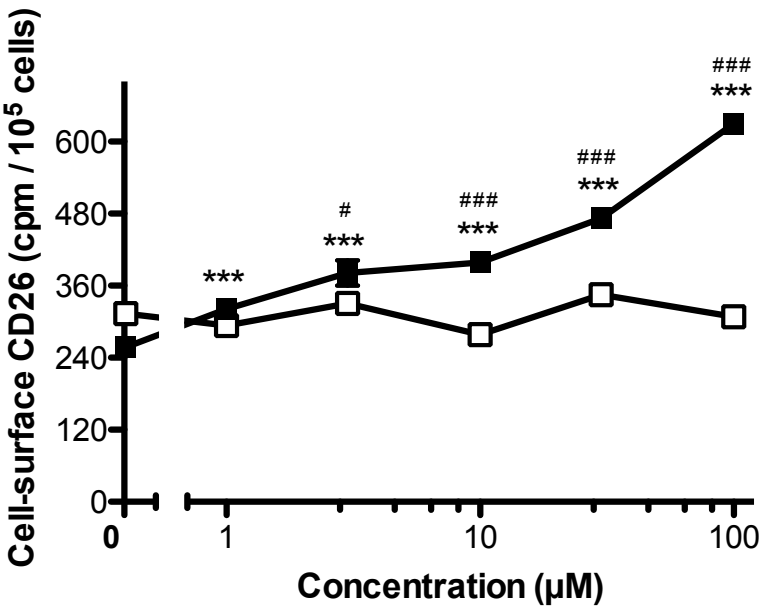

Cell-surface CD26 (cpm/10^5 cells)
600
500
400
300
200
100
0
*
0
100
[Apigenin] (μM)

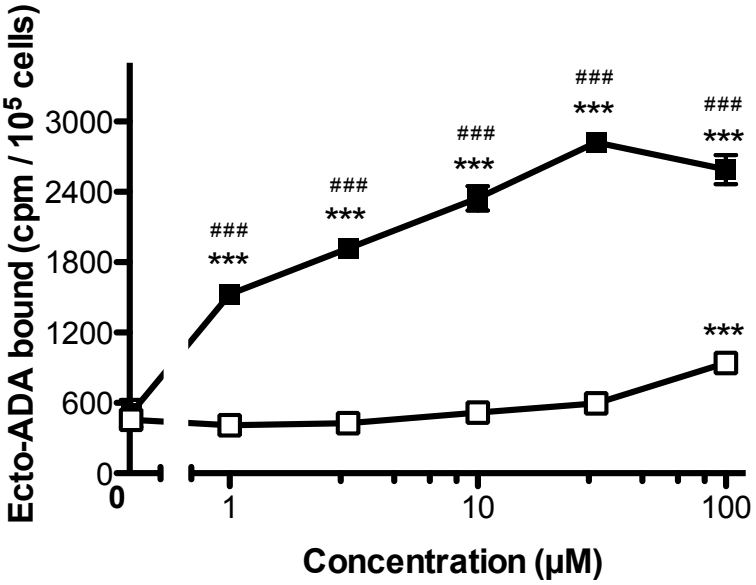
Figure 2.1 Apigenin and genistein upregulate CD26 and ecto-adenosine deaminase (eADA).

(A,B) HT-29 cells were treated with vehicle control or flavonoids (50μM); and **(A)** CD26 cell-surface protein or **(B)** eADA binding capacity were measured following 48 h of treatment. The data are means + SE (n=4), statistical analyses were by 1-way ANOVA with Dunnett's comparison test. Where * $P<0.05$ and ** $P<0.01$ show significant upregulation by the respective flavonoids. **(C)** HRT-18 cells were treated with vehicle control or 100μM apigenin, and CD26 protein measured after 48 h of treatment. The data are means + SE (n=7 independent experiments), statistical analyses were by t-test, where * $P<0.05$ for significant upregulation by apigenin.

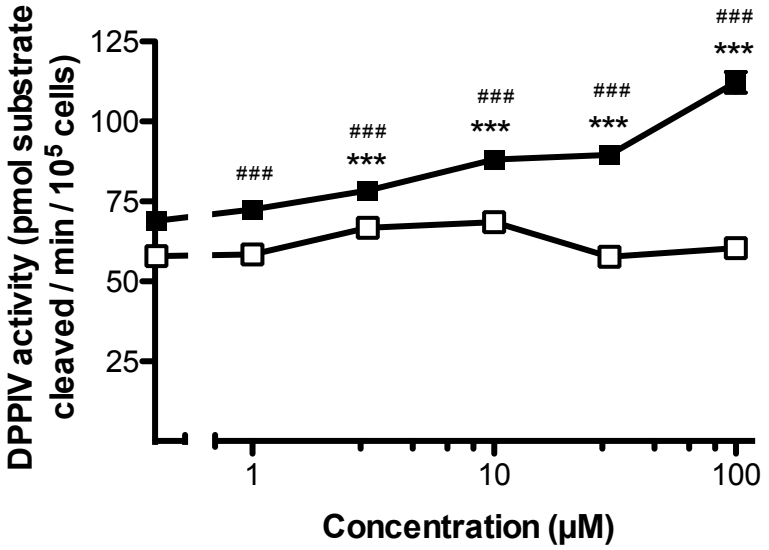
(A)


Cell-surface CD26 (cpm / 10^5 cells)
600
480
360
240
120
0
0
1
10
100
Concentration (μM)
***
#
***
###
***
###
***
###
***

(B)


Ecto-ADA bound (cpm / 10^5 cells)
3000
2400
1800
1200
600
0
0
1
10
100
Concentration (μM)
###
***
###
***
###
***
###
***
***

(C)


DPPIV activity (pmol substrate cleaved / min / 10^5 cells)
125
100
75
50
25
1
10
100
Concentration (μM)
###
***

(D)

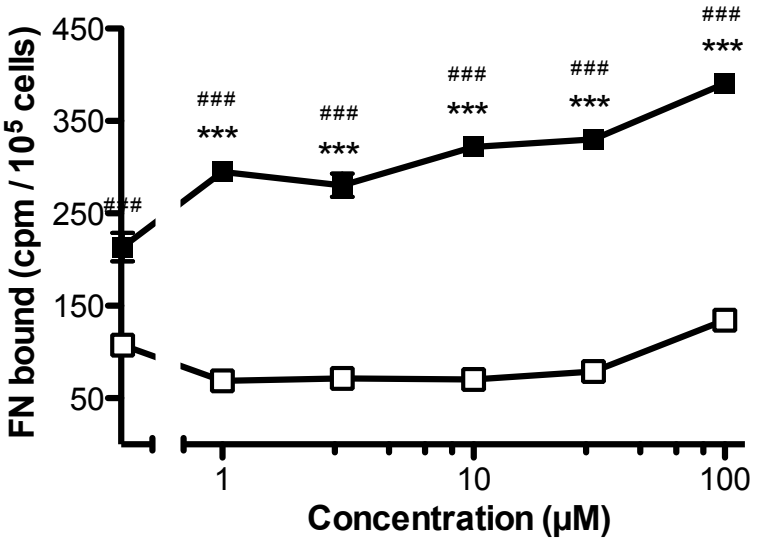
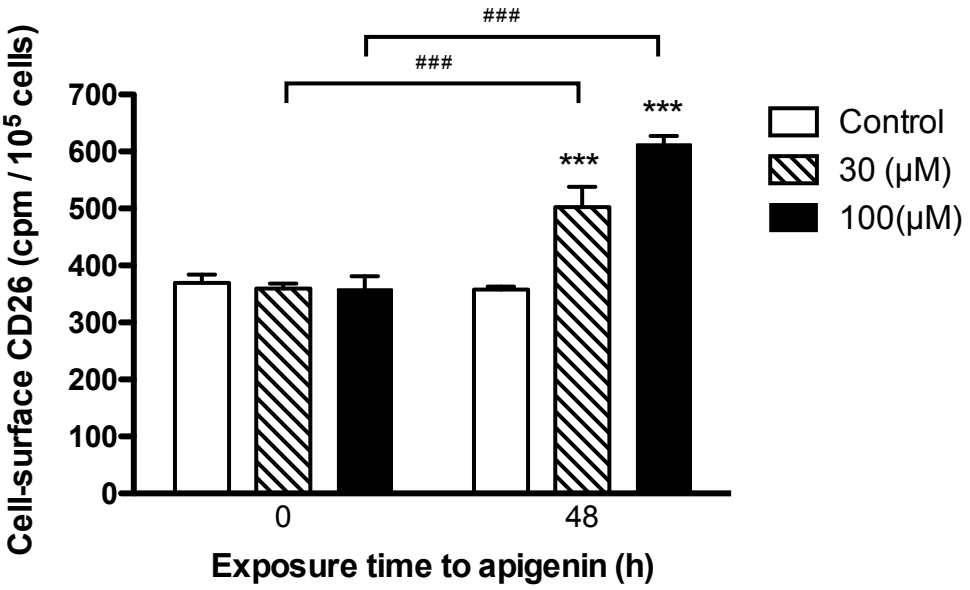

FN bound (cpm / 10^5 cells)
450
350
250
150
50
1
10
100
Concentration (μM)
###
***

Figure 2.2 Apigenin upregulates the multiple functions of CD26 in a dose-dependent manner.

HT-29 cells were treated with apigenin (closed symbols) or 7-ortho-neohesperidoside (A-7-O-N) control (open symbols) at the indicated concentrations. Forty-eight h following these additions, **(A)** CD26, **(B)** eADA binding capacity, **(C)** dipeptidyl peptidase IV (DPPIV) enzyme activity and **(D)** fibronectin (FN) binding capacity were measured. The data are means + SE (n=4), statistical analyses were by 2-way ANOVA with Bonferroni's comparison test. Where * $P<0.05$ and ** $P<0.01$ show significant upregulation by apigenin as compared to the control treated cells.

(A)


###
###
***
***
Control
30 (μM)
100(μM)
Cell-surface CD26 (cpm / 10^5 cells)
700
600
500
400
300
200
100
0
0
48
Exposure time to apigenin (h)

(B)

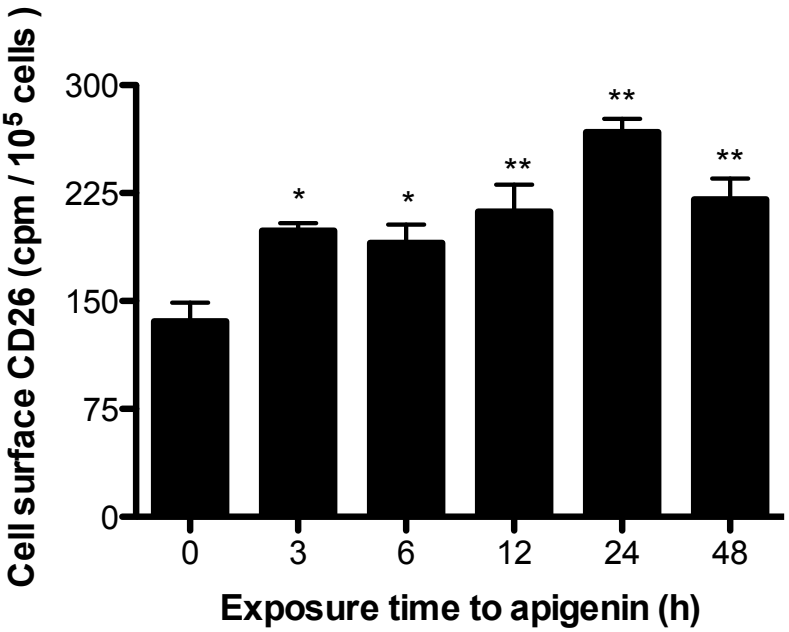

Cell surface CD26 (cpm / 10^5 cells)
300
225
150
75
0
*
*
**
**
**
0
3
6
12
24
48
Exposure time to apigenin (h)

Figure 2.3 Apigenin upregulates CD26 cell-surface expression in a time-dependent manner.

(A) HT-29 cells were treated with apigenin (30 or 100μM) or vehicle control, and cell-surface CD26 was measured at 0 and 48 h. **(B)** Cells were treated with apigenin (30μM) for the indicated time points and cell-surface CD26 was measured at 48 h. The data are means + SE (n=4), statistical analyses were by 2-way ANOVA with Bonferroni's comparison test. Where * $P<0.05$ and ** $P<0.01$ show significant upregulation by apigenin.

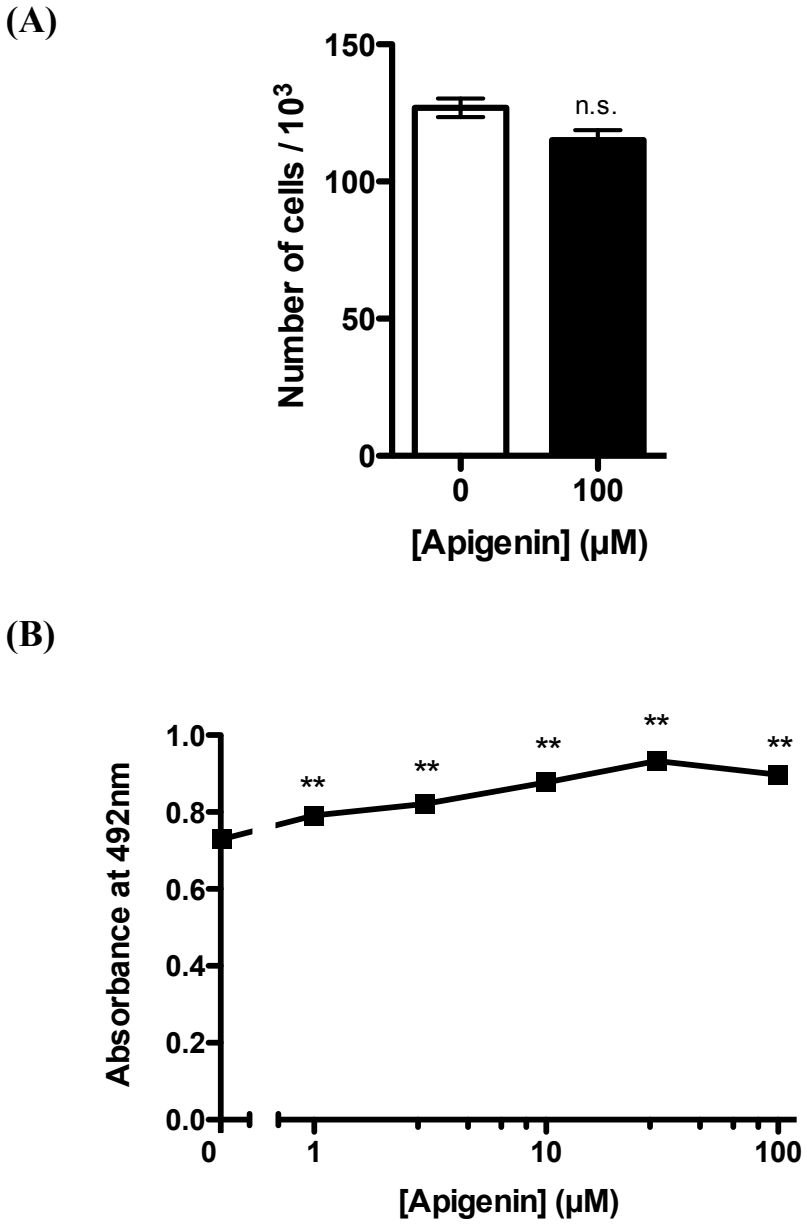
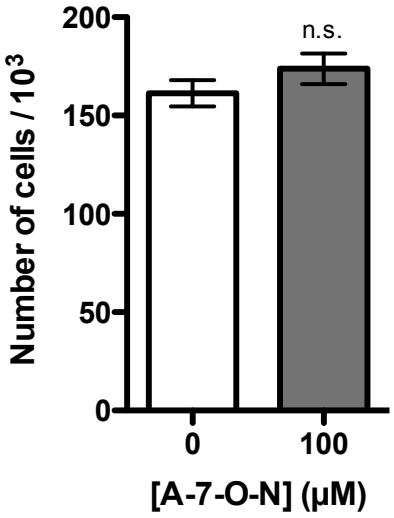


Figure 2.4 Acute apigenin exposure is not cytotoxic to HT-29 cells.

Cells were treated with apigenin, where cell count **(A)** and MTT assay **(B)** were performed after 8h. The data are means ± SE (n=4), statistical analyses were by 1-way ANOVA with Dunnett's comparison test. Where **P<0.01 shows a significant decrease by apigenin. The response to the treatment in panel **(A)** is not significantly different (n.s.) from the control (P>0.05, Student's t-test).

(A)



(B)

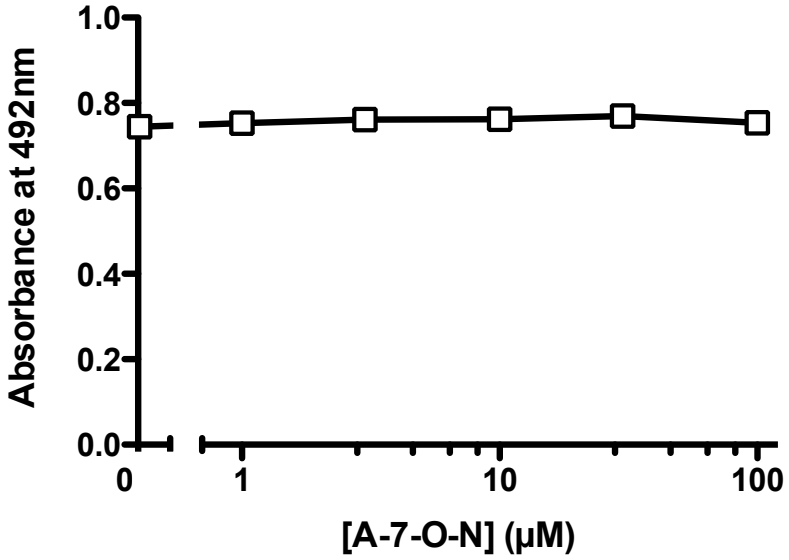
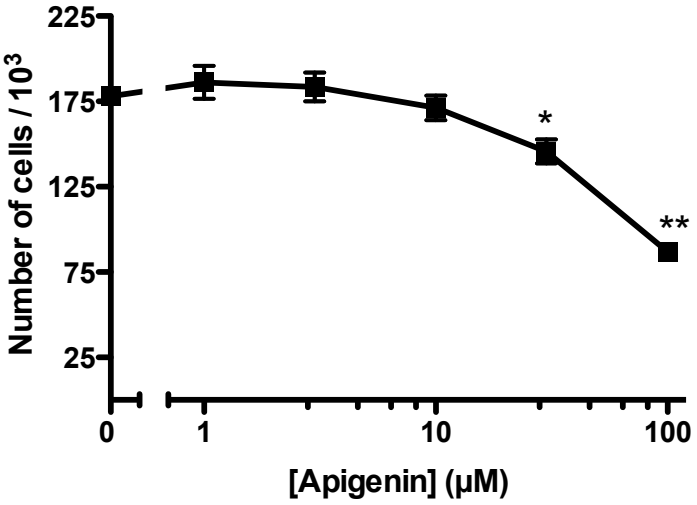


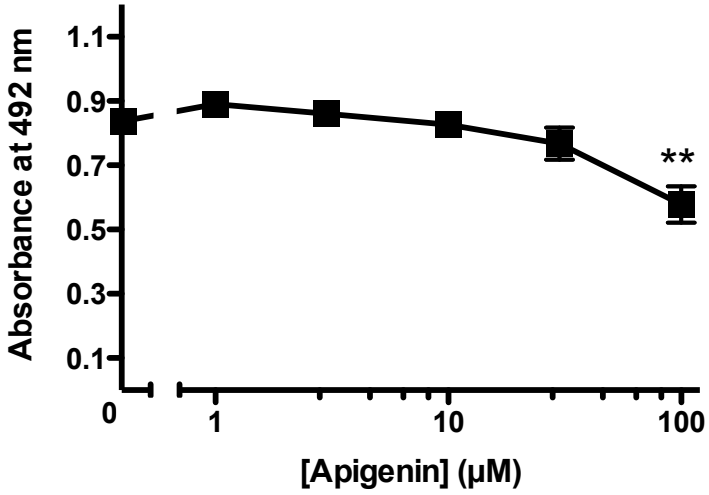
Figure 2.5 Acute A-7-O-N exposure is not cytotoxic to HT-29 cells.

Cells were treated with A-7-O-N, where cell count **(A)** and MTT assay **(B)** were performed after 8 h. The data are means ± SE (n=4), statistical analyses were by 1-way ANOVA with Dunnett's comparison test. Where *P<0.05 and **P<0.01 shows a significant decrease by apigenin.

