COMPARATIVE PRE-CLINICAL EVALUATION OF RESVERATROL AND 3,4,5,4'-TETRAMETHOXYSTILBENE (DMU212) AS COLORECTAL CANCER CHEMOPREVENTIVE AGENTS

Thesis submitted for the degree of Doctor of Philosophy
at the University of Leicester

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June 2005

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Comparative pre-clinical evaluation of resveratrol and 3,4,5,4'-tetramethoxystilbene (DMU212) as colorectal cancer chemopreventive agents

Stewart Sale

Resveratrol is a naturally occurring polyphenol with cancer chemopreventive properties in preclinical models of carcinogenesis. Recently a variety of analogues of resveratrol have been synthesised and investigated in *in vitro* assays. One analogue, 3,4,5,4'-tetramethoxystilbene (DMU212), showed preferential growth-inhibitory and pro-apoptotic properties in transformed cells, when compared with their untransformed counterparts. Cyclooxygenase enzymes are important mechanistic targets of resveratrol. As part of this study the pharmacokinetic properties of DMU212 were compared with those of resveratrol. Both agents were also compared in terms of abilities to prevent adenoma development in the Apc^{Min+} mouse, a model of human intestinal carcinogenesis, and to interfere with the expression and activity of COX in human-derived colon cells. Pharmacokinetic data showed that resveratrol achieved significantly higher levels than DMU212 systemically, whilst DMU212 exhibited superior availability in the gastrointestinal tract. Apc^{Min+} mice received either compound with the diet (0.05, 0.2 or 0.5 %), and adenomas were counted after animals were killed. Resveratrol and DMU212 at 0.2 % decreased adenoma load by 27 and 24 %, respectively. DMU212 is a better inhibitor of proliferation of human colon cancer cells than resveratrol. Incubation of HCA-7 colon cancer cells for 24-96 h with either compound (1-50 μM) decreased PGE_2 production, but only resveratrol decreased COX-2 protein expression. Whilst resveratrol inhibited enzyme activity in purified COX preparations, DMU 212 failed to do so. The results suggest that chemical alteration of the resveratrol molecule to generate DMU212 does not alter its ability to decrease adenoma number in Apc^{Min+} mice or to interfere with PGE_2 generation in cells. However, only resveratrol directly inhibits COX activity and expression. These results further our understanding of the *in vitro* and *in vivo* pharmacology and cancer chemopreventive activity of resveratrol and DMU212, and provide evidence supporting the exploration of analogues of resveratrol as cancer chemopreventive agents.

Acknowledgements

I would like to take this opportunity to say how indebted I am to both my supervisors, Prof **Andreas Gescher** and Prof **William Steward**, whose support and assistance has been inspiring throughout my PhD and certainly made it very enjoyable and it has been a real pleasure to work along side both of them.

I would also like to thank the whole of the Biomedical Sciences staff, especially **Colin, Alice** and **Donna**, for the total care and pampering of my PhD pets and also to Dr **Richard Verschoyle** for his assistance during experiments. Your assistance has been excellent and is very much appreciated. I would also like to thank members of the Pathology staff, notably Dr **Peter Greaves** and Mrs **Jenny Edwards**, for their assistance in histological matters.

My PhD though wouldn't have been what it was without the continual lab banter that went on with my colleagues of the department. Special thanks must go to all the medic boys-you know who you are!! (For those that don't, it was **Giuseppe** (spelt right I hope) **Garcea, Sanjeev** "never told a lie in my life" **Pathak** and **Hari** "Generally pleasant chap" **Ratan**). Also I would like to mention all the old skool of PhD kids **(Sarah Perkins, Sarah Donald, Lynne Howells, Paul** "Chief Faff" **Atherfold**, and **Toryn** "Podman" **Poolman)** that have since left. These guys brought many laughs and certainly great nights out, many, of which, me and a certain other character, who shall stay unnamed till later rarely remember. All you guys have made my PhD very memorable.