(A)


225
175
125
75
25
Number of cells / 10^3
*
**
0
1
10
100
[Apigenin] (μM)

(B)


1.1
0.9
0.7
0.5
0.3
0.1
0
Absorbance at 492 nm
**
1
10
100
[Apigenin] (μM)

(C)

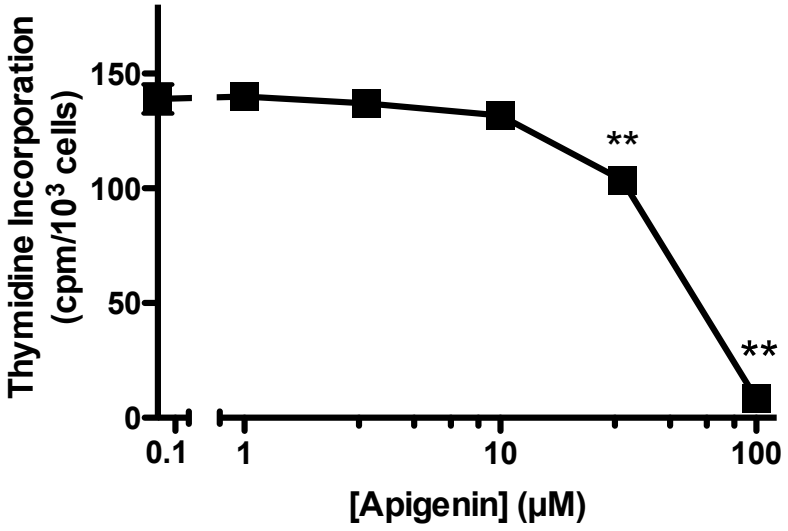
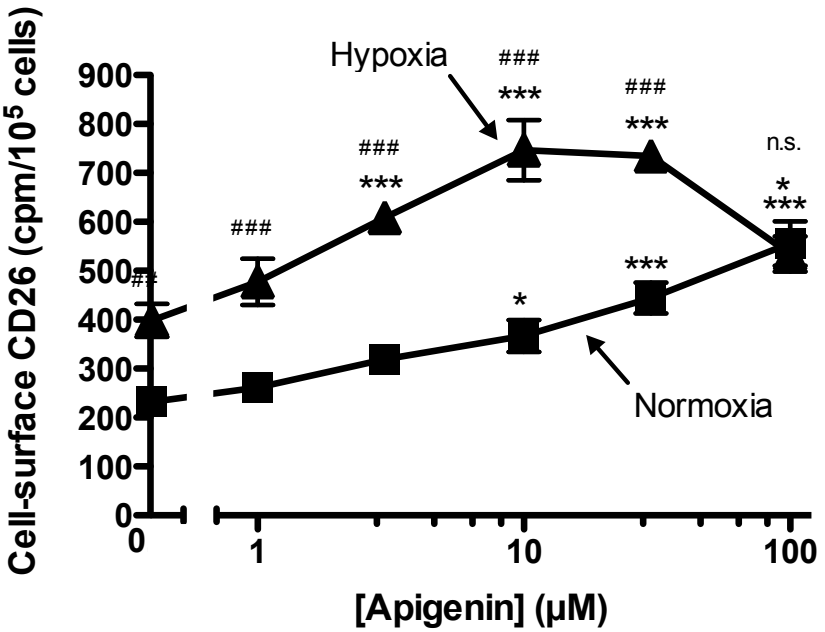


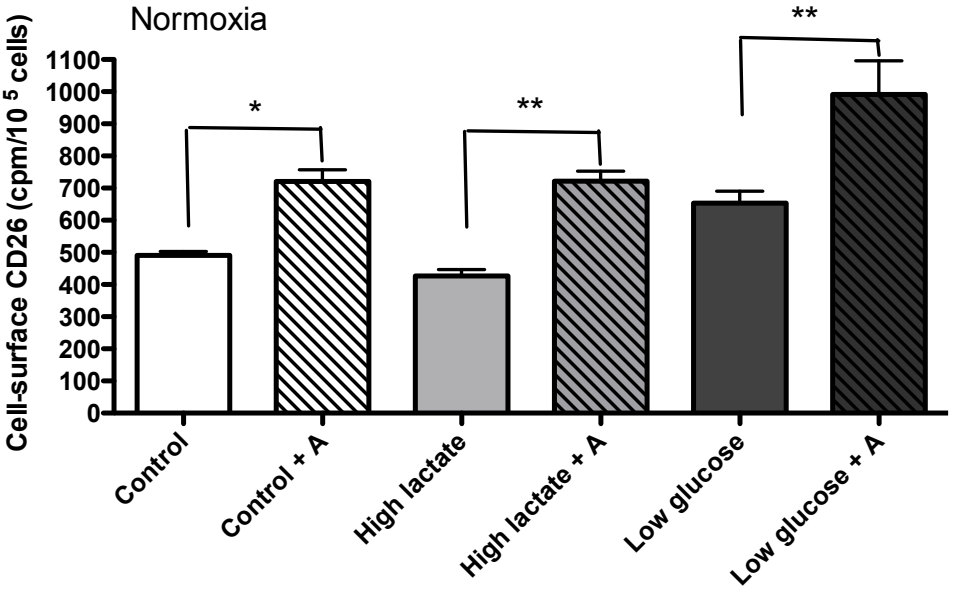
Figure 2.6 Chronic apigenin exposure suppresses cell growth and proliferation.

HT-29 cells were treated with apigenin for a 48 h period. Cellular proliferation was assessed through **(A)** measuring cell number, **(B)** MTT assay and **(C)** thymidine incorporation assay. The data are means ± SE (n=4), statistical analyses were by 1-way ANOVA with Dunnett's comparison test, where * $P<0.05$ and ** $P<0.01$ show a significant decrease in response to apigenin.

(A)


Cell-surface CD26 (cpm/10^5 cells)
900
800
700
600
500
400
300
200
100
0
0
1
10
100
[Apigenin] (μM)
Hypoxia
Normoxia
###
***
n.s.
*
##

(B)


Normoxia
Cell-surface CD26 (cpm/10 ^{5} cells)
1100
1000
900
800
700
600
500
400
300
200
100
0
*
**
**
Control
Control + A
High lactate
High lactate + A
Low glucose
Low glucose + A

(C)

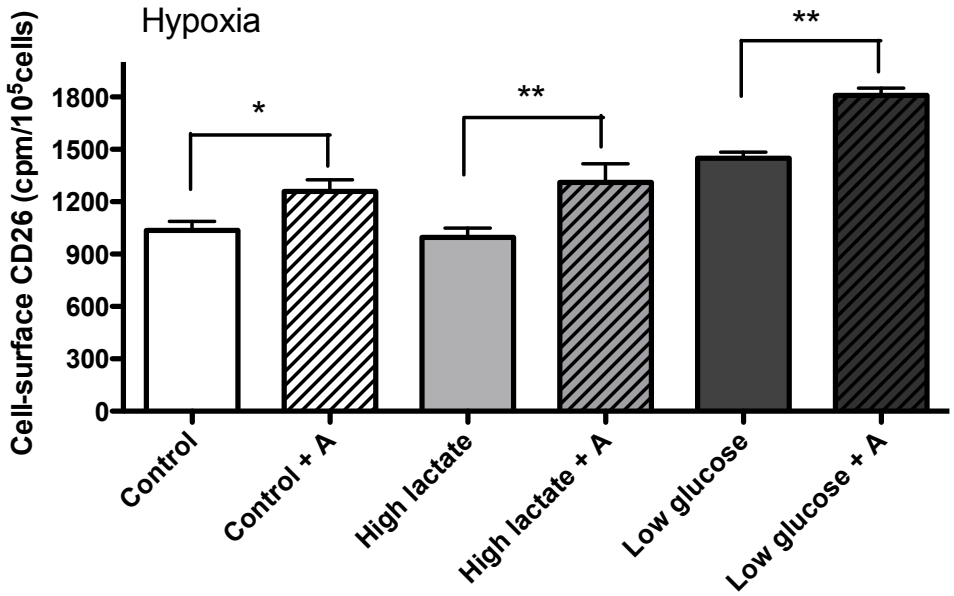
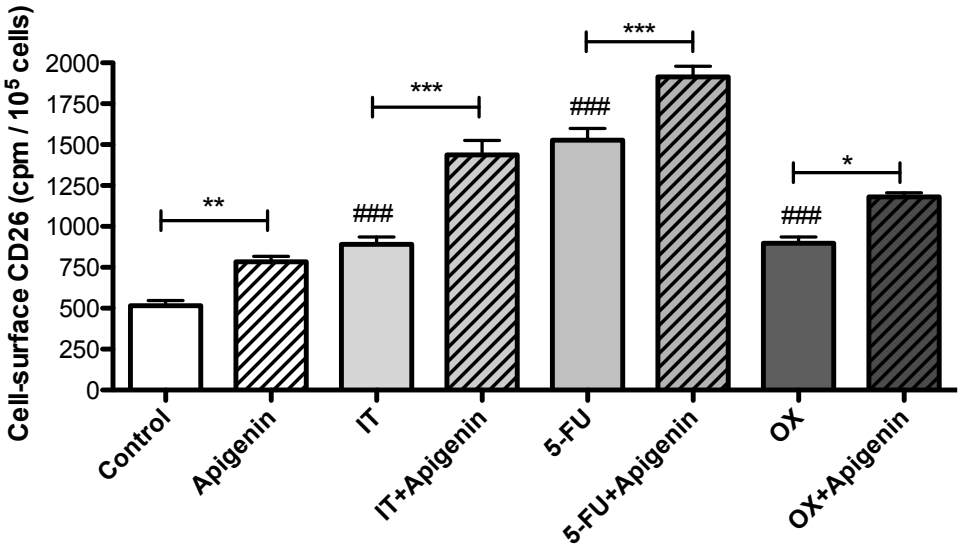


Figure 2.7 Apigenin upregulates CD26 under conditions found within solid tumours.

HT-29 cells were treated with apigenin (A), the concentrations of which was 30μM in panels (b) and (c). The cells were subjected to (A) hypoxia (1% v/v oxygen), (B) conditions found within the tumour microenvironment, i.e., elevated (20mM) lactate or reduced (0.5g/l) glucose., or (C) hypoxia (0.2%, v/v oxygen). Cell-surface CD26 was measured 48 h following these treatments. The data are means + SE (n=4), statistical analyses were by 2-way ANOVA with Bonferroni's comparison test. Significance is shown as *P<0.05 or **P<0.01 for significant upregulation by apigenin; and ###P<0.001 for a significant difference due to hypoxia. Hypoxic conditions were achieved by placing HT-29 cells in a hypoxic chamber for a period of 48 h.

(A)


Cell-surface CD26 (cpm / 10^5 cells)
2000
1750
1500
1250
1000
750
500
250
0
**
***
***
*
###
###
###
Control
Apigenin
IT
IT+Apigenin
5-FU
5-FU+Apigenin
OX
OX+Apigenin

(B)

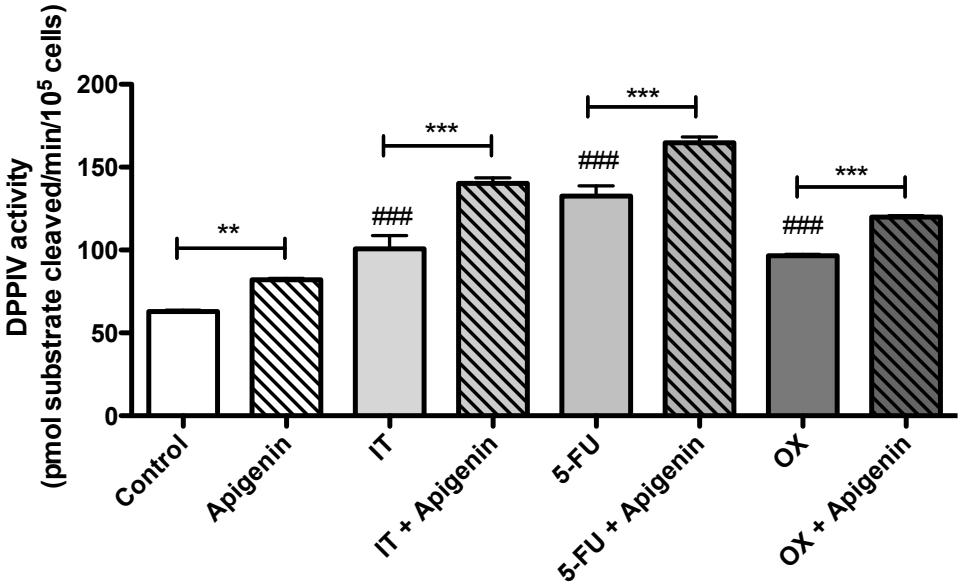
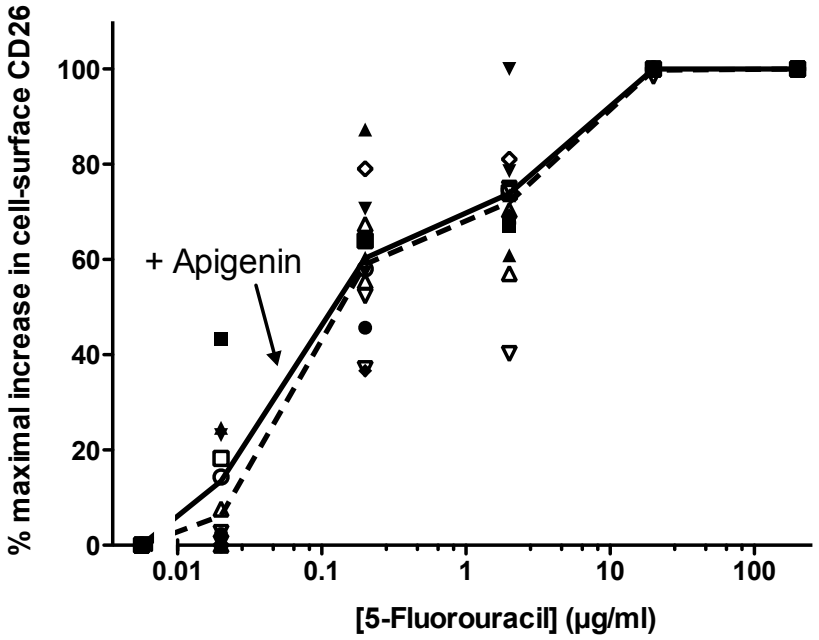

DPPIV activity
(pmol substrate cleaved/min/10^5 cells)
200
150
100
50
0
**
***
***
***
###
###
###
Control
Apigenin
IT
IT + Apigenin
5-FU
5-FU + Apigenin
OX
OX + Apigenin

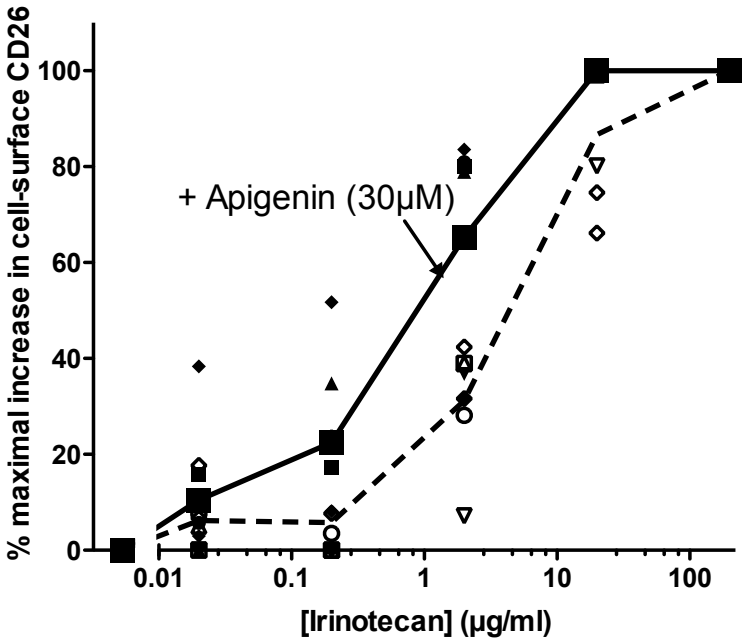
Figure 2.8 Apigenin enhances the effect of chemotherapeutic agents in upregulating CD26 and DPPIV.

HT-29 cells were treated with irinotecan (IT; 2.0μg/ml), 5-fluorouracil (5-FU; 2.0μg/ml) or oxaliplatin (OX; 2.0μg/ml) (solid bars) alone or together with apigenin (30μM; hatched bars). Following 48 h treatment, cells were assayed for **(A)** cell-surface CD26 and **(B)** DPPIV enzyme activity. The data are means + SE (n=4). Statistical analyses were by 2-way ANOVA with Bonferroni's comparison test. Significance is shown as **P<0.01 or ***P<0.001 for significant upregulation by apigenin; and $^{###}$P<0.001 for a significant increase due to the chemotherapeutic agent relative to the control.

(A)


% maximal increase in cell-surface CD26
100
80
60
40
20
0
+ Apigenin
0.01
0.1
1
10
100
[5-Fluorouracil] (µg/ml)

(B)


% maximal increase in cell-surface CD26
100
80
60
40
20
0
+ Apigenin (30µM)
0.01
0.1
1
10
100
[Irinotecan] (µg/ml)

(C)

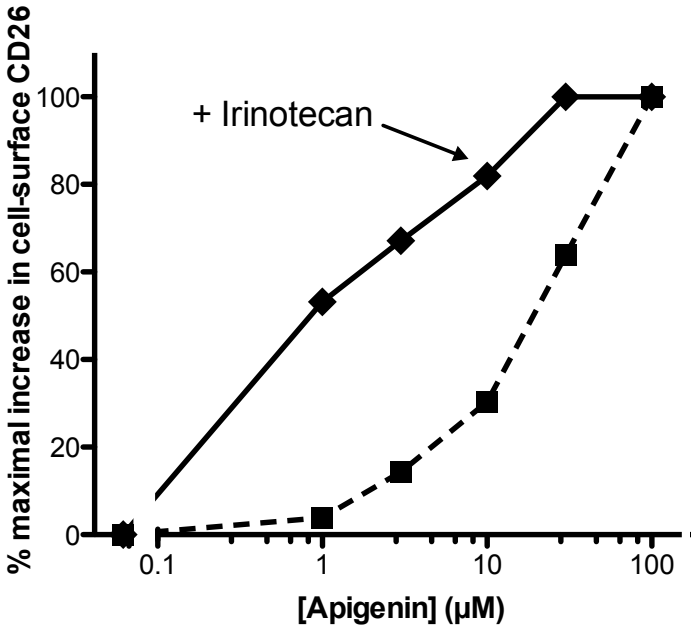


Figure 2.9 Apigenin selectively enhances the potency of irinotecan in upregulating CD26.

HT-29 cells were simultaneously treated with **(A)** 5-fluorouracil or **(B)** irinotecan, alone or together with apigenin (30μM). Following 48 h exposure, the cell-surface CD26 was assayed. Panels (**A)** and (**B)** show individual and averaged data from 7 and 6 independent experiments, respectively (4 replicates within each experiment). Open symbols and dotted lines represent the individual and mean responses to drugs alone, and closed symbols and solid lines the corresponding responses with apigenin. Panel **(C)** shows the mean data from two independent experiments (4 replicates within each experiment and the above result was generated by pooling the data), in which cells were treated with apigenin in the absence (dotted line) or presence (solid line) of irinotecan (2.0μg/ml).

CHAPTER 3

APIGENIN ACTS THROUGH CASEIN KINASE 2 AND TOPOISOMERASE I TO UPREGULATE CD26 ON HUMAN COLORECTAL CARCINOMA CELLS

This chapter appeared in the following submission:

Lefort, É.C., Bentley, V. and Blay, J. 2013. Apigenin acts through casein kinase 2 and topoisomerase I to upregulate CD26 on human colorectal carcinoma cells. Submitted March 4, 2013 to Molecular Cancer Therapeutics.

(É.C. Lefort did greater than 90% of the experimental work in this publication, and directly supervised V.B. for the remainder of experiments. E.C.L. was principally responsible for the experimental design, and had the major role in preparation and submission of the manuscript.)

3.1 Introduction

CD26, alternatively known as DPPIV, is a 110-kDa membrane glycoprotein monomer that undergoes dimerization in order to become enzymatically active at the cell-surface (De Meester, Vanhoof et al. 1992; Gorrell, Gysbers et al. 2001). CD26 is expressed on a variety of cell types, but is most abundant at the epithelial surface of tissues such as that of the human colon (Balis 1985; Kotackova, Balaziova et al. 2009). Expression levels of CD26 vary according to differentiation, where differentiated colonic epithelial cells at the mucosal surface express higher CD26 levels than their progenitors in the mucosal crypts (Dinjens, ten Kate et al. 1989; Darmoul, Rouyer-Fessard et al. 1991).

As a multifunctional protein, CD26 possesses an intrinsic hydrolase activity known as DPPIV, which cleaves N-terminal dipeptides from certain families of polypeptides (De Meester, Korom et al. 1999; Havre, Abe et al. 2008). This allows biological regulation of key peptides such as chemokines and therefore can influence the metastatic process of cancer, including that of CRC cells. Specifically, DPPIV has the greatest affinity for CXCL12, the ligand for CXCR4 and CXCR7, two chemokine receptors implicated in cell survival and malignant spread (Salmaggi, Maderna et al. 2009; Luker, Lewin et al. 2012). CD26 also acts as the major cellular binding protein for ADA, an ecto-enzyme catalyzing the hydrolytic deamination of the tumorigenic and immunosuppressive metabolite compound adenosine (Dinjens, van der Boon et al. 1986; Schrader, West et al. 1990; Dong, Tachibana et al. 1997).

Expression levels of CD26 are frequently dysregulated in cancer (Moehrle, Schlagenhauff et al. 1995; Tsuji, Sugahara et al. 2004; Kikkawa, Kajiyama et al. 2005; Wesley, McGroarty et al. 2005). Downregulation of CD26 is likely to contribute to the aggressive and metastatic phenotype of tumours via enhanced concentrations of the nucleoside adenosine and the chemokine CXCL12 (Tan, Mujoomdar et al. 2004; Richard, Tan et al. 2006). Indeed, reintroducing CD26 into cancer cells expressing low levels of this protein has been reported to reverse the malignant phenotype of a variety of cancers of epithelial origin (Wesley, Albino et al. 1999; Kajiyama, Kikkawa et al. 2002; Wesley, Tiwari et al. 2004; Kikkawa, Kajiyama et al. 2005; Wesley, McGroarty et al. 2005).

Apigenin (4′,5,7-trihydroxyflavone) is a flavone present in the leafy herb parsley and in the dried flowers of chamomile, as well as other sources. Apigenin has a broad spectrum of activities that are beneficial in the context of gastrointestinal cancers, as reviewed by (Lefort and Blay 2013). Most significantly, apigenin has been found to exert inhibitory actions against several steps of the malignant process, by inhibiting molecules such as HIF-1, MMP-9, FAK and CK2. We recently reported that apigenin has the greatest promise amongst a number of flavonoids tested in terms of its ability to upregulate CD26 and therefore potentially oppose CRC progression. We also reported that apigenin could be successfully combined with chemotherapeutic agents employed in the management of metastatic CRC. Notably, apigenin was found to enhance the potency of topo I inhibitor irinotecan with respect to CD26 (Lefort and Blay 2011). We extended our studies to determine whether apigenin can interact with other topoisomerase

inhibitors in upregulating CD26. We also explored the cellular responses for other proteins affected by apigenin, which could explain the regulation of CD26.

3.2 Materials and Methods

3.2.1 Culture of human colorectal cancer cells

HT-29, Caco-2, HRT-18, HCT 116, SW480 and SW620 CRC cells were obtained from (ATCC, Manassas, VA. USA) and were maintained at 37°C, in a humidified atmosphere of 90% air/10% CO_2. All responses have been confirmed in cells within 4 passages of receipt from ATCC. Colorectal cancer cells were cultured in 80-cm^2 flasks (Corning, Nepean, ON. Canada), containing DMEM, supplemented with 5% (v/v), 10% (v/v) or 20% (v/v) heat-inactivated NCS and 1mM sodium pyruvate (Life technologies, Burlington, ON. Canada). Cultures were regularly tested for mycoplasma using a PCR-based approach and were negative for contamination. Once a sub-confluent monolayer was reached, cells were detached with brief exposure to TrypLE^{TM} Express (Life technologies, Burlington, ON. Canada). For experimental purposes, cells were seeded at density of 90,000 cells/ml, unless otherwise indicated. Once cultures reached ≈ 60-70% of confluent density, they were treated with compounds of interest or with control vehicle, as specified in figure legends. Chemotherapeutic treatments included irinotecan (Sandoz, Montreal, Qc. Canada), etoposide (Novopharm, Toronto, ON. Canada) and doxorubicin (Mayne Pharma, Montreal, Qc. Canada). Apigenin, luteolin (3′,4′,5,7-tetrahydroxyflavone), BSA, DMSO as well as the CK2 inhibitors 6-methyl-1,3,8-trihydroxyanthraquinone (Emodin), 4,5,6,7-Tetrabromobenzotriazole (TBB) and 5,6-

Dichlorobenzimidazole 1-β-D-ribofuranoside (DRB) were obtained from Sigma-Aldrich (St. Louis, MO. USA). The MAPK inhibitors PD-98059, SP-600125 and SB-203580 were from (Tocris Bioscience, BS. UK.). Flavonoids, CK2 inhibitors and the MAPK inhibitors were dissolved in DMSO to make stock solutions and then further diluted in medium to give a final DMSO concentration of less than 0.02 % (v/v), a concentration that does not affect CD26 levels (Tan, Mujoomdar et al. 2004).

3.2.2 Topoisomerase I mRNA expression using q-RT-PCR

Total RNA was extracted from HT-29 cells grown in 6-well plates from (VWR International, Mississauga, ON. Canada) using the TRIzol® reagent (Life technologies, Burlington, ON. Canada), as indicated by the manufacturer. RNA concentrations were quantified by spectrophotometric analysis at 280/260nm wavelengths. RNA (1µg) was reverse-transcribed using an M-MLV reverse transcriptase enzyme, 5mM deoxyribonucleotide triphosphate, 0.5µM oligo(Dt), dithiothreitol, 5X First Strand buffer, all obtained from (Life technologies, Burlington, ON. Canada) and DEPC-H_2O (GE Healthcare Life Sciences, Baie D'Ufré, QC. Canada) in a total volume of 20µl. Custom primers (Life technologies, Burlington, ON. Canada) were designed to amplify specific regions in the transcript and the sequences used were: topo I forward primer 5′-TCCGGAACCAGTATCGAGAAGA-3′ and reverse primer 5′-CCTCCTTTTCATTGCCTGCTC-3′. For each of the samples, mRNA levels were normalized to cyclophilin A, sequence forward: 5′-TTCATCTGCACTGCCAAGAC-3′, reverse: 5′-TCGAGTTGTCCACAGTCAGC-3′. Amplification reactions included an

initial cycle of denaturation for 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 18 s, and extension at 70°C for 30 s, with brilliant SYBR Green detection, and a final melting curve cycle of 95°C for 1 min, 65°C for 30 s, and 95°C for 30 s in a Stratagene Mx3000P thermocycler (Cedar Creek, TX. USA). Relative topo I expression as indicated by the fluorescence was analyzed using the comparative cycle threshold method.

3.2.3 Radioimmunoassay for cell-surface CD26

Following a 48 h treatment, cell-surface CD26 was quantified using a radioimmunoassay. Briefly, the culture plates were placed on ice and all subsequent washes and incubations were performed at 4˚C. First, the original medium was aspirated and wells from a 48- or 24-well plate were washed once with 500µl (750µl for 24-well plate) of ice-cold PBS, containing 0.2% (w/v) BSA. Cells were then incubated for 1 h with 125µl (200µl for a 24-well plate) PBS containing 1% (w/v) BSA and 1µg/ml mouse anti-human CD26 (clone M-A261) mAb or mouse IgG isotype-matched control (clone W3/25). Following the incubation period, cells were washed twice with 500µl (750µl for a 24-well plate) PBS containing 0.2% (w/v) BSA and were incubated for 1 h with 125µl (200µl for a 24-well plate) PBS containing 1% (w/v) BSA and 1µCi/ml ^{125}I-labeled goat anti-mouse IgG Ab obtained from (PerkinElmer Life Sciences, NEN, Boston, MA. USA). Cells received two final washes with 500µl (750µl for a 24-well plate) PBS containing 0.2% (w/v) BSA. Finally, 500µl of 0.5M NaOH was added to each well in order to solubilize the cells, and radioactivity was assessed using a gamma counter (Model 1480

Wizard™ 3, Wallac Co. Turku, Finland). Radioactive counts were corrected for both non-specific binding with an isotype control and any difference in cell number was assessed by a Coulter® Model ZM151183 particle counter (Beckman Coulter, Mississauga, ON. Canada). For total CD26 quantification in a 48-well plate, HT-29 cells were first washed with ice-cold PBS and subsequently incubated for 10 min with 500µl 4% formaldehyde. Following permeabilization, cells were washed with ice-cold PBS once and re-washed with PBS containing 0.2% (w/v) BSA. Total CD26 levels were quantified alongside intact cells, using the above-described method.

3.2.4 Western Blot analysis for topoisomerase I and CK2

HT-29 cells were seeded into 6-well plates and were grown until ≈ 60-70% confluency, after which cells were exposed to apigenin (60µM) over a time course of 0-60 h. Total cellular protein was isolated by first washing the cells twice with ice-cold PBS and then dissolved in lysis buffer [50mM Tris-HCL (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150mM NaCl, 1mM EDTA, 1mM NaF, 1mM phenylmethyl sulfonyl fluoride and 1X protease inhibitor cocktail set 1 (EMD Canada Inc. Mississauga, ON. Canada). Samples were incubated on a plate rotator for 20 min at 4°C and cell lysates were clarified by centrifugation (12,000 x g for 20 min). Total cellular protein was collected and quantified by colorimetric assay using Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories Inc. Mississauga, ON. Canada).

Fifteen micrograms of protein extract per lane was first denatured by the addition of 4X Laemmli buffer [150mM Tris-HCl (pH 8.8), 2% β-mercaptoethanol (v/v), 4% glycerol (v/v), 0.05% bromophenol blue (w/v) and 8% SDS (w/v)]. Extracts were then heated to 95°C for 5 min and subsequently loaded and separated by SDS-PAGE using a 10% acrylamide resolving gel, containing 125mM Tris-HCl (pH 6.8), 0.1% SDS (w/v), 0.1% ammonium persulfate (APS, w/v) and 0.15% N,N,N,N-tetramethylethylenediamide (TEMED, v/v) with a 4% acrylamide stacking gel containing 125mM Tris-HCl (pH 6.8), 0.1% SDS (w/v), 0.1% APS (w/v) and 0.3% TEMED (w/v). Gels were electrophoresed at 50milli Amps for 1h30 in SDS-PAGE running buffer [25mM Tris-HCl, 192mM glycine, 2% methanol (v/v) and 0.1% SDS (v/v)] and then electroblotted to a nitrocellulose membrane at 60V for 1h30 at room temperature. Membranes were blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween 20 for 1 h at room temperature. Membranes were subsequently probed overnight at 4°C with a primary Ab, specific for topo I or CK2, using the recommended concentrations. Primary Abs included IgM mouse anti-human DNA topo I (clone C-21) and IgG mouse anti-human casein kinase II α/α′ (clone 31) (BD Pharmingen, San Diego, CA. USA). Membranes were washed five times (7 min each) with Tris-buffered saline with 0.1% Tween 20 and then incubated with an Horseradish Peroxidase (HRP)-conjugated IgG goat anti-mouse secondary mAb from (Cedarlane Laboratories Ltd. Burlington, ON. Canada) or HRP-conjugated polyclonal Ab IgM goat anti-mouse secondary (BD Pharmingen, San Diego, CA. USA), for 1 h at room temperature. Protein expression was detected using an enhanced chemiluminescence detection system (Thermo scientific, Ottawa, ON. Canada). To confirm equal protein loading in each sample, the membrane was re-probed with a IgG rabbit anti-human α–

tubulin (11H10) primary mAb (Cell Signaling Technology®, Pickering, ON. Canada), followed by an IgG HRP-conjugated goat anti-rabbit secondary mAb (BD Pharmingen, San Diego, CA. USA).

3.2.5 Silencing of topoisomerase I and CK2 using siRNA transfection

Small interfering RNA (siRNA) transfection of HT-29 cells was carried out according to the manufacture's instructions for reverse transfection, using 3.0μl/well siPORT™ *Amine* Transfection Agent (AM4503) in 24-well plates. *Silencer* ® Select negative control #1 siRNA were used as a negative control. Cells were seeded in 10% NCS DMEM at a density of 240,000 cells/ml and were transfected with an optimized concentration of 7.5nM, using validated silencer® Select siRNA specific for CK2 or topo I. 24 h following seeding, cells received a medium change in order to reduce cellular cytotoxicity and 36 h or 48 h following siRNA transfection for CK2 and topo I, respectively (time points where the knock-downs became established, as evaluated through western blot), cells were treated with 60μM apigenin or its equivalent DMSO control. To examine the effect of apigenin on CK2 or topo I knock-down cells, cell-surface CD26 levels were subsequently assessed through a radioimmunoassay 48 h following treatment; 84 h and 96 h for CK2 and topo I, respectively.

3.2.6 Statistical analysis

Unless otherwise noted, figures show data that are representative of independent experiments conducted on at least three separate occasions. Statistical analyses were performed using Prism 5.0 software (GraphPad, San Diego, CA). Comparisons of data were performed using one- or two-way ANOVA followed by Dunnett's or Bonferroni's comparison tests, as indicated in the figure legends For all analyses a *P*-value < 0.05 was considered as the minimum for statistical significance.

3.3 Results and Discussion

3.3.1 Apigenin upregulates CD26 in colorectal cancer cells showing high basal CD26

We have previously documented the ability of apigenin to upregulate the cell-surface expression of CD26 on HT-29 and HRT-18 human CRC cells (Lefort and Blay 2011). This increase in CD26 reached a maximum after 24-48 h, with a mean elevation of 56.3% in response to an apigenin EC_{50} of 3-3μM (Lefort and Blay 2011). We wished to examine the consistency of this response in a range of human CRC cells that have different degrees of differentiation (Lea, Ibeh et al. 2010; Schneider, Huber et al. 2012). The results are shown in Fig. 3.1; data are corrected for non-specific binding of the anti-CD26 mAb and are expressed relative to the number of viable cells.

Of the more differentiated cell lines and in addition to HT-29 and HRT-18 cells, Caco-2 cells showed sensitivity to apigenin in upregulating CD26. It is notable that although these three cell lines have substantially different basal levels of surface CD26 (the relative levels for HRT-18:HT-29:Caco-2 cells are ~ 30:50:100), the magnitude of the effect of apigenin was similar in each case, an approximate doubling of CD26 (Fig. 3.1).

In contrast to these cell lines, less-differentiated SW480, SW620 and HCT 116 human CRC cells showed much lower basal levels of CD26 and no effect of apigenin (Fig. 3.1). Our assay measures immunoreactive CD26 on the surface of viable cells and is corrected for background binding in the presence of an isotype mAb. It is likely that SW480 and HCT 116 cells do display low levels of CD26 protein. It is notable that HCT 116 and HRT-18 cells, which have comparable basal levels of CD26 differ completely in their response to apigenin. HRT-18 cells show a ~ 99.7% increase in CD26, whereas the level of cell-surface CD26 on HCT 116 cells remains unaltered by 30μM apigenin. Although unresponsive to apigenin, it is noticeable that in the paired patient cell lines SW480 and SW620, CD26 levels are lower in the lymph node metastasis-derived cell line SW620 than in the primary tumour-derived SW480 counterpart (Fig. 3.1).

It is notable that the basal level of CD26 expression at the surface of cells in these CRC lines increases in proportion to the ability of these cells to differentiate, as evidenced by a more flattened morphology and capacity to express differentiation markers such as sucrose isomaltase (SI), alkaline phosphatase (ALP) and aminopeptidase

N (APN) (Pinto, Appay et al. 1982). This is consistent with the anticipated role of CD26 as a marker of differentiation, by virtue both of its ADA-binding capacity and expression of DPPIV activity (Ten Kate, Wijnen et al. 1985; Darmoul, Rouyer-Fessard et al. 1991; Siavoshian, Blottiere et al. 1997). It conflicts with the more recent assertion that CD26 expression is a feature of stemness within a cancer cell population (Pang, Law et al. 2010). Whether the responsiveness to apigenin, seen with 'HRT-18 and more differentiated lines' indicates a cellular target that is only acquired as the cell becomes more differentiated, remains to be answered.

3.3.2 Apigenin upregulates cell-surface CD26

The level of CD26 at the cell-surface in colorectal cells is highly dependent upon its trafficking between different cellular compartments (Matter, Stieger et al. 1990 ; Klumperman, Boekestijn et al. 1991). It therefore seemed highly possible that changes in CD26 at the cell-surface due to apigenin, which are more evident in CRC lines that have higher levels of differentiation, could reflect an ability of apigenin to mobilize CD26 to the cell surface from intracellular pools. The retention of CD26 through endocytosis has been observed and this creates an internal pool, where CD26 is present in intracellular vesicles without being degraded (Matter, Stieger et al. 1990). Therefore CD26 mobilization from intracellular pools to the cell-surface could account for CD26 enhancement observed by apigenin. Alternatively, CD26 can also become redistributed from the basolateral surface, where basolateral CD26 membrane-bound protein would be trancytosed to the apical surface of cells and result in CD26 enhancement at the cell-

surface (Matter, Stieger et al. 1990). We therefore evaluated this possibility. Permeabilization of the cells with mild fixation to distinguish total protein from cell-surface protein and therefore distinguish intracellular material (Jalal, Jumarie et al. 1992; Andre-Garnier, Robillard et al. 2003) failed to reveal significant intracellular pools of CD26 in HT-29 cells in addition to that detectable at the cell surface (Fig 3.2). Indeed, all of the CD26 in HT-29 cells is displayed at the cell surface. Apigenin therefore acts to increase total cellular CD26 protein, which is reflected by increased CD26 at the cell surface, in a fully functional form (Lefort and Blay 2011). We therefore explored the possible pathways that might lead to this cellular upregulation.

3.3.3 Apigenin partially acts through casein kinase 2 to elevate CD26

Apigenin – as for many small organic molecules, including some that have been adopted for clinical use – has many different activities when tested for its effects on cellular biochemistry, as reviewed by (Lefort and Blay 2013). One of its better known properties is an ability to inhibit the broad-specificity kinase, CK2. Protein kinase CK2 (formally known as casein kinase II), is a tetrameric protein complex, composed of two catalytic subunits (α 42kDa or α' 38kDa) and two regulatory subunits (β 28kDa), forming the tetramer combinations $\alpha_2\beta_2$, $\alpha'_2\beta_2$ and/or $\alpha\alpha'\beta_2$ (Litchfield 2003; Ahmad, Wang et al. 2008). CK2 is widely distributed in different cellular compartments and therefore able to influence diverse cellular processes and molecules, as reviewed by (Litchfield 2003). We evaluated whether apigenin might be acting through its known CK2 inhibitory ability to upregulate CD26.

If apigenin acts through this route, other CK2 inhibitors should enhance levels of CD26 at the cell surface. We tested three different CK2 inhibitors for their ability to elevate cell-surface CD26: Emodin (6-methyl-1,3,8-trihydroxyanthraquinone), TBB (4,5,6,7-Tetrabromo-2-azabenzimidazole) and 5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside). The three inhibitors are structurally diverse (Figs 3.3 A-C) and distinct from apigenin (4′,5,7-trihydroxyflavone, 5,7-Dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one; Fig. 3.3, D).

Each of the CK2 inhibitors led to an increase in cell-surface CD26 that was dose-dependent and maximal in the concentration range 20-60μM (Fig. 3.4). The maximum elevations of CD26 in multiple independent experiments were: Emodin, 95.5%; TBB, 71.4% and DRB, 77.4%. Although different molecules, and with different activities noted for other targets (Seifeddine, Dreiem et al. 2008; Song, Kim et al. 2013), this shows remarkable congruency in both molar concentration range and maximal effect on CK2 for four very different agents.

If apigenin were acting through the same pathway as these CK2 inhibitors, we would anticipate that each would attain the same maximal effect on CK2 (corresponding to maximum CK2 inhibition), and that individual effects would sum to a fixed ceiling. Additive effects at maximally-effective dose, or synergism between apigenin and CK2 inhibitors, would point to apigenin acting through a non-CK2 pathway to elevate CD26. We therefore evaluated the consequence of combining CK2 inhibitors with apigenin and

observing the effect on CD26. At no point did we detect additive effects of combining apigenin with these CK2 inhibitors. Combining the CK2 inhibitors at concentrations producing sub-maximal elevation in cell-surface CD26 (which did not lead to desensitization or significant effects on cell viability) did not increase the elevation of CD26 beyond that which could be attained with a maximally-effective dose of apigenin (Fig. 3.5). This would be consistent with apigenin also acting through CK2, and a maximum CK2 inhibition already being in place. We therefore pursued the possibility that apigenin was acting through inhibition of CK2.

Apigenin had no effect on the overall protein level of CK2, over the 60 h treatment period (Fig. 3.6, A). To decrease the amount of CK2 and evaluate its role as the cellular target for apigenin action in upregulating CD26, an siRNA approach was employed, which combined 4 validated siRNA constructs. The negative control was a validated negative control siRNA from the same manufacturer, used at the same final RNA concentration and ratio with transfection reagent as the CK2-silencing combination. Figure 3.6, B shows that we were able to achieve a substantial CK2 knock-down that reached 95.4% after 36 h and remained at 75.7% 84 h after the transfection step. Interestingly, there was a substantial reduction in CK2 protein (44.9% after 36 h, 14.3% 84 h) in the presence of the negative control siRNA (Fig. 3.6, B). It is unclear why a commercial, validated siRNA control should have this effect. Many studies of CK2 knock-down do not include a negative or scrambled control, e.g. (Olsen, Issinger et al. 2010). However, other CK2 knock-down studies do not show any effect of a scrambled sequence, e.g. (Smolock, Wang et al. 2007). This did not interfere with our overall

conclusions (see below) and serendipitously gave rise to an intermediate (relative to transfection control) level of CK2 reduction, so we accepted these data.

We then measured the effect of apigenin on cell-surface expression of CD26 protein in cells, following CK2 knock-down or treatment with control transfection reagent alone or with a negative control siRNA (Fig. 3.7). Apigenin retained its ability to substantially upregulate CD26 in spite of the altered seeding and culture conditions necessary for the siRNA approach. Relative to the transfection control, CK2 siRNA led to a significant increase in CD26, a further observation consistent with the view that CK2 activity serves to constrain the level of CD26 at the cell surface (Fig. 3.7).

The effect of CK2 knock-down was to eliminate the apigenin response, although CD26 levels were reduced to the knock-down control rather than the transfection control in the absence of apigenin (Fig. 3.7). This indicates that apigenin acts through CK2 to upregulate CD26. The response to apigenin was retained in the negative siRNA control but was substantially diminished. The reduced response corresponded with the partial decrease in CK2 expression, providing further evidence that the CD26 response of apigenin is mediated through CK2.

3.3.4 Mitogen-activated protein kinase pathways also serve to elevate CD26

Although CK2 inhibition appears necessary for the ability of apigenin to upregulate CD26, it is not sufficient for a maximal apigenin response. The elevation in CD26 due to CK2 inhibitors across all of our independent experiments was remarkably consistent at about 35% over control: Emodin, 35.3 ± 8.2 % relative to vehicle control (mean ± SEM, n=9); TBB, 37.1 ± 10.2 % (n=4); DRB 34.5 ± 17.0 % (n=4). This is substantially lower than the elevation achievable with apigenin using these same HT-29 cells, which is 56.3 ± 0.03% (n=10) for CD26 binding, and 55.0 ± 0.06% (n=7) for the functional measure of eADA binding (Lefort and Blay 2011). This strongly suggests that for apigenin itself, there is a further effector pathway in addition to CK2 inhibition.

Further evidence that CK2 inhibition is not the sole route of action comes with the observation that CK2 inhibitors such as Emodin do not produce a leftward shift in the dose-response curve of the ability of irinotecan to elevate CD26 (Fig. 3.8). This contrasts markedly with the ability of apigenin to enhance the potency of irinotecan (Lefort and Blay 2011). Apigenin led to a 4.2-fold enhancement of irinotecan potency, with a reduction in its EC_{50} from 4.68 ± 1.17μg/ml to 1.11 ± 1.26μg/ml in the presence of 30μM apigenin (mean ± SEM, n=6, statistically different with $P< 0.01$) (Lefort and Blay 2011).

We investigated the possibility that apigenin might be acting through kinases other than CK2. We combined inhibitors of the different mitogen-activated protein kinase (MAPK) pathways and examined the effect on the CD26 response to apigenin. When we combined submaximal inhibitory concentrations of the MEK1 (ERK1/2 pathway) inhibitor PD-98059, JNK inhibitor SP-600125 and p38 inhibitor SB-203580, we saw no

further enhancement in CD26 with apigenin beyond that achieved in its absence (Fig. 3.9). However, the combination of inhibitors itself substantially elevated CD26. We examined each individual inhibitor for an ability to further elevate CD26 beyond the increase seen with apigenin alone (Fig. 3.10). Inhibition of each of the three MAPK activities was able to increase cell-surface CD26 relative to the vehicle control and the maximal level of CD26 attained was not greater than that for apigenin alone (Fig. 3.10). This strongly suggests that MAPK pathways are inhibited by apigenin. However, the involvement of multiple phosphorylation pathways and cross-talk prevented practical intervention through siRNA without excessive cytotoxicity, we therefore concentrated on a downstream target of MAPK and CK2 signalling, topo I.

3.3.5 Apigenin acts through topoisomerase I to elevate CD26

The cellular distribution of CK2 in a cancer cell differs from that of a normal cell. In the former, CK2 is mainly localized in a diffuse pattern in the nucleus and the cytoplasm whereas in a cancer cell, CK2 levels are substantially higher in the nuclear compartment (Faust, Niehans et al. 1999; Laramas, Pasquier et al. 2007). This favours a nuclear target for apigenin action through CK2. Furthermore, we have shown that the ability of apigenin to upregulate CD26, while in most cases simply additive the modulatory effects of chemotherapeutic agents such as 5-FU or OX is interactive with that of irinotecan, such that irinotecan causes a ~30-fold increase in the potency of apigenin to enhance CD26 levels (Lefort and Blay 2011).

Both irinotecan, which is a topo I inhibitor and etoposide, which is a topo II inhibitor, enhanced cell-surface CD26 levels (Fig. 3.11, A and B). However, topo II inhibitors did not enhance the potency or action of apigenin and at higher doses of the inhibitors enhancement of CD26 by apigenin was decreased or lost (Figs. 3.12 and 3.13). This is shown for different doses of etoposide in Fig. 3.12 and illustrated for two topo II inhibitors, etoposide and doxorubicin, relative to irinotecan in Fig. 3.13. Furthermore, the apigenin metabolite luteolin (3',4',5,7-tetrahydroxyflavone), which like apigenin acts as an inhibitor of CK2 (Li, Liu et al. 2009; Lolli, Cozza et al. 2012) was itself able to upregulate CD26 (Fig. 3.14, A) but was unable to substitute for apigenin in enhancing the effect of irinotecan (Fig. 3.14, B). In fact, luteolin completely failed to elevate the irinotecan effect. The specificity of the apigenin link to topo I, and the observation that a structurally closely-related metabolites is devoid of the unique ability of apigenin to interact with the topo I-targeted agent irinotecan, argue for an additional effect of apigenin through the topo I pathway and suggest that there may also be direct interaction, rather than it simply occurring through CK2.