Special thanks must go to my main man in business and general trouble side kick **Mark** "Always well behaved" **Anderton**. You have been a sound friend throughout my PhD as well as some others previously mentioned **(Garcea**, and **Pathak)** and for that I am very grateful. One thing I have noticed since your departure is the fact I tend to remember more from nights out and early mornings (who will forget the World Cup with early morning lager shandy sessions in the local), which highlights the bad influence you had on me!!!

I also am truly indebted to a current work colleague of mine, Dr. **Richard** "Beer Buddy" **Tunstall** whose excellent knowledge and understanding of science has been of utmost importance to me when seeking advice and assistance. Thank you so much. And his understanding of real ale, ciders, single malt whiskies and hi-fi equipment has provided many relaxing evenings.

Although you guys have been there for me during my PhD I must say a special thank you to my parents and brother for their continued support (especially financial!!) not only during my PhD but through my whole life, I hope I have made you proud. But most of all I would like to thank my lovely fiancée, **Jane**, who has been there through all the ups and downs of the PhD roller coaster. She has been my rock and I hope one day I can provide her will all the support and love that she has shown me.

Thank you all. I am truly grateful.

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Abbreviations

ACF	Aberrant crypt foci
ANOVA	Analysis of variance
AOM	Azoxymethane
AP-1	Activator protein 1
Apc	Adenomatous polyposis coli
AUC	Area under the curve
BSA	Bovine serum albumin
bw	Body weight
COX	Cyclooxygenase
CRC	Colorectal cancer
CV	Coefficient of variance
CYP	Cytochrome P450
Caspase	Cysteinyl aspartate-specific proteinase
DCC	Deleted in colorectal cancer (gene)
DMBA	7,12-dimethylbenzanthracene
DMEM	Dulbecco's minimum essential medium
DMSO	Dimethyl sulphoxide
DMU	De Montfort University
ECL	Enhance chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin gallate
EGFR	Epidermal growth factor receptor
ERK	Extracellular regulated kinase
FAP	Familial adenomatous polyposis
FasL	Fas ligand
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GSK-3β	Glycogen synthase kinase 3β
Hi-QC	High quality control
HPLC	High performance liquid chromatography
HRP	Horse radish peroxidase

IC_{50}	Dose at which the number of live cells is 50% of that in untreated cultures
IKK	IκB kinase
IS	Internal standard
iNOS	Inducible nitric oxide synthase
LC-MS	Liquid chromatography/ mass spectrometry
LLOQ	Lower limit of quantification
LOD	Limit of detection
LOH	Loss of heterozygosity
Lo-QC	Lower quality control
Min/+	Multiple intestinal neoplasis
NFκB	Nuclear factor κB
NO	Nitric oxide
NOS	Nitric oxide synthase
NSAIDs	Non steroidal anti-inflammatory drug
PAR	Peak area ratio
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pd	Per day
PG	Prostaglandin
PI	Propidium iodide
PI (3) K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
QC	Quality control
ROS	Reactive oxygen species
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
TBE	Tris borate EDTA
tBID	Truncated bid
TBS	Tris buffered saline
TBST	Tris buffered saline tween

TCF	T-cell factor
TE	Trypsin EDTA
TEMED	N, N, N', N,'-tetramethylethylenediamine
TNF	Tumour necrosis factor
VEGF	Vascular epithelial growth factor

CHAPTER 1

INTRODUCTION

This thesis describes research into the pharmacokinetics, metabolism, efficacy and mechanism of action of resveratrol and DMU212, both stilbene analogues. The following introduction lays out the background relevant to these compounds. It describes concepts pertinent to colorectal carcinogenesis and chemoprevention, and introduces the chemistry and pharmacology of polyphenols derived from stilbenes.

1.1 Carcinogenesis

Carcinogenesis occurs via multiple distinguishable events of molecular and cellular alterations, which can be separated into three distinct phases: initiation, promotion and progression (Figure 1.1.1) (Farber, 1968). Initiation, is the primary step of carcinogenesis. In genotoxic carcinogenesis this involves a carcinogen directly binding to the target DNA and inducing DNA damage. Unless DNA repair mechanisms are activated or the cell undergoes apoptosis, the carcinogen can go on to cause irreversible genetic mutations. The resulting somatic mutation in the damaged cell can be reproduced during mitosis, giving rise to a clone of mutated cells (Surh, 1999). Apart from the genotoxic carcinogenesis explained above there are other known routes for carcinogenesis initiation, for instance viral carcinogenesis and initiation by non-genotoxic carcinogens. In these cases the blocking agents shown in Figure 1.1.1 may prove less effective than the suppressing agents as preventative agents. Promotion is a process that takes place over many years and involves the expansion of the damaged cells to form an actively proliferating multi-cellular premalignant tumour cell population (Surh, 1999). Progression is much quicker and is the irreversible process that produces a new clone of tumour cells with increased proliferative potential, invasiveness, and metastatic capacity and also additional mutations (Surh, 1999) (Figure 1.1.1.). These alterations lead to the final stage of this process which is a malignant neoplasm.