Apigenin had no effect on topo I mRNA expression (Fig. 3.15). Topo I mRNA levels were somewhat lowered at times of 36 h and beyond, but this was also seen in the vehicle control treatments and likely reflects changes in topo I expression rate due to progressive cell growth. This slight decline was somewhat evident in measurements of topo I protein over time but relative to that background, apigenin had no effect on the levels of topo I protein assessed by western blot (Fig. 3.16, A).

To evaluate the importance of topo I in apigenin action, we knocked-down topo I using siRNA which combined 3 validated siRNA constructs, and then subsequently tested the cells to see whether apigenin would enhance CD26 levels. As shown in Fig. 3.16, B we were able to substantially (>75.0%) knock-down topo I protein at 48 h while retaining good cellular viability, as indicated by cell numbers part of our routine radioimmunoassay. As shown in Fig. 3.17, the effect of removing topo I was to eliminate the apigenin-induced upregulation of CD26, arguing that topo I is indeed involved in that mechanism.

However, it is also apparent that knocking down topo I itself raises CD26 levels, in the same way that direct inhibition of CK2 or MAPK pathways leads to an increase in CD26. This somewhat confounds interpretation. Nevertheless, coupled with the fact that apigenin itself has a greater effect than direct CK2 inhibition or knock-down, and that it has a structurally selective activity toward topo I alongside the topo I inhibitor irinotecan, we feel that there is sufficient evidence to implicate topo I as involved in the apigenin-induced upregulation of CD26.

3.4 Conclusion

Taken together, our findings provide direct evidence for the involvement of topo I in CD26 upregulation by the dietary flavone apigenin, in HT-29 human CRC cells. We have also demonstrated that kinase activities upstream of topo I, notably those of CK2 and MAPKs, are relevant in mediating the apigenin effect on cell-surface CD26. This route of CD26 upregulation, which interacts with effects of the topo I inhibitor irinotecan,

is highly selective to apigenin. Substitution of apigenin with its hydroxylated metabolite luteolin, or the CK2 inhibitor Emodin, failed to result in a similar potency enhancement as with apigenin. This suggests that apigenin relies on other cellular pathways to be synergistically combined with irinotecan, perhaps by also directly targeting topo I. These findings help to distinguish a unique molecular mechanism for the action of apigenin on CD26. This may point to a novel approach to elicit a more differentiated cellular phenotype and dampen the metastatic process of CRC cells.

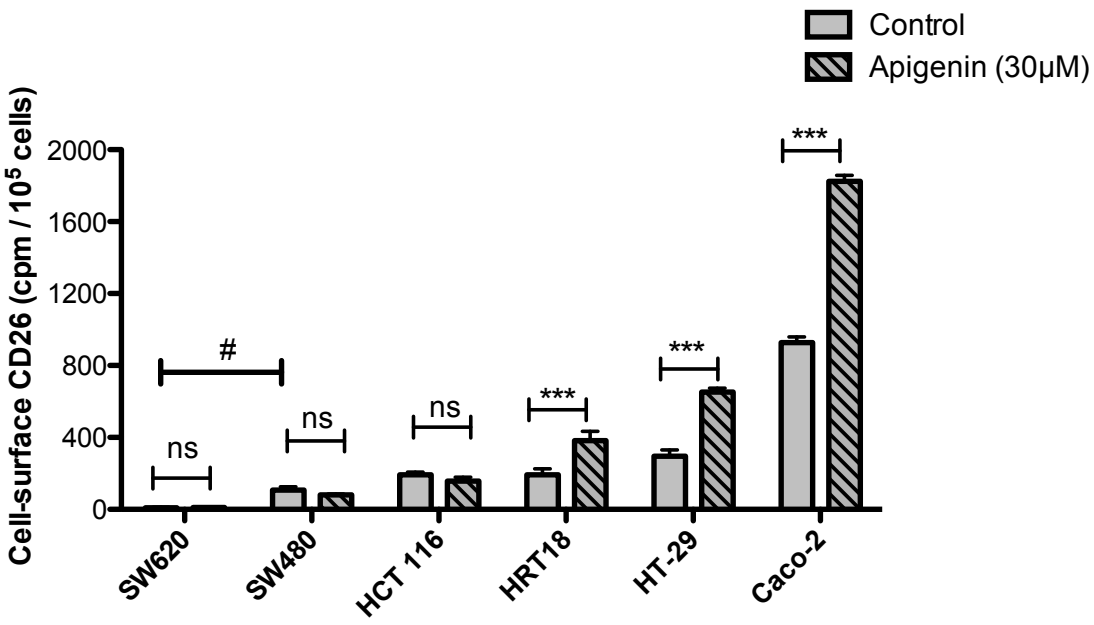


Figure 3.1 Apigenin upregulates CD26 in human colorectal carcinoma cells showing higher basal CD26.

Monolayer cultures of the human colorectal carcinoma cell lines were evaluated for cell-surface CD26 after 48 h in the presence and absence of apigenin (30μM). The values are means + SE (n=4), comparisons of data were performed using two-way ANOVA with Bonferroni's comparison test. Statistical differences are shown as ***P<0.001, indicating significant enhancement of CD26 by apigenin as compared to the respective control treated cells, and # P< 0.05, indicating significant difference in basal CD26 expression between the SW620 and SW480 paired cell lines.

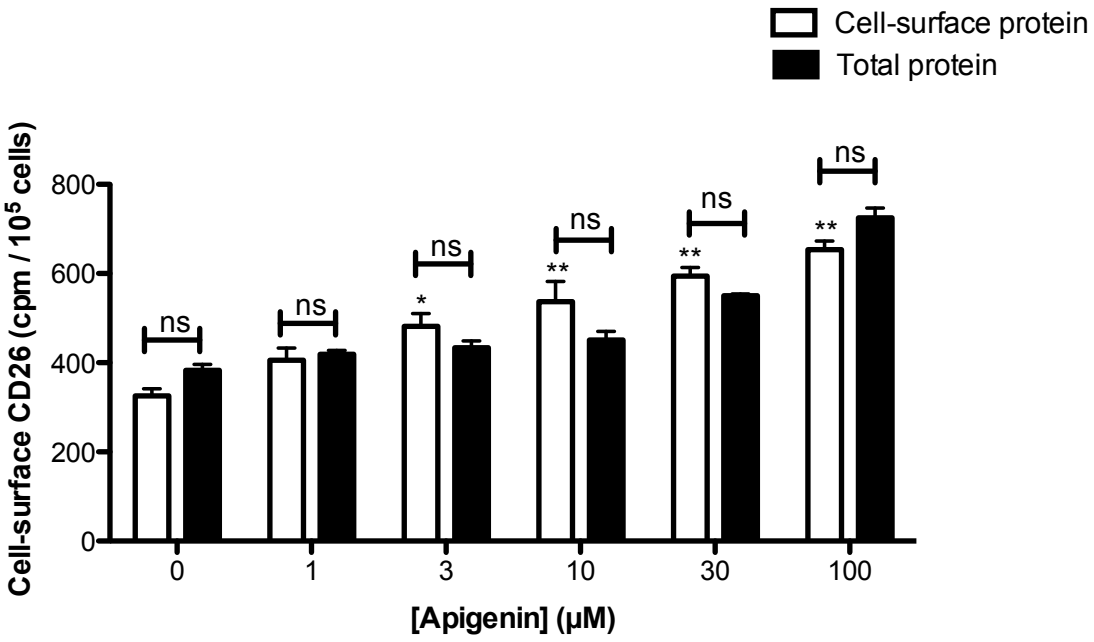
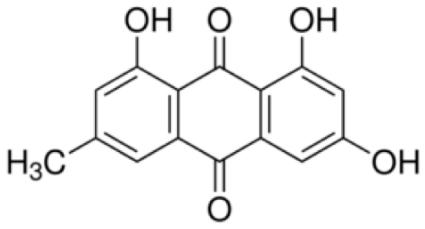


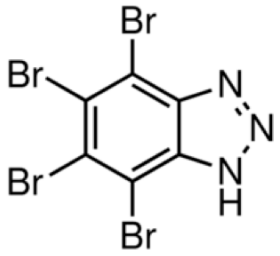
Figure 3.2 CD26 enhancement by apigenin occurs entirely at the cell-surface.

HT-29 cells were examined for cell-surface CD26 levels (white bars) or total CD26 levels (black bars), in the presence and absence of apigenin (0-100μM). The data are means + SE (n=4), comparisons of data were performed using two-way ANOVA with Bonferroni's comparison test. Statistical significance is shown as *P<0.05, **P< 0.01, indicating significant enhancement of cell-surface CD26 levels by apigenin as compared to control-treated cells and ns, indicates no significant difference between cell-surface and total levels.

(A)



(B)



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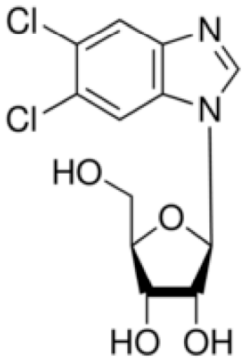


(D)

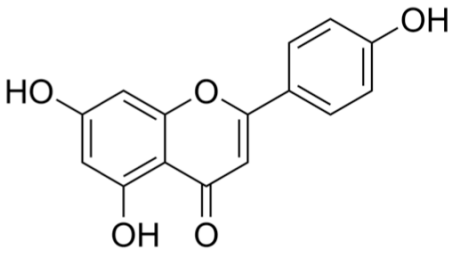


Figure 3.3 Structures of CK2 inhibitors.

A) Emodin (6-methyl-1,3,8-trihydroxyanthraquinone). **B)** TBB (4,5,6,7-Tetrabromo-2-azabenzimidazole). **C)** DRB (5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside). **D)** Apigenin (4′,5,7-trihydroxyflavone; 5,7-Dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one).

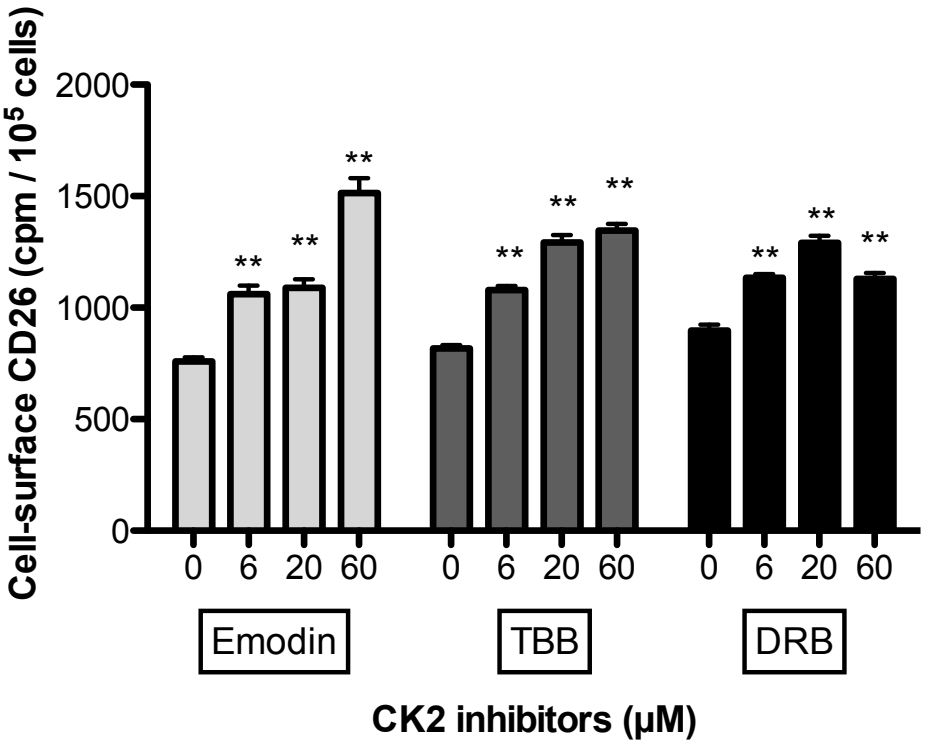


Figure 3.4 CK2 inhibitors enhance cell-surface CD26 levels.

HT-29 cells were treated with Emodin (6-methyl-1,3,8-trihydroxyanthraquinone), TBB (4,5,6,7-Tetrabromo-2-azabenzimidazole) and DRB (5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside) at a (0-60μM) concentration and cell-surface CD26 was measured 48 h following treatment. The values are means + SE (n=4), analyzed by one-way ANOVA followed by Dunnett's comparison test. Statistical significance is shown as **P<0.01, indicating significant enhancement by CK2 inhibitors as compared to the relative control.

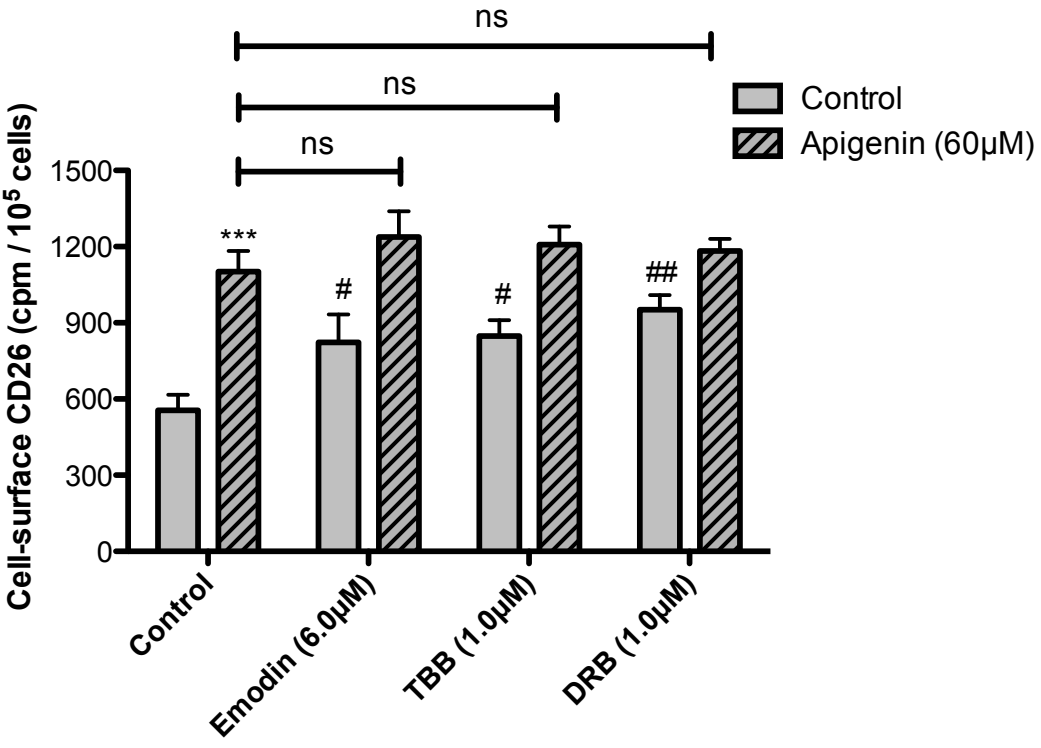
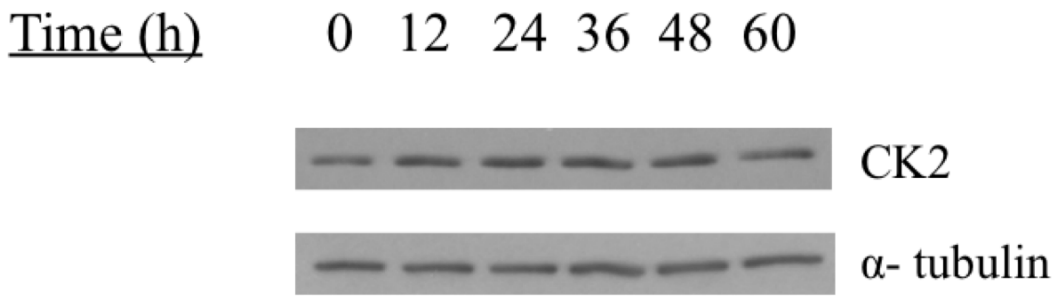


Figure 3.5 CK2 inhibitors do not enhance the maximum increase in CD26 above that achieved with apigenin.

HT-29 cells were treated with CK2 inhibitors at the indicated concentrations in the presence and absence of apigenin (60μM). The values are means + SE (n=4), showing the average of 5 independent experiments, comparisons of data were performed using two-way ANOVA with Bonferroni's comparison test. Statistical significance is shown as ***P<0.001, indicating significant increase by apigenin, and #P<0.05 and ##P<0.01, indicates significant CD26 enhancement by CK2 inhibitors, as compared to the relative control.

(A)



(B)

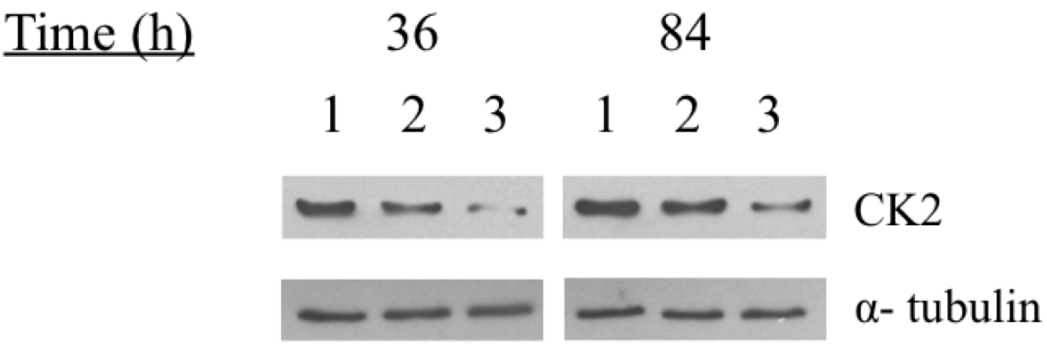


Figure 3.6 CK2 protein studies

(**A) Lack of effect of apigenin on CK2 protein levels**. HT-29 cells treated over a 60 h time course in the presence of apigenin (60μM). CK2 protein levels were evaluated through western blot. (**B) Successful knock-down of CK2 levels using an siRNA approach**. Lane 1, transfection control; lane 2, scramble siRNA; lane 3, CK2 siRNA.

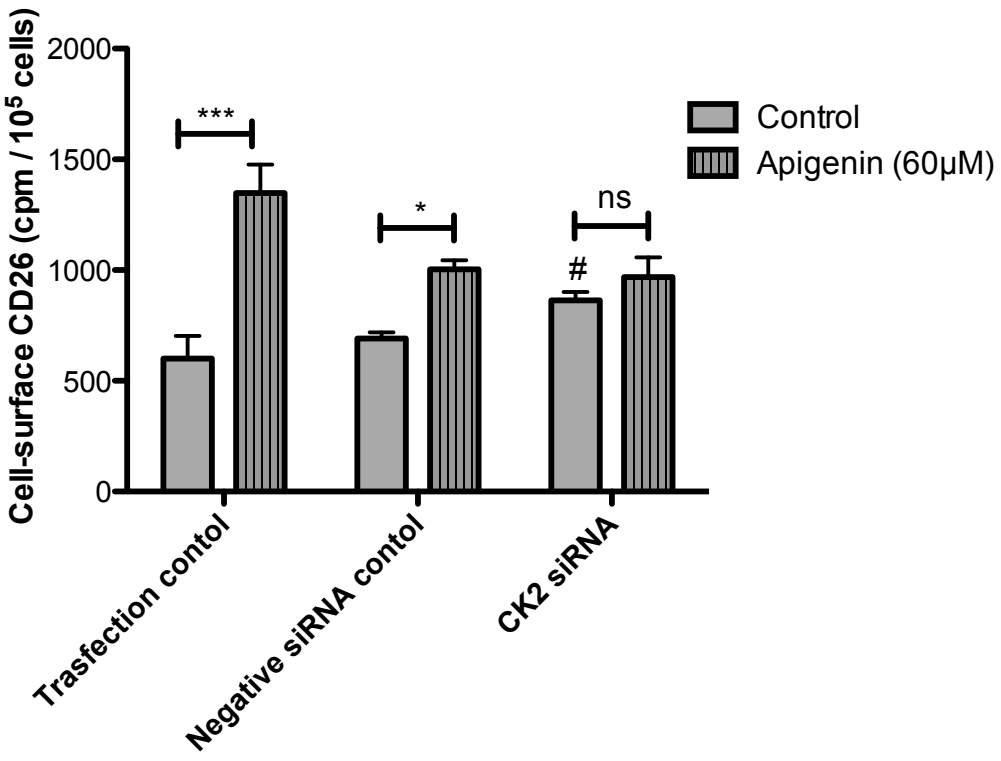


Figure 3.7 Knock-down of CK2 abrogates the effect of apigenin on CD26, but does not reduce cell-surface CD26 to control levels.

48h following apigenin (60μM) treatment or its representative control, CD26 levels were evaluated. The values are means + SE (n=4), comparisons of data were performed using two-way ANOVA with Bonferroni's comparison test. Statistical significance is shown as #P<0.05, indicating significant enhancement by CK2 knock-down and *P<0.05 and ***P<0.001, indicating significant CD26 enhancement by apigenin as compared to the relative control.

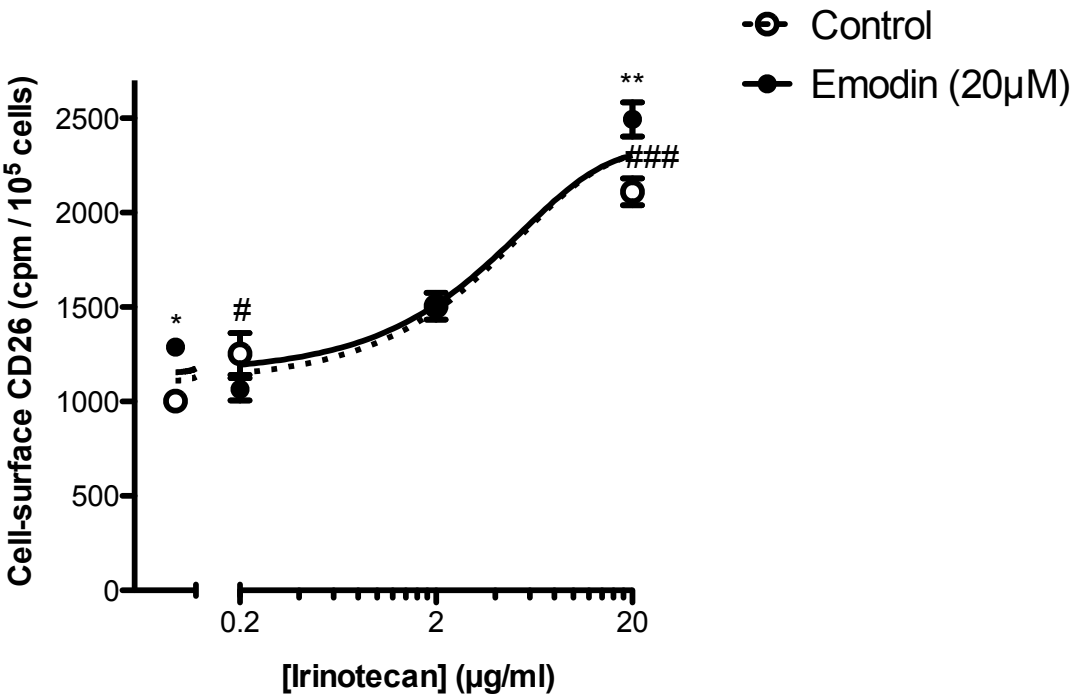


Figure 3.8 The CK2 inhibitor Emodin does not enhance the potency of the topoisomerase I inhibitor irinotecan in increasing CD26.

HT-29 cells were treated with irinotecan at the indicated concentrations, in the presence (closed circles) and absence (open circles) of Emodin (20µM), and CD26 was assessed 48 h following treatment. The data are means ± SE (n=4). Comparisons of data were performed using two-way ANOVA with Bonferroni's comparison test. Statistical significance is shown as *P<0.05 and **P<0.01 indicates significant enhancement by emodin and #P<0.05 and ###P<0.001, indicates significant CD26 enhancement by irinotecan as compared to the relative control.

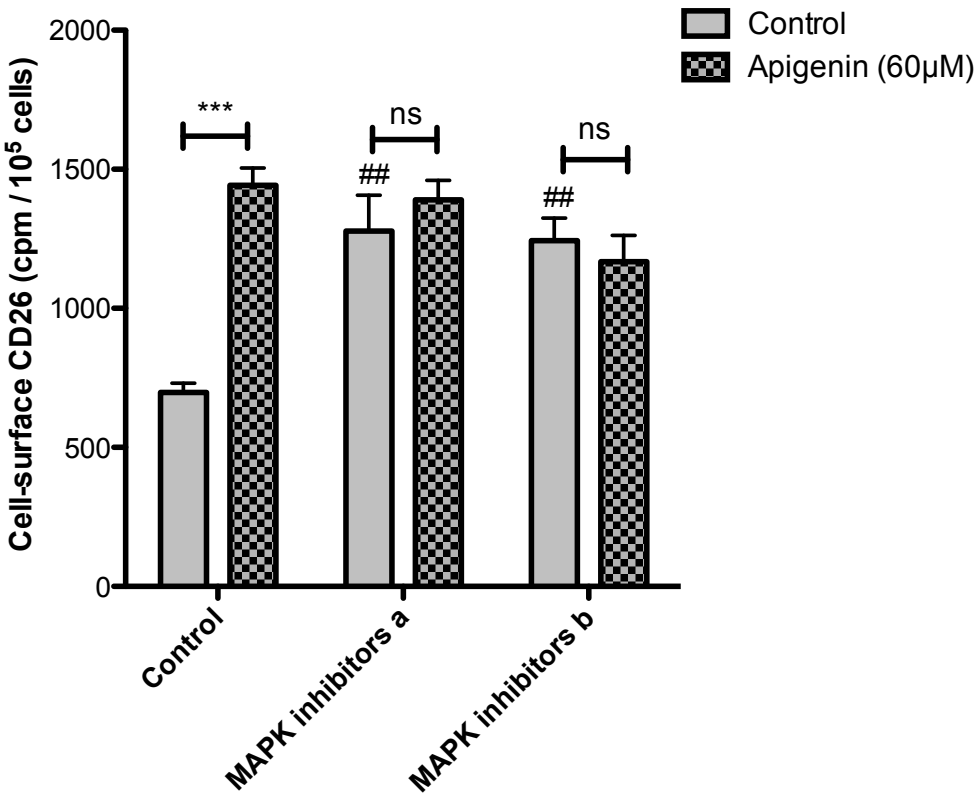


Figure 3.9 MAPK inhibitors block the apigenin upregulation on CD26.

HT-29 cells were pretreated for 30 min with appropriate vehicle control or with the MAPK inhibitors PD-98059 (ERK1/2), SP-600125 (JNK) and SB-203580 (p38) added in combination, at the respective concentrations of (4, 2 and 1μM) MAPK inhibitors **a** or (10, 5 and 2.5μM) MAPK inhibitors **b**, in the presence or absence of apigenin (60μM). The data are means + SE (n=4), comparisons of data were performed using two-way ANOVA with Bonferroni's comparison test. Significance is shown as ##P<0.01 for significant upregulation by MAPK inhibitors, and ***P<0.001 for a significant upregulation by apigenin as compared to the relative control.

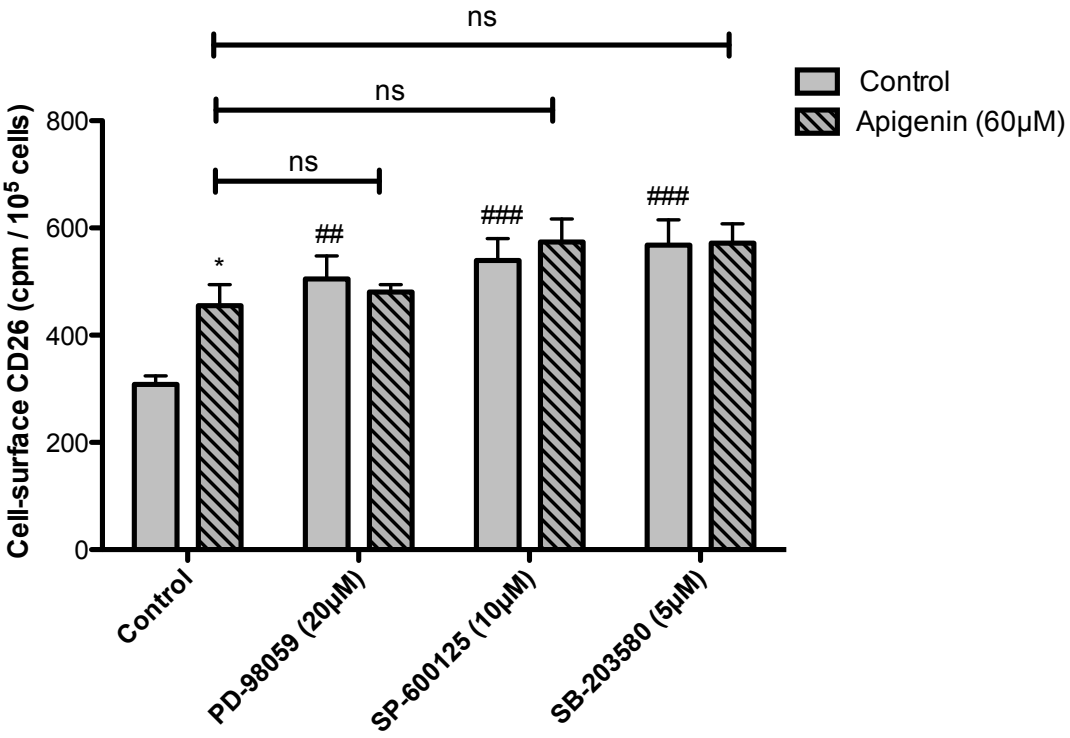


Figure 3.10 Individual MAPK inhibitors enhance cell-surface CD26 levels but apigenin does not produce a further increase.

HT-29 cells were pretreated with the MAPK inhibitors PD-98059 (ERK1/2), SP-600125 (JNK) and SB-203580 (p38), at the indicated concentrations for 30 min, followed by vehicle control or apigenin treatment (60μM). The data are means + SE (n=4), comparisons of data were performed using two-way ANOVA with Bonferroni's comparison test. Significance is shown as ##P<0.01 or ###P<0.001 for significant upregulation by MAPK inhibitors, and *P<0.05 for significant upregulation by apigenin as compared to the relative control.

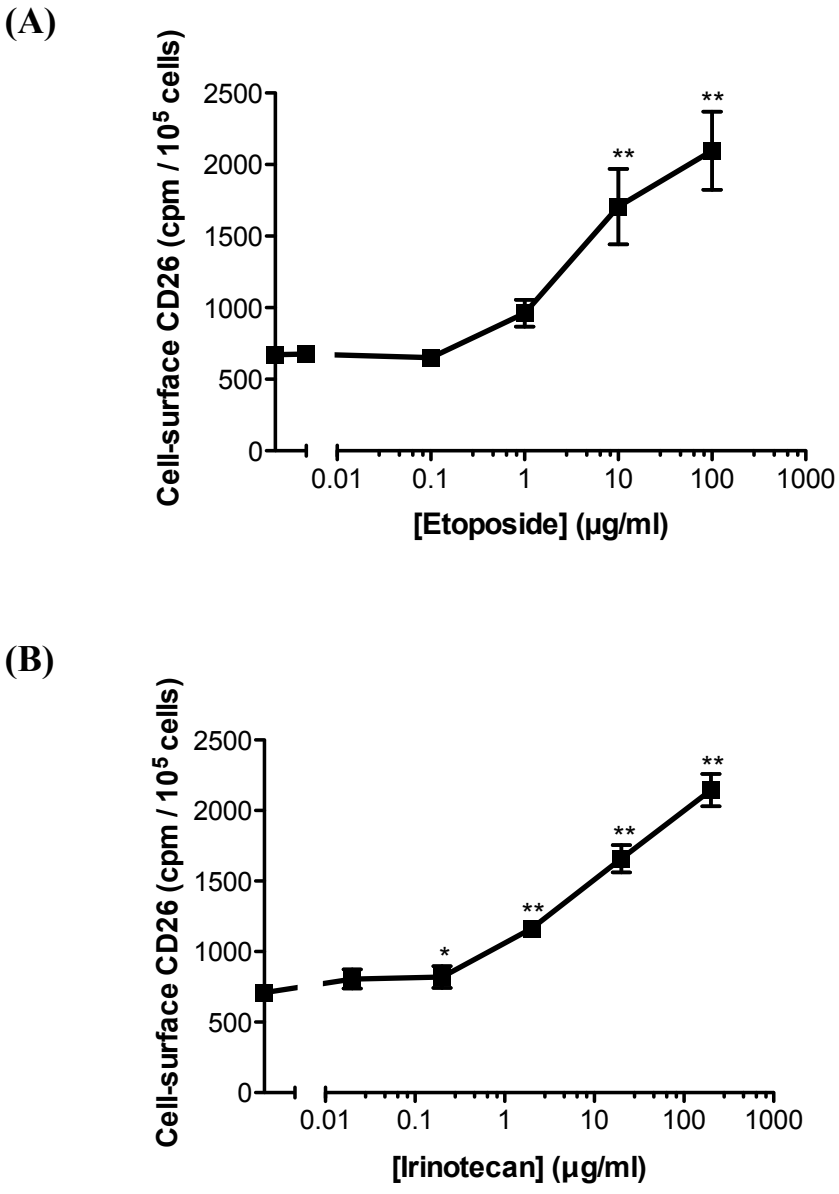


Figure 3.11 Topoisomerase I and II inhibitors enhance cell-surface CD26.

HT-29 cells were treated with **(A)** irinotecan, a topoisomerase I inhibitor or **(B)** etoposide, a topoisomerase II inhibitor at the indicated concentrations and a radioimmunoassay was performed 48h following treatment. The data are means ± SE (n=4), analyzed by one-way ANOVA followed by Dunnett's comparison test. Significance is shown as *P<0.05 and **P<0.01, indicating significant enhancement in CD26 by topoisomerase inhibitors as compared to control treated cells.

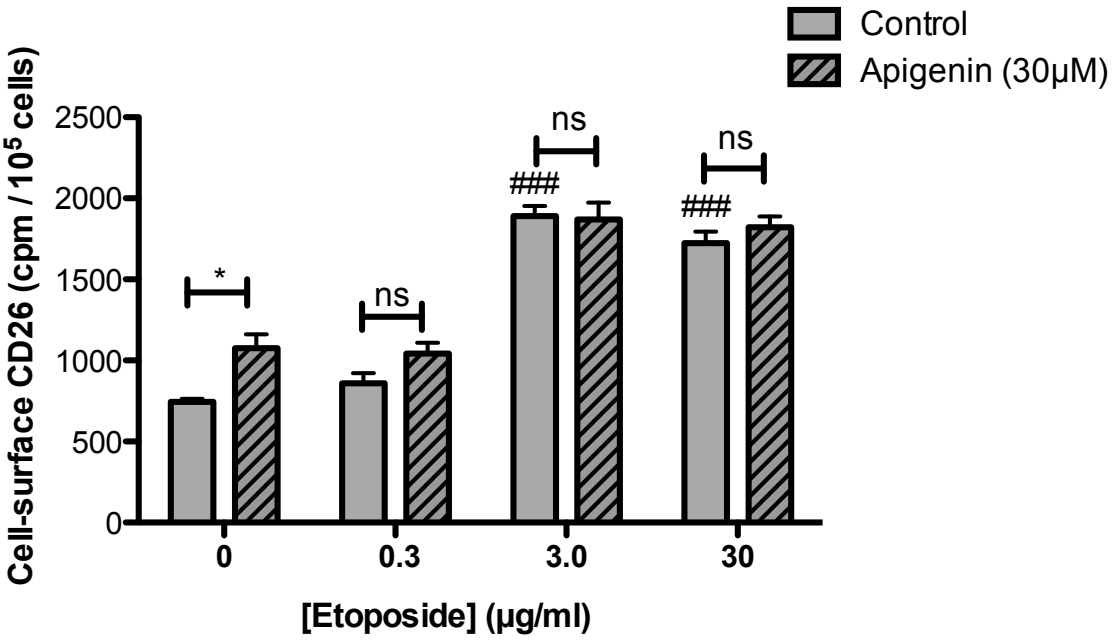


Figure 3.12 Apigenin does not enhance the action or potency of the topoisomerase II inhibitor etoposide.

HT-29 cells were treated with etoposide (0-30μg/ml) in the presence and absence of apigenin (30μM). The data are means + SE (n=4), comparisons of data were performed using two-way ANOVA with Bonferroni's comparison test. Statistical significance is shown as *P<0.05 indicating significant CD26 enhance by apigenin, and ###P<0.001 shows significant upregulation in CD26 by etoposide as compared to the relative control.

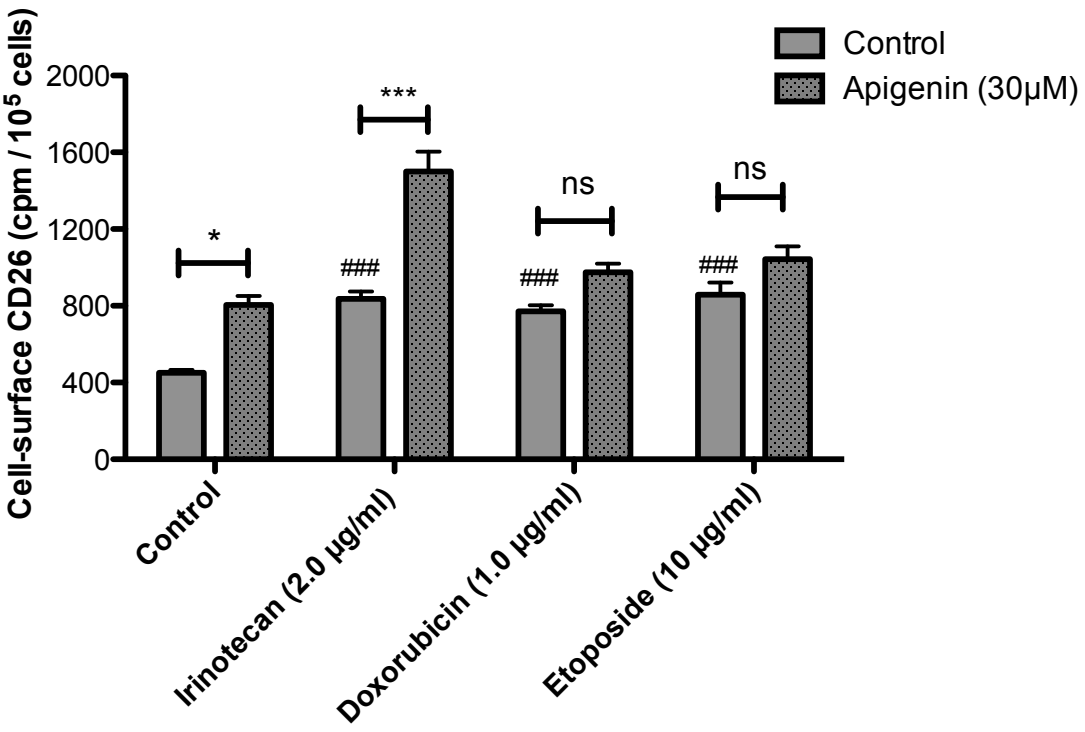
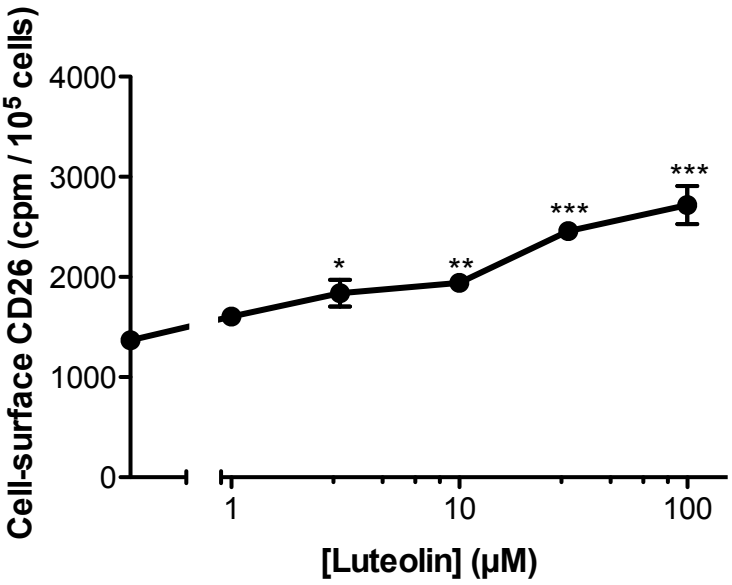


Figure 3.13 Apigenin solely enhances the effects of the topoisomerase I inhibitor irinotecan on CD26 levels.

HT-29 cells were treated with topoisomerase I or topoisomerase II inhibitors in the presence and absence of apigenin (30μM). The data are means + SE (n=4), comparisons of data were performed using two-way ANOVA with Bonferroni's comparison test. Statistical significance is shown as *P<0.05, ***P<0.01 indicating significant enhancement by apigenin, and ###P<0.001 shows significant CD26 upregulation by topoisomerase inhibitors, as compared to the relative control.

(A)


Cell-surface CD26 (cpm / 10^5 cells)
4000
3000
2000
1000
0
*
**
***
***
1
10
100
[Luteolin] (μM)

(B)

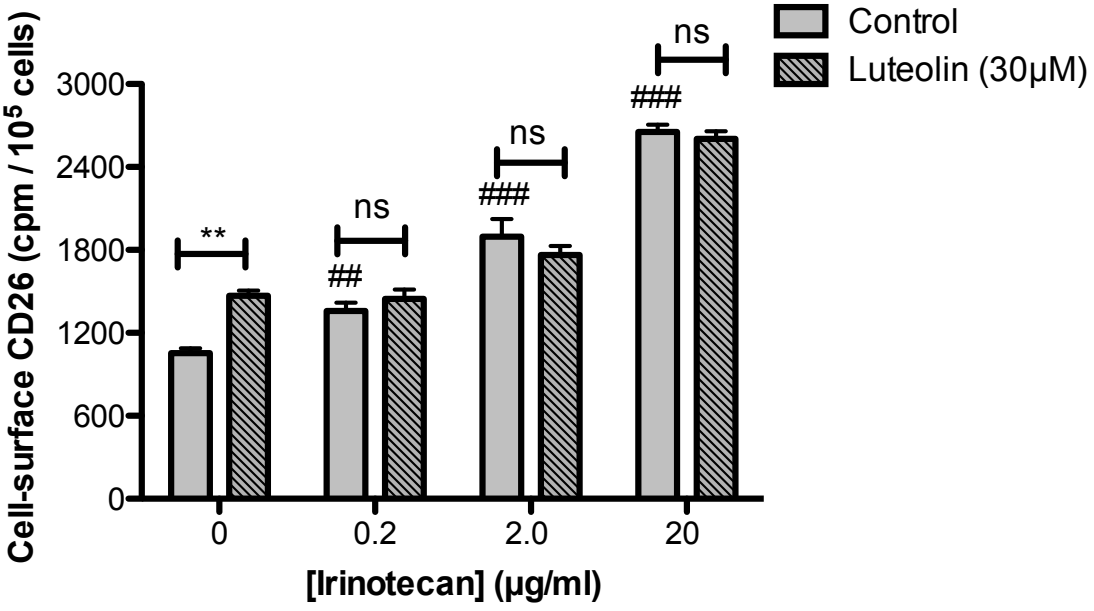

Control
Luteolin (30μM)
Cell-surface CD26 (cpm / 10^5 cells)
3000
2400
1800
1200
600
0
**
ns
##
ns
###
ns
###
0
0.2
2.0
20
[Irinotecan] (μg/ml)

Figure 3.14 The apigenin hydroxylated metabolite luteolin enhances cell-surface CD26 in a dose-dependent manner, but does not increase the potency of irinotecan.

(A) HT-29 cells were treated with the apigenin metabolite luteolin (0-100μM) and 48 h following treatment, cell-surface CD26 was assessed by radioimmunoassay. The data are means ± SE (n=4), analyzed by one-way ANOVA followed by Dunnett's comparison test. Significance is shown as *P<0.05, **P<0.01 and *** P<0.001, indicating significant upregulation in CD26 by luteolin. (**B)** Cells were treated with a dose-response of irinotecan in the presence and absence of luteolin (30μM) and CD26 was assessed 48h following treatment. The data are means + SE (n=4), comparisons of data were performed using two-way ANOVA with Bonferroni's comparison test. Significance is shown as **P<0.01, indicating significant enhancement in CD26 by luteolin and ##P<0.01 and ###P<0.001 indicates significant enhancement by irinotecan as compared to the relative control.