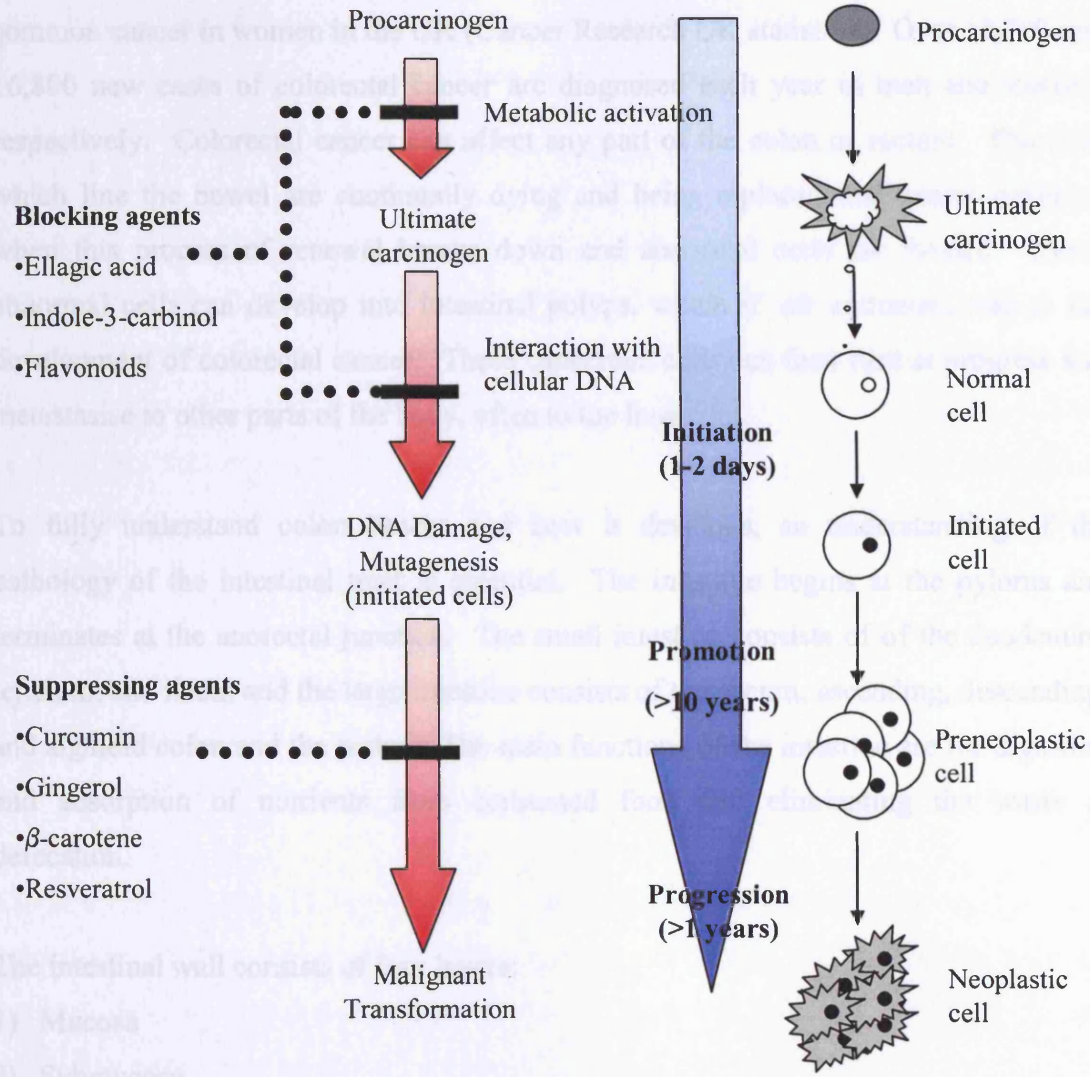


Figure 1.1.1. Schematic representation of the three stage process of carcinogenesis and also the classification of chemopreventive phytochemicals based on their mechanism of action. Adapted from (Surh, 1999).

1.2 Colon Cancer

Colorectal cancer is the third most common cancer in men, and the second most common cancer in women in the UK (Cancer Research UK statistics). Over 18,700 and 16,800 new cases of colorectal cancer are diagnosed each year in men and women, respectively. Colorectal cancer can affect any part of the colon or rectum. The cells which line the bowel are continually dying and being replaced and cancer develops when this process of renewal breaks down and abnormal cells are formed. These abnormal cells can develop into intestinal polyps, which if left untreated, lead to the development of colorectal cancer. These cancerous cells can then further progress and metastasise to other parts of the body, often to the liver.

To fully understand colon cancer and how it develops, an understanding of the pathology of the intestinal tract is essential. The intestine begins at the pylorus and terminates at the anorectal junction. The small intestine consists of of the duodenum, jejunum, and ileum and the large intestine consists of the cecum, ascending, descending, and sigmoid colon and the rectum. The main functions of the intestine are the digestion and absorption of nutrients from consumed food and eliminating the waste at defecation.

The intestinal wall consists of four layers:

1) Mucosa
2) Submucosa
3) Muscularis externa
4) Serosa

The crypts (invaginations of the epithelium), within the mucosal layer, effectively increase the surface area of the intestine. The small number of stem cells, which are located at the base of each crypt, form by asymmetric division of four cellular types:

1) columnar absorptive cells
2) goblet (mucus-secreting) cells
3) neuroepithial cells
4) paneth cells

These forementioned cells are primarily located in the upper two thirds of the crypts and are continually shifting upwards until they are eventually shed into the lumen by an apoptotic process. This mechanism of epithelial cell renewal takes between 3-6 days, however, the rate of replacement of these cells is equal to the rate of epithelial cell loss. It is only when this process becomes unbalanced and increases, favouring replacement of cells that intestinal tumours develop.

The initial appearances of neoplasia are the aberrant crypt foci (ACF), which can be visualised by methylene blue staining or microscopy. ACF usually include few crypts and can be composed of either normal (nondysplastic) or abnormal (dysplastic) cells. However, it is only the dysplastic cells that tend to progress to become a polyp, defined as a benign tumour mass that projects into the lumen from the intestinal epithelium. These adenomatous polyps can be divided into three subtypes based on their epithelial architecture

1) Tubular adenomas: tubular glands
2) Villous adenomas: villous projections
3) Tubulovillous adenoma: a mixture of tubular glands and villous projections.

Familial Adenomatous Polyposis is the achetypal example of an adenomatous polyposis syndrome. In the region of 500-2500 colonic adenomas, which line the mucosal surface, develop between the ages of 10-20 years old in those affected. Colon carcinoma (malignant epithelial neoplasm) emerge in 100% of cases. The mean age for carcinoma development is 35-40 years.

Geographically, the incidence of colorectal cancer varies immensely. The incidence is highest in Australia and New Zealand, North America, Western Europe and in Japan, where numbers exceed 40 cases per 100,000 of the population in males and 25-30 cases per 100,000 in females (Parkin et al, 1999). These figures are much higher than those seen in Africa, Central and South America, and South Central Asia, where the incidence among males is between 5-10 per 100,000 population, and lower still in their female counterparts (Parkin et al, 1999). Efforts to reduce the mortality from this disease are primarily focussed on early detection of the precursor lesions, polyps, and early diagnosis of established cancers, as advanced colorectal cancer, when metastases have

formed, is incurable (Krishnan et al, 2000). Hence, chemopreventive intervention studies have been undertaken to delay or inhibit the development of colorectal cancer.

1.3 Chemoprevention and colon cancer

Cancer chemoprevention is a recent and rapidly expanding area of oncology. The term "chemoprevention" was first used by Sporn and Newton, in 1979, who defined it as "the prevention of cancer by the use of pharmacological agents that inhibit or reverse the process of carcinogenesis" (Sporn & Newton, 1979) a notion also supported by others (Wattenburg, 1985). Carcinogenesis is a complex and multi-stage process that involves the interactions between genes and environmental factors that ultimately affect cell proliferation and death (Decensi & Costa, 2000).

Several naturally occurring and synthetic agents have been shown to have cancer chemopreventive properties in a variety of bioassay systems and animal models (Aziz et al., 2003). To be an effective chemopreventive agent certain criteria need to be fulfilled: (i), little or no toxic, or adverse effects in normal and healthy cells; (ii), high efficacy against target sites; (iii), possibility of oral consumption; (iv), known mechanisms of action; (v), low cost; and (vi), acceptance by humans (Aziz et al., 2003).

As cancer is a multi-step process, it offers numerous potential targets for chemopreventive agents to intervene in the progression of the disease. The stage of the carcinogenic process on which these compounds act, determines how they are classified. Those compounds that interfere with initiation are referred to as "blocking agents" and those that inhibit and prevent promotion and progression are known as "suppressing agents" (Wattenburg, 1985). Figure 1.1.1, highlights some of the diet derived chemopreventive agents identified to date.

Colorectal cancer development is known to be associated with increased genetic mutations in the mucosal cells, activation of tumour promoting genes and inactivation of genes which suppress tumour formation (Kerr et al., 2002). The role of several risk factors in the aetiology of colorectal cancer is now becoming apparent (Fearon & Vogelstein, 1990) and the genetic events involved in the susceptibility to this disease are being rapidly uncovered (Kerr et al., 2002). Few specific risk factors, other than those of dietary origin, have been identified for colorectal cancer. Inflammatory bowel

diseases and familial adenomatous polyposis syndromes constitute an increased risk of colorectal cancer in affected individuals, but these risk factors account for only a limited percentage of the overall incidence of colorectal cancer (Kerr et al., 2002)

1.3.1 Mechanisms of colon carcinogenesis

The success of chemoprevention studies is dependant on the mechanistic understanding of carcinogenesis at the molecular, cellular and tissue level (Sporn & Suh, 2002). In 1990, Fearon and Vogelstein proposed a model of successive genetic changes that occur in the development of colorectal cancer (Fearon & Vogelstein, 1990) (Figure 1.3.1.). The model put forward the idea that numerous genes were involved in the development of colorectal cancer, primarily APC (adenomatous polyposis coli), K-ras, Smad 2 and Smad 4, and p53 (Fearon & Vogelstein, 1990). The original model emphasised that mutations in these genes were essential for the development of colorectal cancer, but did not specify the exact sequence of alterations that takes place. However, 10 years on, light has been shed on some of the functions of these key genes (Arends, 2000).