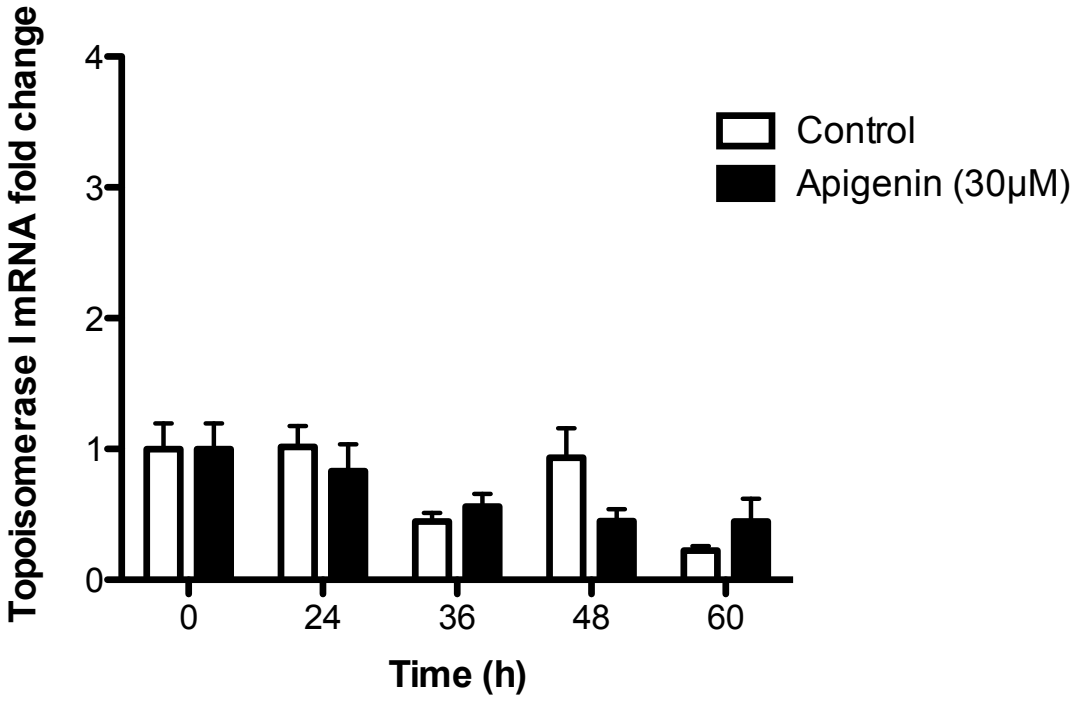
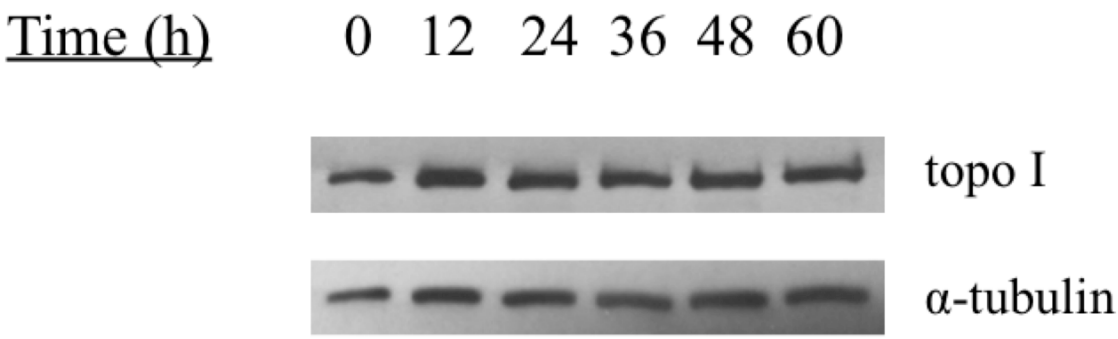


Figure 3.15 Apigenin does not affect topoisomerase I mRNA levels.

HT-29 cells were treated with apigenin (30μM) or vehicle control for the indicated times and topoisomerase I mRNA levels were quantified by q-RT-PCR. The values are means + SE (n=3), comparisons of data were performed using two-way ANOVA with Bonferroni's comparison test. Results were not statistically significant as assessed by two-way ANOVA, followed by Dunnett's comparison test as compared to control treated cells.

(A)



(B)

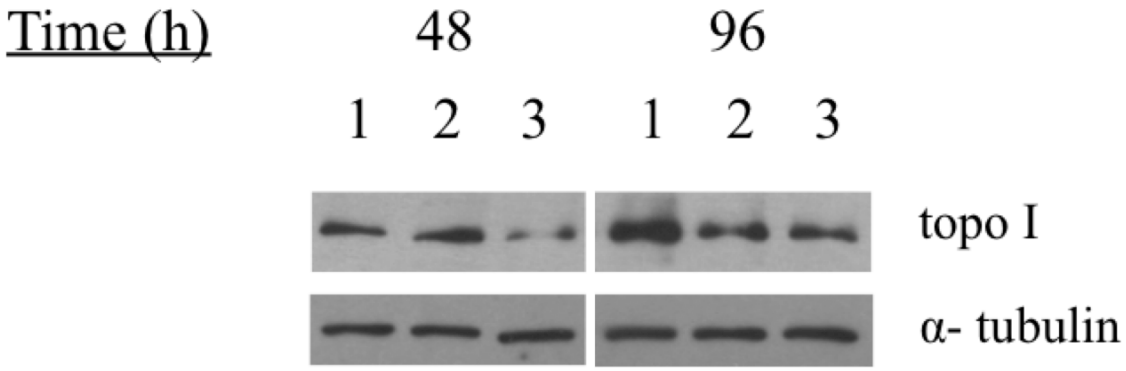


Figure 3.16 Topoisomerase I protein studies

(A) Apigenin does not affect topoisomerase protein levels. Cells were treated over a 60 h period with apigenin (60μM) and topoisomerase I protein levels were evaluated through western blot. **(B) Successful knock-down of topoisomerase I levels using an siRNA approach as indicated by western blot**. Lane 1, transfection control; lane 2, scramble siRNA; lane 3, topo I siRNA.

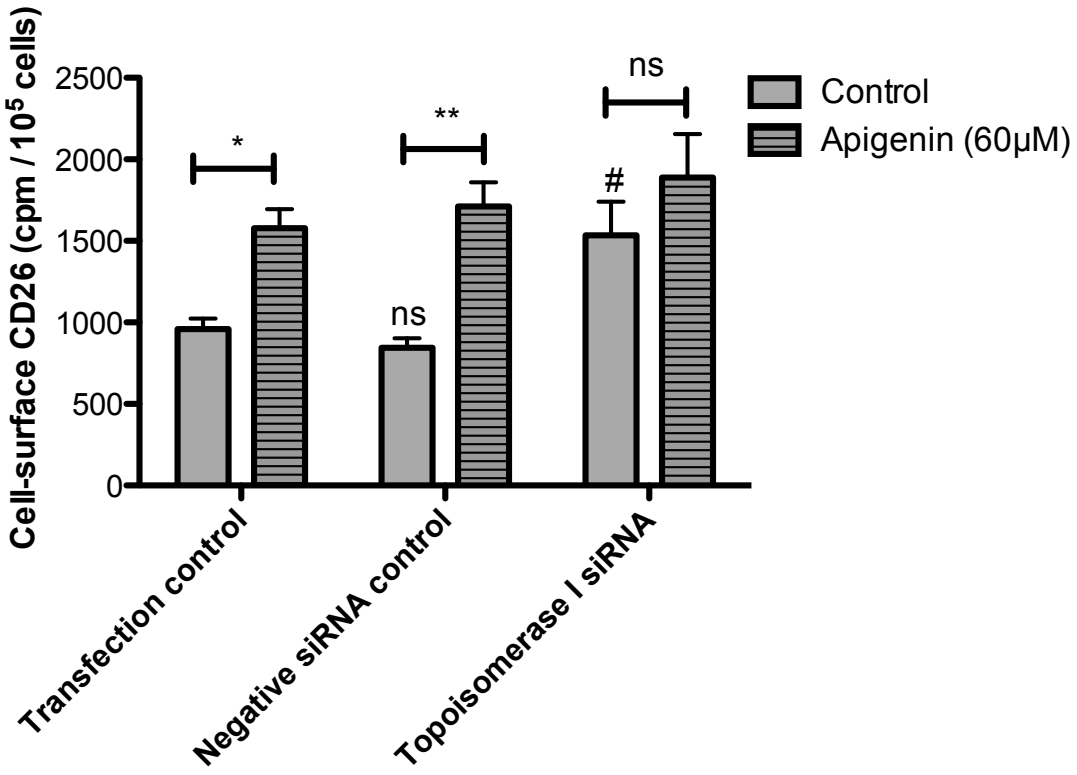


Figure 3.17 Knock-down of topoisomerase I significantly enhance cell-surface CD26 and abrogates the apigenin upregulation on CD26.

48 h following apigenin (60μM) treatment or its representative control, CD26 levels were evaluated through a radioimmunoassay. The values are means + SE (n=4), representing the average of 3 independent experiments, comparisons of data were performed using two-way ANOVA with Bonferroni's comparison test. Statistical significance is shown as #P<0.05, indicating significant enhancement by topoisomerase I knock-down, and *P<0.05 and **P<0.01, indicates significant CD26 enhancement by apigenin as compared to the relative control.

CHAPTER 4

GENERAL DISCUSSION

4.1 Preamble

The mechanism by which apigenin mediates its anti-cancer effects has only recently become clearer through careful studies *in vitro* and in pre-clinical animal models. These newer data suggest that apigenin's mechanism of action is diverse; and in the situation of cancer it affects proteins implicated in cellular proliferation, angiogenesis, apoptosis, invasion and metastasis. Findings in the last few years have also demonstrated that apigenin can interact with chemotherapeutic agents to enhance their cytotoxic actions, further linking this flavonoid to the pathways that are important in cancer regulation. The exact mechanism by which apigenin affects these diverse molecules to inhibit cancer progression is unlikely to depend on direct interaction with each component. We suggest that these findings are partly explained by apigenin's regulation of key regulators such as the multifunctional protein CD26, which is involved in both modulation of various cell behaviours and regulation of the therapeutic response to chemotherapeutic drugs.

The objectives of this concluding chapter are four-fold:

1. To discuss how apigenin might regulate CD26, both by itself and in combination with the anticancer drug irinotecan, evaluated within a model system of human CRC cells.

2. To review the technical limitations of this research.

3. To consider how our findings might relate to possible use of apigenin with human patients.

4. To summarize the overall findings of my thesis research.

4.2 Apigenin and its action on CD26 in colorectal cancer cells

4.2.1 CD26 and tumour differentiation

As outlined in the INTRODUCTION, CD26 is generally regarded as a marker of increased differentiation in epithelial enterocytes. This correlation between levels of CD26 and differentiation is also reflected in colorectal cell lines. Whereas poorly differentiated (CRC grade IV) SW480 do not express detectable levels of CD26 for example, well-differentiated (CRC grade I) HT-29 cells do (Masur, Schwartz et al. 2006). The findings of our study have been consistent with this view and other reports in the literature. In our work, Caco-2 cells expressed CD26 at the highest level, followed by HT-29, HRT-18, HCT 116, SW480 and SW620 cells. In different published reports, Caco-2 and HT-29 cells were found to express elevated levels of cell-surface CD26 while SW480 and SW680 (which is derived from a metastatic site in the same patient from who the SW480 cells were obtained) express lower levels (Chantret, Barbat et al. 1988; Bohm, Gum et al. 1995; Masur, Schwartz et al. 2006; Schneider, Huber et al. 2012). It is

interesting that levels of cell-surface CD26 vary so drastically among the different CRC cell lines. Although we have not examined factors responsible for this phenomenon, the spectrum of CD26 expression in these cells likely relates to differences in transcription. A recent study indicated that the expression pattern of CD26 is indeed regulated at the RNA level, in particular due to the fact that the promoter for the CD26 gene can be hypermethylated, an epigenetic modification affecting transcription (McGuinness and Wesley 2008). The methylation pattern of the CD26 gene appears to be regulated during the differentiation process and under normal circumstances, differentiated colonic epithelial cells at the mucosal surface express greater CD26 levels (5-fold higher), than their progenitors located in the bottom of the mucosal crypt (Dinjens, ten Kate et al. 1989; Darmoul, Rouyer-Fessard et al. 1991). This suggests that the expression level of cell-surface CD26 is correlated with the methylation status of the CD26 gene (Lea, Ibeh et al. 2010).

To definitively correlate levels of CD26 mRNA to the methylation status of the CD26 gene in our cells, this phenomenon would need to be explored. A sensitive and specific method commonly employed to examine the methylation of genes is methylation-specific PCR (MSP), which uses specific primers to distinguish methylated from unmethylated DNA present in the gene of interest, thus establishing the degree of methylation (Herman, Graff et al. 1996). Results of MSP are likely to demonstrate an inverse relationship between levels of gene methylation and CD26 mRNA expression.

The fact that high CD26 levels reflect a more differentiated state of the epithelium and of certain CRC cells, conflicts with the recent notion that CD26 expression might characterize a CRC population with stem cell characteristics (Pang, Law et al. 2010). CD26 is not conventionally viewed as a marker of stemness, which is usually associated with the cell-surface expression of other proteins such as the chemokine receptor CXCR4 and the antigen CD133 (O'Brien, Pollett et al. 2007; Jung, Rho et al. 2013). The presence of CXCR4 together with CD133 on CRC cells has been found to predict the metastatic potential of CRC cells as well as to indicate a poor prognosis in CRC patients (Zhang, Han et al. 2012). The proposition of CD26 as a marker of stemness also conflicts with several previous studies in the Blay laboratory in the context of CRC, which have consistently indicated that the expression pattern of cell-surface CD26 runs in opposition to that of the known stemness marker CXCR4. For instance, adenosine downregulates CD26 in HT-29 cells but upregulates the cell-surface expression of CXCR4 (Tan, Mujoomdar et al. 2004; Richard, Tan et al. 2006). CXCR4 upregulation and the converse downregulation of CD26 promote CRC progression in experimental models (Tan, Mujoomdar et al. 2004; Richard, Tan et al. 2006). While we can currently offer no explanation for the conflict with the work of Pang and colleagues, our results are firmly consistent with the view that CD26 expression correlates with the differentiated state, not stemness.

There is a functional link between the stemness marker CXCR4 and CD26 through CXCL12, the chemokine ligand for the CXCR4 receptor. CD26, through its DPPIV activity, regulates (inactivates) the activity of CXCL12 and as such, the

enzymatic function of CD26 interferes with the migration of CXCR4-expressing cancer cells to distant locations plentiful in CXCL12. CXCR4 is clearly implicated in tumour growth and metastasis, therefore increased levels of CD26 would oppose cancer spread through this mechanism (Matsusue, Kubo et al. 2009; Shin, Moon et al. 2012), (Fig. 4.1). Indeed, it is entirely contradictory for CD26 to also be implicated in tumour seeding alongside CXCR4 since both CXCR4 and CD26 compete for the CXCL12 chemokine (Zeelenberg, Ruuls-Van Stalle et al. 2003).

4.2.2 Apigenin and its upregulation of CD26

As fully explained in CHAPTER 2 and CHAPTER 3, apigenin upregulates CD26 in selected CRC cell lines. The apigenin upregulation of cell-surface CD26 in the Caco-2, HT-29 and HRT-18 lines as opposed to the lack of upregulation in the HCT 116, SW480 and SW620 is striking. While our current method of CD26 quantification strictly measures cell-surface levels (as this is the functional form and location of the protein) it is possible that apigenin modulates the overall pool of CD26 intracellularly in those other cell lines. As noted in early studies by Ten Kate and colleagues, the expression pattern of CD26 in CRC cells can range between cell-surface, nuclear and cytoplasmic (Ten Kate, Wijnen et al. 1985). To determine the level of whole-cell CD26 regulation by apigenin, a modified radioimmunoassay was performed using both permeabilized cells and intact cells in order to distinguish between cell-surface and intracellular CD26 levels. Our results demonstrated that the majority of CD26 in CRC cells is in fact located at the cell surface and that the apigenin upregulation occurs at the cell surface. To ensure that the

expression pattern of CD26 was consistent with that indicated by our radioimmunoassay, especially for the cell types expressing very low levels such as SW480 and SW620 cells, immunofluorescence studies were performed. Our findings suggested that SW480 and SW620 lines do indeed express very low levels of CD26, and (because the expression pattern did not significantly differ between permeabilized and non-permeabilized cells) in these cell types, CD26 was found predominantly at the cell surface while being undetectable intracellularly (data not shown). These results suggest that in all of these CRC cell lines, CD26 is predominantly expressed at the cell surface and that the apigenin regulation occurs at the cell surface.

There are other phytochemicals that can induce the expression of differentiation markers in CRC, particularly using the Caco-2 cell line, which is an established model for studies of epithelial differentiation (Lea, Ibeh et al. 2010). For instance, both the quercetin aglycone and its glucoside (25μM for 72 h) upregulate the enzymatic activities of CD26 and ALP in Caco-2 cells (Lea, Ibeh et al. 2010). Along with CD26, ALP is a brush border membrane-associated hydrolase, associated with a differentiated phenotype (Chantret, Barbat et al. 1988). Lea and colleagues also reported that apigenin (25μM) upregulated ALP enzymatic activity, to about the same extent as quercetin. The fact that apigenin upregulated ALP is further circumstantial evidence that the increase it elicits with CD26 is a response consistent with differentiation.

It is interesting to note that the quercetin glycoside exerted a biological effect as well as the aglycone, despite the bulky sugar appended to the molecule (Lea, Ibeh et al.

2010). In the situation of apigenin upregulation of CD26, we found that the glycoside A-7-O-N had no modulating effect on cell-surface CD26 and DPPIV activity, unlike the aglycone. This would indicate the need for apigenin to be deglycosylated by metabolism in the gut prior to exerting its biological function. Although Lea and colleagues reported that rutin (another quercetin glycoside) had no effect on the hydrolase enzymes in Caco-2 cells, the rutin molecule differs from the aforementioned quercetin glycoside by having a rutinose as opposed to a rhamnose sugar, respectively. This suggests that the exact structure of the sugar moiety attached to the flavonoid molecule may be an important factor for biological activity.

The positive regulation of CD26 does not appear to be a universal response shared by phytochemicals. In our studies, we found that kaempferol had no effect on cell-surface CD26. Other work reported in the research literature points to conflicting findings. A grape seed-derived procyanidin extract (GSPE) was found to downregulate DPPIV enzymatic activity (10 and 100mg/L) and CD26 mRNA (100mg/L), following a 72 h treatment in Caco-2 cells (Gonzalez-Abuin, Martinez-Micaelo et al. 2012). These results were recapitulated in an animal model, whereby the oral administration of 1g GSPE/kg and 25mg GSPE/kg to healthy female rats for 1h or 45 days, respectively, led to a decrease in CD26 gene expression and DPPIV enzyme activity in intestinal tissues (Gonzalez-Abuin, Martinez-Micaelo et al. 2012). The exact mechanism by which GSPE modulated CD26 in this study was undetermined, although regulation occurred at the level of transcription, since CD26 mRNA levels were reduced in the presence of GSPE. Clearly, the nature of a phytochemical effect on CD26 varies and is dependent upon the

exact molecule that is acting on the cell rather than being a general nutritional or antioxidative action.

4.2.3 Apigenin upregulation of CD26: Transcription or translation?

To determine if the apigenin CD26 upregulation in our CRC cells occurred at the level of transcription, we performed q-RT-PCR, while also including the positive control butyric acid (Appendix Figure A4.1). Our results demonstrate that unlike the GSPE, apigenin does not modulate CD26 mRNA. Meanwhile butyric acid enhanced CD26 mRNA by greater than 10 fold, showing that upregulation through transcription was possible. These results were exclusively obtained using HT-29 cells, thus extending this method to other CRC cell lines positively affected by apigenin would be helpful in definitively pinpointing the regulatory mechanism of apigenin on CD26.

Butyric acid itself is a four-carbon fatty acid that is naturally produced through microbial fermentation of dietary fibers, particularly in ruminants (Leonel and Alvarez-Leite 2012). Butyric acid has previously been reported to enhance CD26 mRNA levels (Bohm, Gum et al. 1995). The mechanism by which this modulation occurs is likely through butyric acid binding to butyrate responsive elements and subsequently enhancing the transcription of the CD26 gene (Bohm, Gum et al. 1995). However, enhanced CD26 transcription by butyric acid has also been reported to occur based on the fact that butyric acid has the ability to modulate histone dynamics, since it has been recognized as a histone deacetylase (HDAC) inhibitor (Davie 2003).

Previous studies in our laboratory have shown that the regulation of cell-surface CD26 in CRC cells may indeed be at the level of transcription. This occurs for example during downregulation of CD26 by the purine nucleoside adenosine, which follows from the ability of adenosine to activate a protein tyrosine phosphatase and therefore reduce ERK1/2 MAPK phosphorylation and activity (Tan, Richard et al. 2006). Considering that ERK1/2 modulates transcription factors implicated in the downregulation of CD26 such as MYC, adenosine likely modulates CD26 through the MYC pathway (Vervoorts, Luscher-Firzlaff et al. 2006). Although the mechanism by which apigenin enhances cell-surface CD26 is not fully determined in our studies, we found that CD26 enhancement by apigenin does not occur at the transcriptional level nor does apigenin modulate ERK1/2 phosphorylation (Appendix Figure A4.2). As such, the regulatory mechanism on CD26 by adenosine and apigenin is likely to be different.

4.2.5 Apigenin upregulation of CD26: A novel link to topoisomerases

Our experimental findings indicate that apigenin appears to be working in part through CK2. This is no great surprise, since apigenin is know to inhibit CK2 activity (Zhao, Ma et al. 2011; Dixit, Sharma et al. 2012). In exploring other possible targets, we have identified MAPK inhibitors as positive regulators of cell-surface CD26. This is also consistent with previous findings from our laboratory (Tan, Richard et al. 2006), and indeed flavonoids such as apigenin seem to be able to inhibit a number of kinases (Ferry, Smith et al. 1996). However, our discovery of an intriguing link with topoisomerases, is

both novel and exciting. We found that cell-surface CD26 was upregulated by both topo I and topo II inhibitors.

While studies on chemotherapeutic agents and their impact on CD26 is limited, our findings are consistent with the findings of Rashid and Basson, in which a 24 h exposure to etoposide (up to 3μM) enhanced DPPIV enzyme activity, while also reducing cellular migration in Caco-2 cells (Rashid and Basson 1996). CD26 enhancement was also reported in MOLT3 (a type of T-acute lymphoblastic leukaemia cells), following a 48 h treatment in the presence of 8-azaguanine (10 and 25μM) (Dourado, Sarmento et al. 2007). Interestingly, CD26 enhancement in the MOL3 cells was not accompanied by DPPIV activity enhancement (Dourado, Sarmento et al. 2007). This finding differs from our own experimental studies and previous findings in the laboratory, where changes associated with CD26 are invariably reflected in the multifunctional properties of the protein including DPPIV activity. Factors which could account for a lack of DPPIV enhancement in the study conducted by Dourado and colleagues could be: (i) the use of a different synthetic substrate to measure the enzymatic activity, (ii) the results for DPPIV activity were not adjusted for cellular viability, which could be a substantial factor, since 8-azaguanine substantially reduced the viability of MOLT3, (iii) T-cells are known to 'shed' CD26 in a soluble form, thus DPPIV enhancement could have been masked by this process.

While effects of doxorubicin and etoposide were at times additive with those of apigenin, at no time did we see a synergism (an increase in the relative potency of either

the chemotherapeutic drug or apigenin) on the increase in CD26 levels with the topo II inhibitors, as we have clearly observed for irinotecan. This observation appears to discount possibilities raised by studies of the ability of apigenin to influence apoptosis in cancer cells, for which it does appear to show synergism with a variety of chemotherapeutic agents. The pathway that is most implicated in that context is the generation of reactive oxygen species. Studies performed on HeLa human cervical cancer cells suggest that apigenin appears to interact synergistically with paclitaxel (Taxol®) by inhibiting the enzyme superoxide dismutase (SOD) (Xu, Xin et al. 2011). SODs are types of antioxidant enzymes that function to catalyze the conversion of superoxide into hydrogen peroxide and oxygen. Although taxanes such as paclitaxel mediate their primary cellular mechanism by stabilizing microtubules, paclitaxel also rely on ROS to induce cellular apoptosis via release of cytochrome c from the mitochondria (Meshkini and Yazdanparast 2012). This study concluded that apigenin acts as a SOD inhibitor to enhance the apoptotic function of paclitaxel (Xu, Xin et al. 2011). Doxorubicin partly relies on ROS in order to mediate its cytotoxic activity on cancer cells. In our research, combining doxorubicin with apigenin on HT-29 cells did not result in significant enhancement in cell-surface CD26, as compared to doxorubicin or apigenin alone. This suggests that apigenin does not exert its effects on CD26 through enhanced ROS production. As flavonoids such as apigenin are thought to exert many of their effects through antioxidant activities (Patel, Shukla et al. 2007; Butt and Sultan 2009; Vasquez-Garzon, Arellanes-Robledo et al. 2009), we did feel it important to further exclude an action of apigenin through ROS pathways. This was particularly so since hydrogen peroxide itself enhances cell-surface CD26 expression in a dose-dependent fashion

(Appendix Figure A4.3). In supplementary exploratory work in which we performed an NBT assay and an amplex red assay to measure superoxide and hydrogen peroxide production, respectively, we concluded that apigenin does not alter ROS levels in HT-29 cells (Appendix Figure A4.4, A and B), arguing that the regulation of CD26 by apigenin does not occur at the level of ROS. This is entirely consistent with the inability of apigenin to synergize with agents based upon ROS dependence, such as doxorubicin.

The lack of interaction between apigenin and doxorubicin with respect to CD26 modulation is somewhat surprising in view of the finding that apigenin has previously been reported to inhibit the DNA enzyme topo II (Constantinou, Mehta et al. 1995). Indeed, combining etoposide with irinotecan in the human malignant glioma (U251) cells was found to be synergistic with respect to cellular toxicity (Janss, Cnaan et al. 1998), suggesting that apigenin's interaction with irinotecan could be due to topo II inhibition. To firmly exclude the possibility that the apigenin interaction with irinotecan was caused by the fact that apigenin acted as a topo II inhibitor, we combined irinotecan with doxorubicin (Appendix Figure A4.5). With respect to CD26, this particular drug combination did not replicate the apigenin-irinotecan combination (results were neither additive or synergistic). This finding indicates that apigenin is not inhibiting topo II to lead to its interaction with the topo I inhibitor irinotecan, with respect to CD26.

4.2.6 The interaction between apigenin and irinotecan

Other flavonoids have also been reported to positively enhance the actions of irinotecan *in measures of cytotoxicity and apoptosis*. Specifically, following the

intraperitoneal administration of naringin or quercetin (100mg/kg) with irinotecan (50mg/kg) to swiss albino mice injected with Ehrlich ascites tumour cells, survival time was enhanced as compared to flavonoids or irinotecan alone. Irinotecan combined with naringin exerted greater DNA damage, while causing minimal damage to healthy cells of the kidney or the liver (Knezevic, Dikic et al. 2011). Significantly, studies on flavonoids do reveal a common theme of effects on topoisomerases, although data on topo I versus II are conflicting (Boege, Straub et al. 1996; Snyder and Gillies 2002; Lopez-Lazaro, Willmore et al. 2010; Bensasson, Zoete et al. 2011). While certain studies suggest that flavonoids inhibit topo I (Boege, Straub et al. 1996; Bensasson, Zoete et al. 2011), others suggest topo II inhibition (Azuma, Onishi et al. 1995; Constantinou, Mehta et al. 1995; Snyder and Gillies 2002) and inhibition of both DNA enzymes has also been documented (Lopez-Lazaro, Willmore et al. 2010).

Given the unique relationship between apigenin and irinotecan we investigated the possibility that an effect on topo I activity could be a further part of apigenin's ability to elevate CD26. To determine first the effect of apigenin on levels of topo I itself, we looked at the effect of apigenin on topo I mRNA and protein through q-RT-PCR and immunoblotting, respectively. Apigenin did not modulate topo I mRNA nor protein levels. The siRNA studies, in which knocking down topo I was both found to upregulate CD26 and to prevent the enhancement in CD26 due to apigenin, thus providing direct evidence that apigenin exerts its effect via topo I in spite of not altering its levels. Because of the lack of regulation of topo I levels, apigenin must instead exert its inhibitory actions on topo I activity. Topo I inhibitors such as irinotecan target the

cleavage complex through which a type 1 topoisomerase enzyme enables the process of DNA strand breakage and rotation. Indeed, other groups have successfully demonstrated through a relaxation assay that flavonoids such as apigenin interfere with topo I activity (Boege, Straub et al. 1996). We attempted to confirm this directly by performing a relaxation assay, in which the enzymatic activity of topo I can be assessed by the use of supercoiled plasmid that become relaxed in the presence of topo I enzyme activity. Despite many efforts at quantifying changes associated with topo I activity following apigenin treatment, this was not accomplished for technical reasons (due to time constraints, the relaxation assay could not be fully optimized to have the sensitivity to detect changes in topo I activity due to apigenin). However, the involvement of topo I was shown definitively using siRNA knockdown.

For completeness of the story, further optimization of the relaxation assay to confirm the findings of apigenin on topo I would be ideal. An alternate method to the relaxation assay is the cleavage complex formation assay. As mentioned previously, the cleavage complex is a structure formed by topo I itself, and topo I inhibitors subsequently bind to and stablilize the cleavage complex to prevent the religation step and generate a single-stranded break. Like irinotecan, apigenin has been reported to stabilize the topo I cleavage complex (Boege, Straub et al. 1996). Although poorly characterized, apigenin appears to stabilize the cleavage complex of topo I in a slightly different manner from that of irinotecan (Boege, Straub et al. 1996). If apigenin and irinotecan both inhibit the topo I induced cleavage complex but in a slightly different manner, this could possibly

explain the synergism between apigenin and irinotecan with respect to cell-surface CD26 enhancement.

4.3 Principal technical limitations of our study

To quantify changes in cell-surface CD26 levels and its functions (eADA binding and cellular fibronectin binding), a cell-based radioimmunoassay was performed. For this particular assay type, primary mAb against the protein of interest was employed, followed by the incubation of a radioactive secondary antibody. Although this particular method is sensitive and has been previously validated in our laboratory, this assay type only measures cell-surface protein levels, while excluding proteins located at the basal and basal lateral surfaces of cells. According to a study performed on Caco-2 cells, it would appear that over 85% of cell-surface CD26 is located at the apical surface, and as such, we may have slightly underestimated the quantitative changes seen with apigenin and chemotherapeutic agent treatment on cell-surface CD26 and its related functions (Matter, Brauchbar et al. 1990). To examine the binding capacity and the expression level of CD26 in all of its dimensions in the HT-29 CRC cells, immunofluorescence studies such as flow cytometry could have been performed. Such a sensitive and specific method has been previously employed in the Blay laboratory. Additionally, employing flow cytometry allows one to distinguish between the percent of positive cells and level of expression on positive cells, and would have eliminated the problem of using a radioactive labeled antibody, with a limited half-life. While other techniques are commonly employed in measuring changes associated with the CD26 protein (such as western blotting and immunocytochemistry (Abe, Havre et al. 2011)), the cell-based

radioimmunoassay has been chosen in our studies based on the fact that this particular assay type is sensitive, specific and rapid, as well as being able to accommodate a large volume of experimental samples.

An additional limitation of our study is the method by which CD26 binding to cellular fibronectin was quantified. Apart from CD26, a number of proteins bind cellular fibronectin. Although not exclusively, integrins bind ECM proteins such as cellular fibronectin. Like most epithelial cells, HT-29 CRC cells also express various integrin subunits, including α1,α2, α3, αV, α6, β1 and β4 (Haier, Nasralla et al. 1999; Gassmann, Kang et al. 2010). Of these specific subtypes, the αV integrin subunit is the major fibronectin receptor in HT-29 cells (von Lampe, Stallmach et al. 1993; Kemperman, Wijnands et al. 1997). Experimental assays measuring cellular adhesion in HT-29 cells found that using an antibody directed against αV, to effectively block αV integrin mediated fibronectin binding, did not completely abolish binding to fibronectin. This suggests that other proteins apart from αV integrin are implicated in cellular fibronectin binding (Ebert 1996; Kemperman, Wijnands et al. 1997; Haier, Nasralla et al. 1999). Although CD26 binds to cellular fibronectin, this particular cell-surface protein is not exclusively responsible for cellular binding to fibronectin, therefore we may have overestimated the binding capacity by flavonoids on CD26. Consequently, an appropriate mode of quantifying fibronectin binding associated with CD26, would have been to subtract αV binding from that of CD26 binding.

4.4 Limitations of apigenin in human studies

Cancer patients frequently seek alternative remedies/treatment in an attempt to eliminate undesired side effects of chemotherapy, enhance their lifestyles and perhaps to potentiate the effects of their current therapy. Although this area of research remains underdeveloped and controversial, exploring the pharmacological interactions of natural products such as apigenin in conjunction with chemotherapeutic drugs is of great importance. As such, it is interesting to consider if apigenin might be combined with current agents employed in the management of metastatic CRC, such as 5-FU, OX and irinotecan. Other experimental work has suggested that apigenin might be useful as an adjunct to chemotherapy in a variety of cancers (Turktekin, Konac et al. 2011; Xu, Xin et al. 2011; Chan, Chou et al. 2012).

4.4.1 Dietary considerations

Circulating levels of apigenin following the consumption of apigenin have been previously studied. Specifically, two human studies have looked at circulating levels of apigenin following the consumption of apigenin-rich foods such as parsley and celery leaves, at an oral intake of approximately 2g/kg of body weight. Both studies reported mean circulating levels of apigenin to be approximately 127-190nM (Meyer, Bolarinwa et al. 2006; Cao, Zhang et al. 2010). This indicates that following ingestion and metabolism of apigenin a significant concentration (0.1-0.2μM) can be found in the human plasma. A major problem of course is that this concentration is not sufficient to positively regulate cell-surface CD26 levels!

Up to this point, no human studies have investigated the benefits of apigenin in the context of cancer progression, rather, most human studies on apigenin have centered on the metabolism and tissue distribution of the flavonoid in normal subjects. There is therefore a need for human studies in order to determine the safety and efficacy of apigenin in cancer patients. In order to progress from in vitro and animal models to human studies, several issues would first need to be resolved, including; (i) establishing a consistent source of apigenin, so that the intake can be quantitatively consistent; and (ii) finding ways to improve overall absorption in order to obtain apigenin concentrations in the range to exert the anticipated biological activity.

4.4.2 The issue of metabolism

In food sources, apigenin is mostly present as a glycoside such as apigenin-O-neohesperidoside, which is more water-soluble than the aglycone apigenin. While the aglycone molecules can be absorbed by the gut through passive diffusion, the glycoside form requires enzymatic cleavage of the pentose sugar prior to intestinal absorption (Manach, Regerat et al. 1996). A major setback is that generally speaking, flavonoids are thought to be poorly absorbed due to their extensive phase II metabolism in the intestine, which primarily involves glucuronidation by uridine 5'-diphospho-glucuronosyltransferase (UGT), particularly UGT1A1 (Galijatovic, Otake et al. 2001; Ng, Wong et al. 2004). Glucuronide derivatives are less effective than the aglycone forms and they are generally excreted in the urine (Birt et al., 2001). Apart from

glucoronidation, apigenin is also subjected to other phase II metabolism such as methylation and sulfation, each of which affect its subsequent distribution and activity (Manach, Scalbert et al. 2004). Molecules such as apigenin are also likely to be pumped out of enterocytes back into the lumen of the gut, through drug transporters such as the multidrug resistance-associated protein 2 (MDRP2) (Lin, Chiba et al. 1999; Tukey and Strassburg 2000).

In this study we indeed found that the apigenin glycoside apigenin-7-O-neohesperidoside was devoid of activity with respect to cell-surface CD26 and its multiple functions. This is consistent with the fact that flavonoid glycosides (as compared to aglycones) require enzymatic cleavage in order to be able to exert biological activity. We also tested the apigenin hydroxylated metabolite luteolin, which also enhances cell-surface CD26 levels. As compared to apigenin, luteolin offers greater solubility and stability; in one study apigenin and luteolin were found to differ in their stability when in a PBS solution for a period of 6 h, the intact molecule being recovered at a percentage of 40.46% and 79.35%, respectively (Ng, Wong et al. 2004). Given that luteolin was approximately 2X more stable than apigenin, this is a positive factor from a drug discovery point of view. Luteolin is more susceptible to glucuronidation as compared to apigenin, making it less attractive to pursue in clinical studies (Ng, Wong et al. 2004).

Our findings indicate that apigenin exerts bioactivity on CD26 in selected human CRC cells, which are from a location that can be more directly accessed through the gut lumen following ingestion. However, the actual concentration in the gut after ingestion

has been reported to be only in the nanomolar range (Meyer, Bolarinwa et al. 2006; Cao, Zhang et al. 2010). To improve the apigenin concentration reaching the gut, several strategies could be employed; (i) apigenin can be given in conjunction with MK-571, a MDRP inhibitor previously shown to inhibit the glucuronidation of apigenin in Caco-2 cells (O'Leary, Day et al. 2003). This particular strategy would render the apigenin molecule less susceptible to glucuronidation, and therefore improve overall absorption and subsequent distribution (ii) the apigenin molecule could be modified directly in order to make it less susceptible to phase II metabolism such as glucuronidation. Studies looking at methylated forms of apigenin such as tricin and PMF (3',4',5',7-pentamethoxyglavone) found that the addition of methylation group(s) onto the flavone core structure not only increased potency but also enhanced stability, and the rates of glucuronidation and sulfonation were found to be greatly reduced as compared to apigenin (Cai, Sale et al. 2011).

4.4.3 The problem of the apigenin source and intake

A problem that applies to all studies of the benefits of nutraceuticals in humans is having a consistent source and being able to be sure of the dose ingested and human compliance. These factors can significantly impact the outcome and the reproducibility of the study results. The stability and concentrations of apigenin found in food sources may vary drastically according to season, supplier and method of preparation (blanching, frying, boiling and microwave) (Nielsen, Young et al. 1999; Kaiser, Carle et al. 2013). If a large intervention study was to be conducted, it would be a challenge to accurately

monitor the exact amounts of apigenin patients were ingesting on a daily basis, particularly if there were variations in compliance. This could potentially result in large sources of error, which might underestimate the benefit of apigenin in cancer patients. Rather then expecting patients to ingest copious amounts of parsley or celery leaves, the accurate consumption of a specific dose would be much simpler if a pill form of 'standard apigenin amount' was to be implemented. A human intervention study using apigenin in a concentrated pill form would achieve higher systemic concentrations, since both parsley and celery leaves contain many other phytochemicals in addition to apigenin. Additionally, ingestion of a pill form would ensure equal ingestion of apigenin, which would improve the accuracy of the study. Compliance may be complicated in patients experiencing therapy-related side effects such as nausea, fatigue, anorexia and vomiting. Overall, several obstacles need to be addressed prior to designing studies to look at the benefit of apigenin to cancer patients.

4.4 Overall summary and conclusions

In this thesis we have demonstrated that apigenin alone and in combination with chemotherapeutic agents such as irinotecan, 5-FU and OX, enhances cell-surface CD26 and its multiple functions in HT-29 human CRC cells, causing phenomena that would oppose the process of cancer metastasis. Apigenin appears to mediate this process through a network of pathways that include CK2, MAPK and topo I inhibition. CD26 through its DPPIV enzymatic activity interferes with the CXCR4:CXCL12 axis, which would impede the metastatic behavior of colon cancer cells and other cancer types. Nonetheless, the issue of timing (at which point should the patients be administered

apigenin supplements) and which patient is likely to respond to the apigenin supplements are questions that remain to be answered at the moment.

We have found the basal level of CD26 expression to be an indicator of apigenin responsiveness, in that CRC cells that expressing higher levels of CD26 are typically good responders to apigenin, but that where CD26 levels are extremely low, apigenin does not induce expression. To translate this observation to CRC patients becomes difficult based on the facts that CD26 expression is dependent on the anatomical location in the colon, and typically very heterogeneous within the tumour (Havre, Abe et al. 2008). Based upon the limited existing information it can be suggested that CRC patients with a well-differentiated tumour located on the right side of the colon are the most likely to benefit from apigenin.

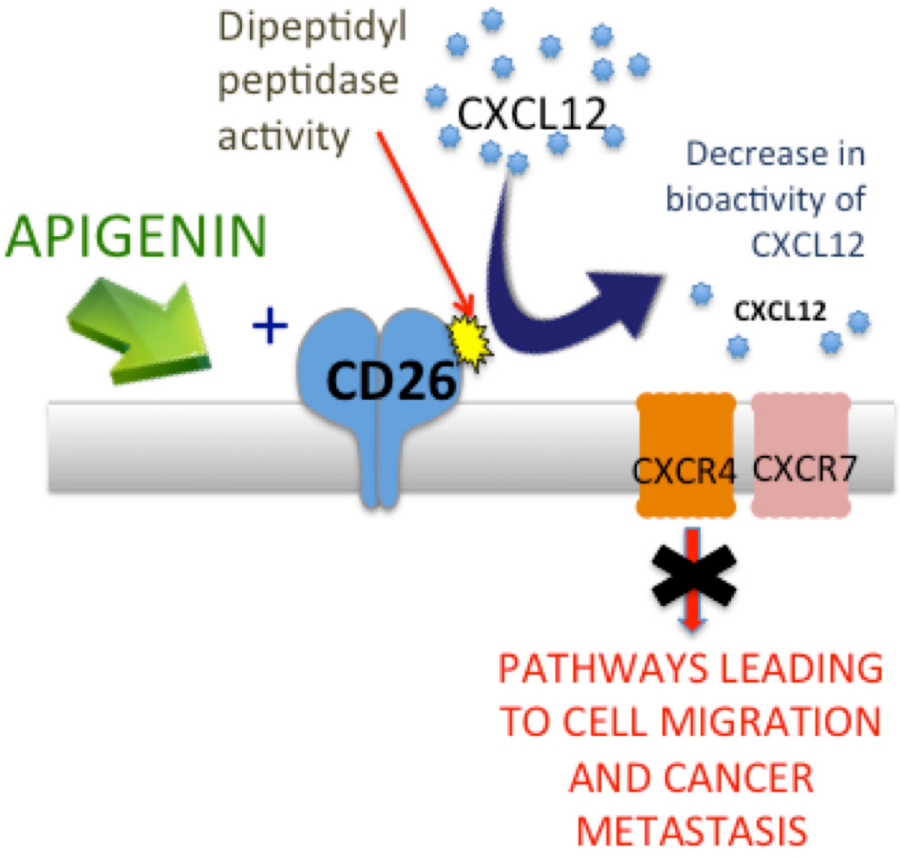


Figure 4.1 Consequences of Upregulation of CD26 by Apigenin on Cancer Cells: Increased Degradation of CXCL12 and Decreased Stimulation of CXCR4 and CXCR7.

Apigenin has been shown to upregulate the levels of CD26 at the surface of cancer cells. This increases the cell-surface activity of dipeptidyl peptidase IV, the enzyme that degrades the chemokine CXC12 to an inactive form. Reduction in the levels of intact, active CXCL12 will reduce activation of the receptors CXCR4 and CXCR7, and decrease stimulation of cell migration and cancer metastasis through CXCR4.

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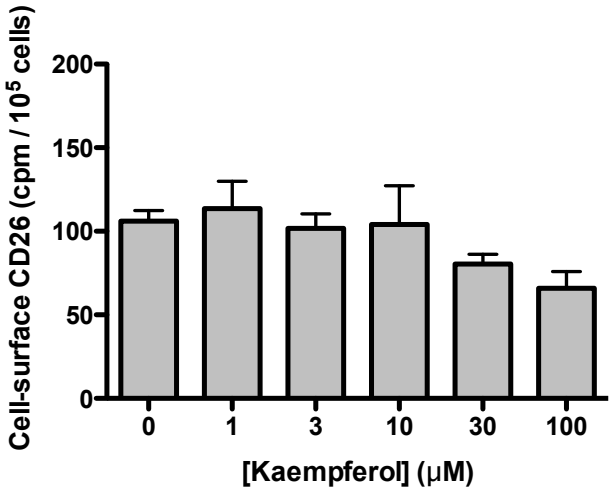
APPENDIX A

(A)



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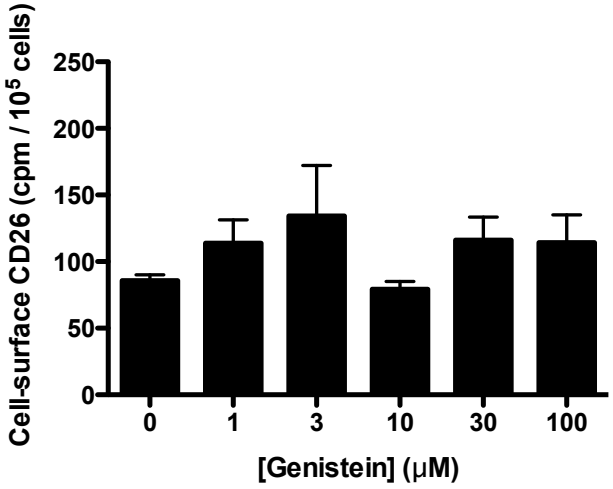


Figure A2.1 Neither kaempferol nor genistein modulate cell-surface CD26 in HRT-18 cells.

HRT-18 cells were treated with kaempferol **(A)** or genistein **(B)** at the indicated concentrations and cell-surface CD26 levels was measured by radioimmunoassay, following 48 h of treatment. The data are means + SE (n=4), although not significant, statistical analyses were performed by 1-way ANOVA with Dunnett's comparison test. The data is representative of one experiment, performed on three separate occasions.

APPENDIX B

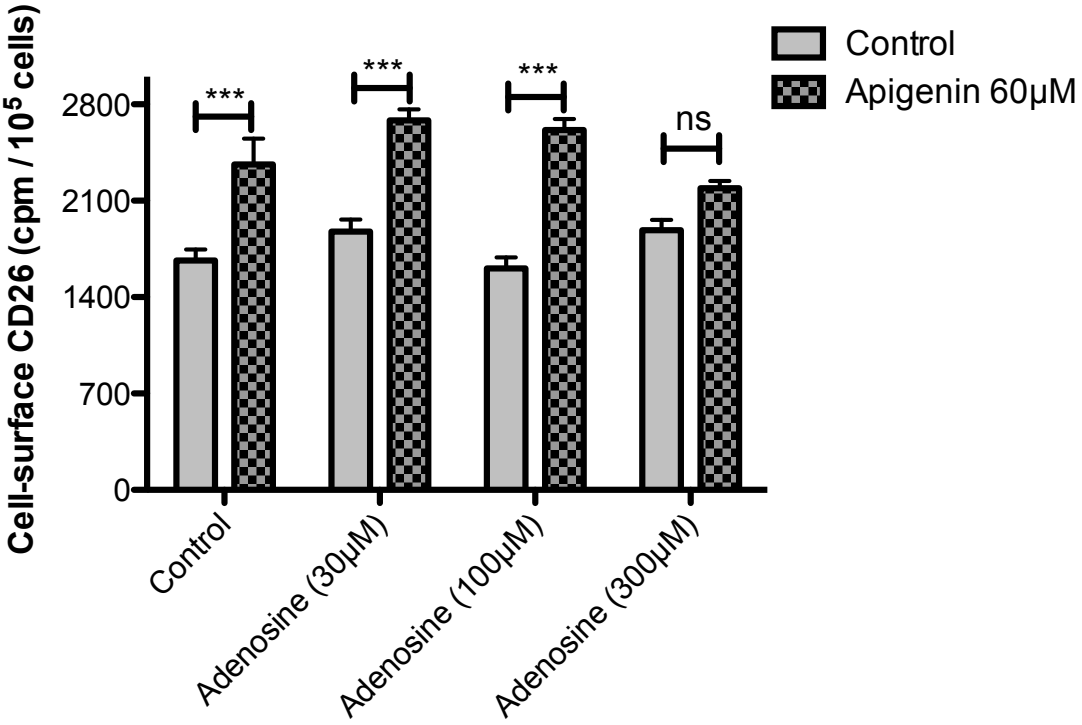


Figure A2.2 Apigenin upregulates cell-surface CD26 in the presence of adenosine.

HT-29 cells were treated with adenosine at the indicated concentrations, in the presence or absence of apigenin (60μM), where cell-surface CD26 levels was measured by radioimmunoassay following 48 h of treatment. The data are means + SE (n=4), statistical analysis was by 2-way ANOVA with Bonferroni's comparison test. Significance is shown as ***P<0.001, indicating significant upregulation by apigenin. The data is representative of one experiment, performed on three separate occasions.

APPENDIX C

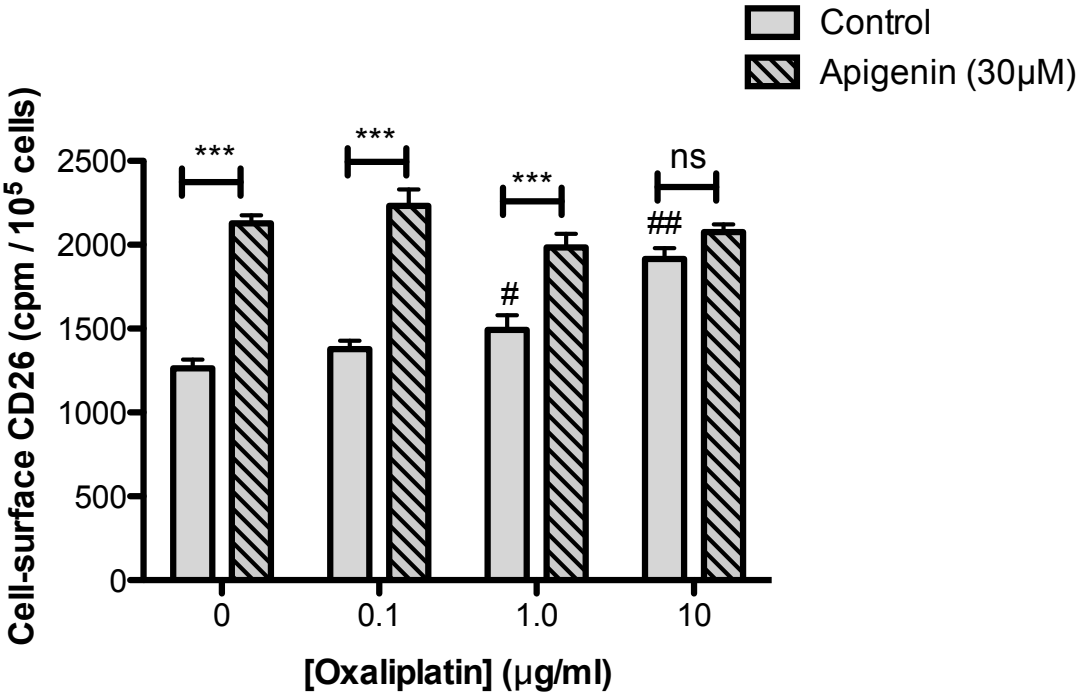


Figure A2.3 Apigenin does not enhance the potency of oxaliplatin in upregulating CD26.

HT-29 cells were treated with oxaliplatin at the indicated concentrations, in the presence or absence of apigenin (30μM), where cell-surface CD26 levels was measured by radioimmunoassay following 48 h of treatment. The data are means + SE (n=4), statistical analysis was by 2-way ANOVA with Bonferroni's comparison test. Significance is shown as # $P<0.05$ and ##$P<0.01$, indicating significant upregulation by oxaliplatin and *** $P<0.001$ show significant upregulation by apigenin. The data is representative of one experiment, performed on four separate occasions.

APPENDIX D

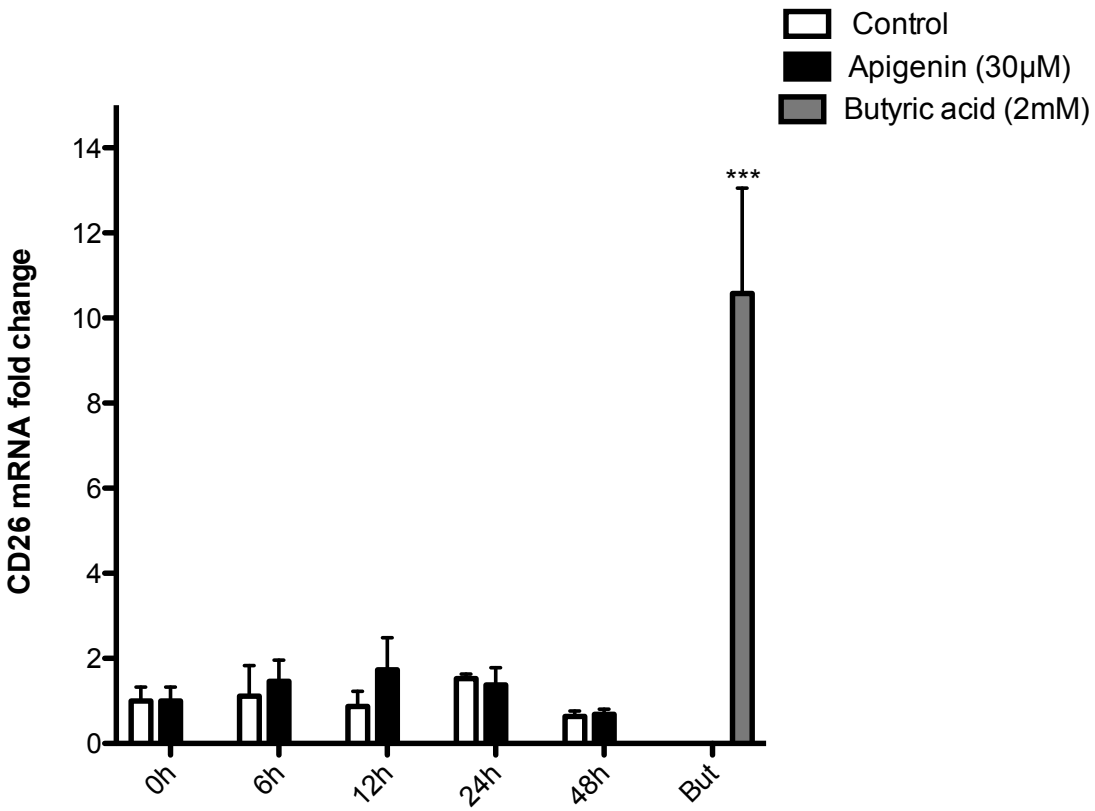


Figure A4.1 Apigenin does not affect CD26 mRNA levels.

HT-29 cells were treated with apigenin (30μM) or vehicle control for the indicated times and CD26 mRNA levels were quantified by q-RT-PCR using custom primers to amplify specific regions in the transcript with the forward 5′-CTGACAGTCGCAAAACTTACACT-3′ and reverse primer 5′-TGAGCTGTTTCCATATTCAGCAT-3′, while also including the positive control butyric acid (2mM). The data are means + SE (n=3), statistical analysis was by 2-way ANOVA with Bonferroni's comparison test. Significance is shown as ***P<0.001, indicating significant upregulation by the positive control butyric acid. The data is representative of one experiment, performed on four separate occasions.

APPENDIX E

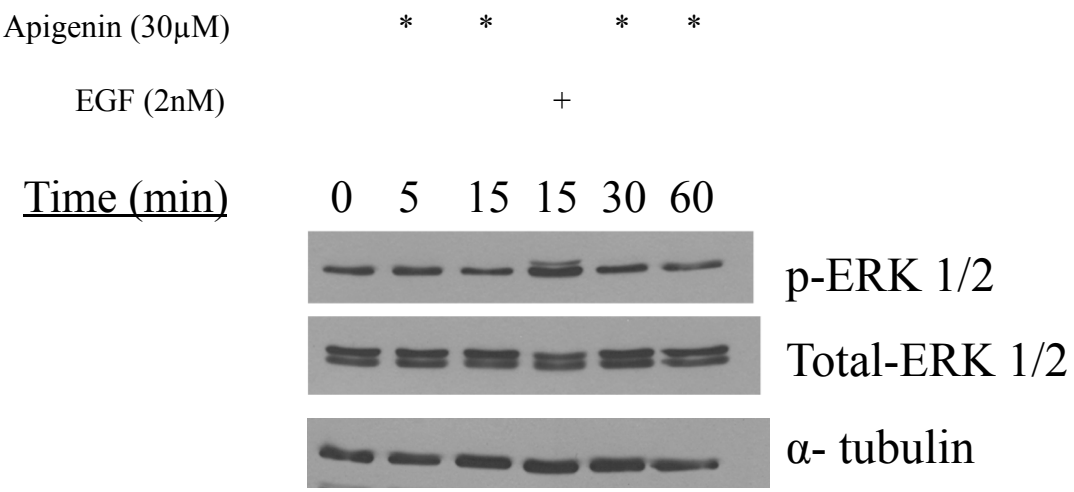


Figure A4.2 Apigenin does not modulate p-ERK 1/2 protein levels.

HT-29 cells were treated over a 60 min period in the presence of apigenin (60μM), while also including the positive control Epidermal Growth Factor (EGF) at a 2nM concentration. Along with phosphoryled ERK1/2, total ERK and alpha-tubulin were also probed to ensure accurate protein loading and were evaluated through western blot, using mouse-anti-human anti-phospho-ERK mAb and rabbit anti-human Erk1/2 (137F5) antibodies from (BD Pharmingen, San Diego, CA. USA). Probing of alpha-tubulin and secondary mAb was performed as previously described. Western blots indicate that apigenin does not affect p-ERK1/2 levels, while EGF is a potent stimulator. The data is representative of one experiment, performed on three separate occasions.

APPENDIX F

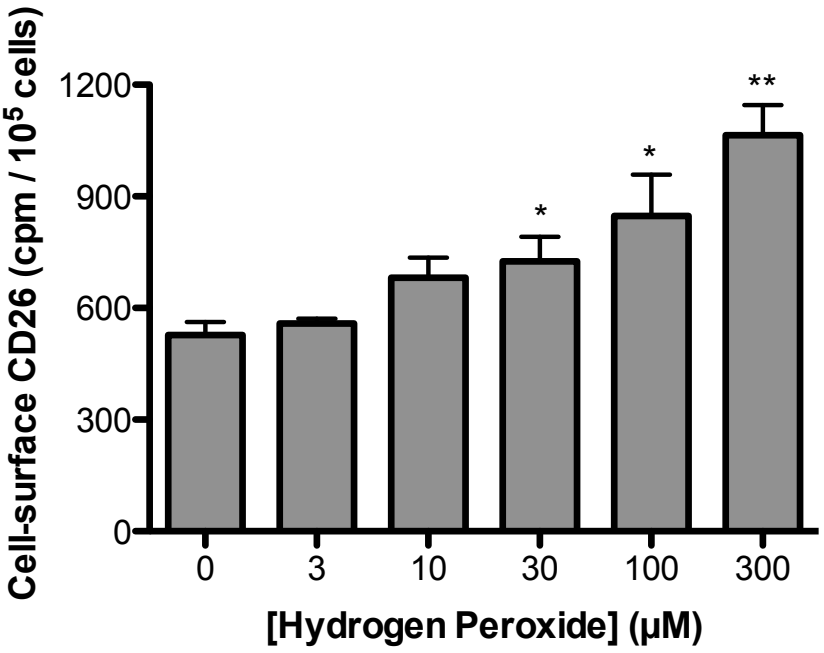
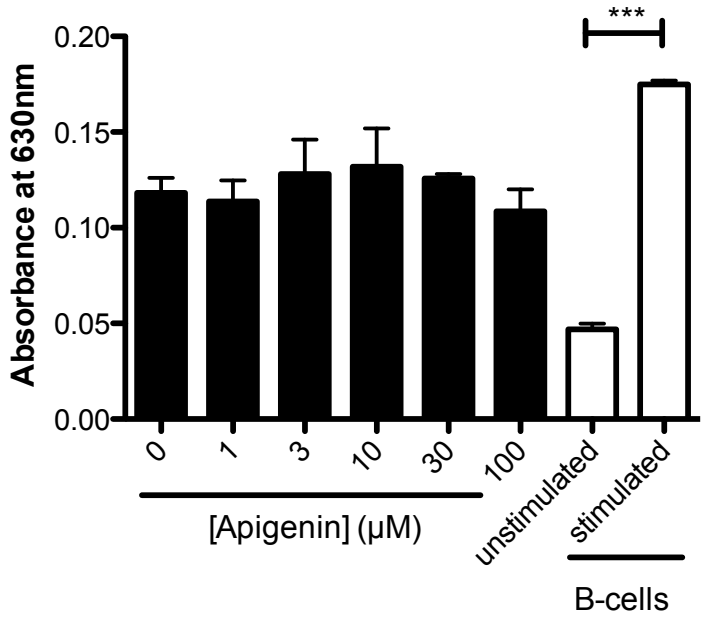


Figure A4.3 Hydrogen peroxide enhances cell-surface CD26.

HT-29 cells were treated with hydrogen peroxide at the indicated concentrations and cell-surface CD26 levels were measured by radioimmunoassay, following 48 h of treatment. The data are means + SE (n=4), statistical analysis was performed by 1-way ANOVA with Dunnett's comparison test. Significance is shown as *P<0.05 and **P<0.01, indicating significant upregulation by hydrogen peroxide. The data is representative of one experiment, performed on three separate occasions.

APPENDIX G

(A)



(B)

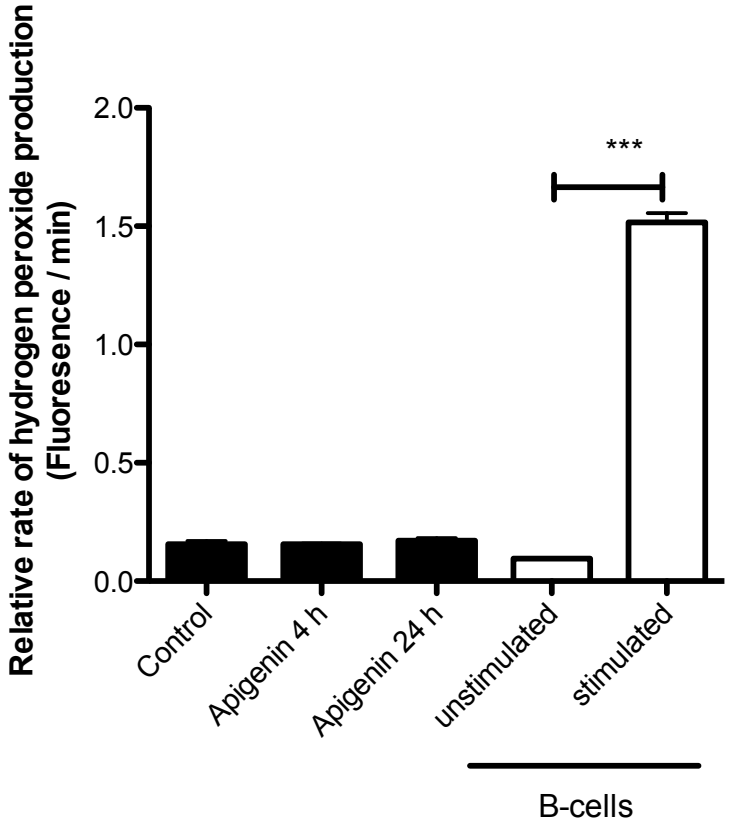


Figure A4.4 Apigenin does not produce reactive oxygen species.

(A) Superoxide anion production was measured using the colorimetric Nitro Blue Tetrazolium (NBT) assay, where the water soluble NBT is converted into a NBT crystal in the presence of superoxide anions. Briefly, HT-29 and the positive control cells known to produce superoxide anion, Epstein-barr Virus (EBV) transformed B-lymphocytes derived from a healthy individual, were seeded in a 96-well plate, and HT-29 cells were treated with apigenin (0-100µM). Transformed B-cells were stimulated with the PKC activator phorbol 12-myristate 13-acetate (100 nM). Cells subsequently received 20µl of 2% NBT and were incubated for 3 h at 37°C. Following the incubation period, cells were washed with 70% ice-cold methanol and the NBT salt was solubilized with 200µl with a combination of 2M KOH and DMSO. Absorbance was measured at the 630nm wavelength, using a spectrophotometer. In this instance, apigenin did not modulate levels of superoxide anion. HT-29 cells were analyzed by one-way ANOVA with Dunnett's comparison test. While the B-cells were analyzed independently by Student's t-test, where ***P<0.001 indicates significant enhancement in superoxide anion production in stimulated B cells as compared to unstimulated cells. The data is representative of one experiment, performed on three separate occasions.

(B) Hydrogen peroxide production was measured with the Amplex Red® fluorescence assay, where amplex red reacts with hydrogen peroxide to form the red fluorescent oxidative product resorufin. Briefly, detached HT-29 and the positive control EBV-transformed B-cells cells were plated in a 96-well plate at a density of 500,000 cells/ml in 200µl of Hanks Buffer saline solution, containing 25µM Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) and 0.005 units/ml horseradish peroxidase. Transformed B-cells were stimulated with the PKC activator phorbol 12-myristate 13-acetate (100nM) and prior to measuring the amount of hydrogen peroxide, the reaction was inhibited with 10µM diphenyleneiodonium. The amount of hydrogen peroxide produced was determined by measuring fluorescence using a microplate reader every 2 min for 30 cycles at 37°C, with excitation and emission wavelengths of 544 nm and 590 nm, respectively. In this case, apigenin did not modulate the amount of hydrogen peroxide produced. HT-29 cells were analyzed by one-way ANOVA with Dunnett's comparison test. While B-cells were analyzed independently by Student's t-test, where ***P<0.001 indicates significant enhancement in hydrogen peroxide production in stimulated B cells as compared to unstimulated cells. The data is representative of one experiment, performed on three separate occasions.

APPENDIX H

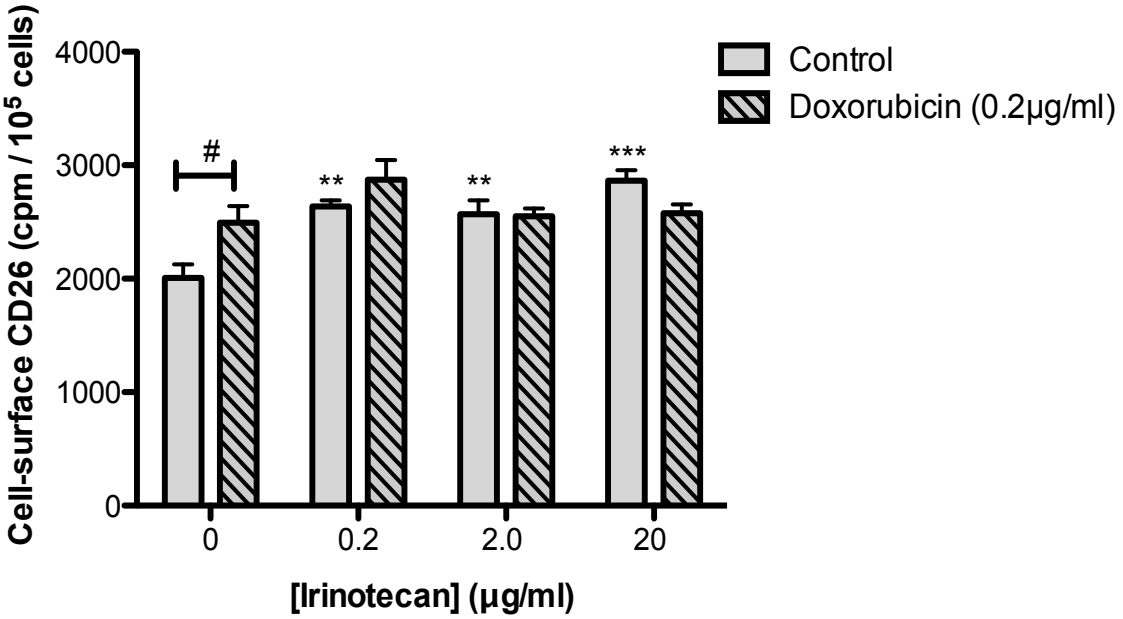


Figure A4.5 Combining the topoisomerase I inhibitor irinotecan with the topoisomerase II inhibitor doxorubicin does not replicate the apigenin-irinotecan interaction.

HT-29 cells were treated with irinotecan at the indicated concentrations, in the presence or absence of doxorubicin (0.2µg/ml), where cell-surface CD26 levels were measured by radioimmunoassay following 48 h of treatment. The data are means + SE (n=4), statistical analysis was by 2-way ANOVA with Bonferroni's comparison test. Significance is shown as ** $P<0.01$ and ***$P<0.001$, indicating significant upregulation by irinotecan and # $P<0.05$ shows significant upregulation by doxorubicin. The data is representative of one experiment, performed on three separate occasions.

APPENDIX I

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[Molecular Nutrition Food Research]
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[Apigenin and its impact on gastrointestinal cancers. Lefort, E.C. and Blay, J. Molecular Nutrition Food Research. 57(126-144) 2013. I am seeking approval to include pages 126-134]

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