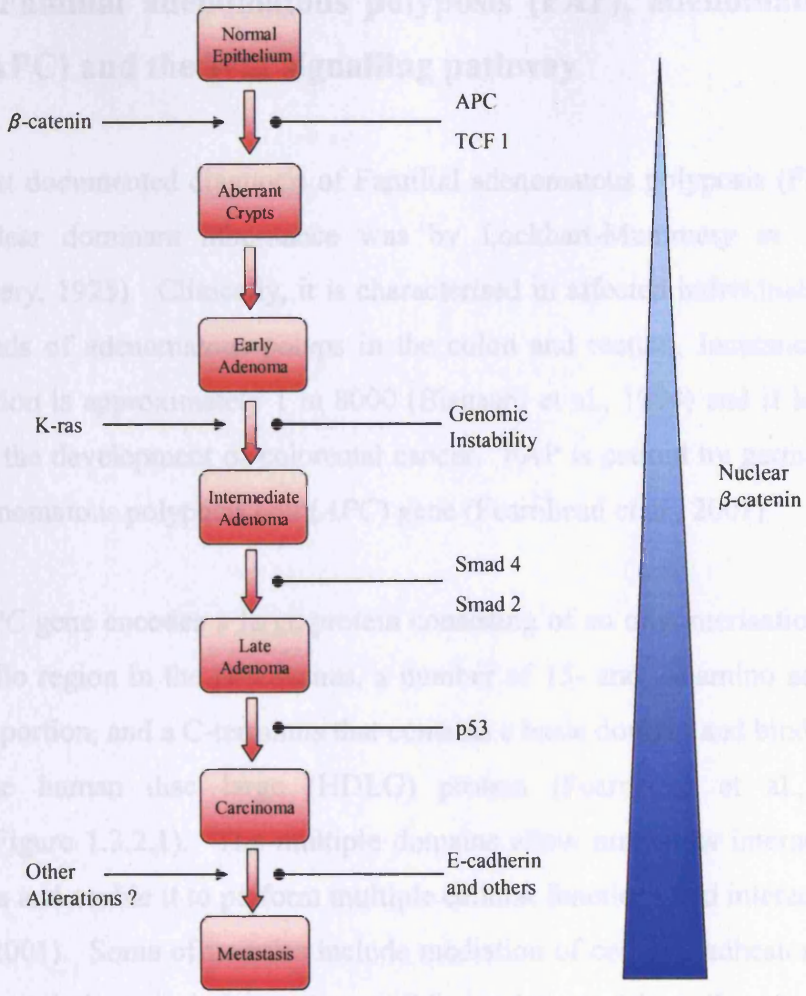


Figure 1.3.1. The adenoma-carcinoma sequence of colorectal cancer. A mutation in APC or β-catenin genes activates the Wnt signalling pathway, triggering tumour formation. Ensuing progression towards malignancy is complemented by sequential mutations in *K-ras*, deletion of chromosome 18q affecting genes encoding *Smad 2* and *Smad 4*, *p53* and genes involved in tumour invasiveness such as E-cadherin. Tumour progression is accompanied with increasing levels of nuclear β-catenin. Adapted from (Giles et al., 2003).

Mutations in the adenomatous polyposis coli (*APC*) gene are found in the majority of colorectal adenomas (Arends, 2000). *Per se*, it is one of the earliest and most commonly mutated genes in colorectal cancer (Kinzler & Vogelstein, 1996). There is also evidence to suggest that it is a mutation in the *K-ras* gene that is one of the first genetic events of colorectal cancer (Arends, 2000). Investigations have shown *K-ras* point mutations in colonic mucosa, as well as in aberrant crypt foci (ACF) (Pretlow et al., 1993), and that their frequency decreases during progression.

1.3.2 Familial adenomatous polyposis (FAP), adenomatous polyposis coli (APC) and the Wnt signalling pathway

The first documented diagnosis of Familial adenomatous polyposis (FAP) as a disease with clear dominant inheritance was by Lockhart-Mummery in 1925 (Lockhart-Mummery, 1925). Clinically, it is characterised in affected individuals by hundreds to thousands of adenomatous polyps in the colon and rectum. Incidence of FAP in the population is approximately 1 in 8000 (Bisgaard et al., 1994) and if left untreated will lead to the development of colorectal cancer. FAP is caused by germline mutations in the adenomatous polyposis coli (*APC*) gene (Fearnhead et al., 2001).

The *APC* gene encodes a large protein consisting of an oligomerisation domain and an armadillo region in the N-terminus, a number of 15- and 20-amino acid repeats in the central portion, and a C-terminus that contains a basic domain and binding sites for EB1 and the human disc large (HDLG) protein (Fearnhead et al., 2001; Polakis, 2000)(Figure 1.3.2.1). The multiple domains allow numerous interactions with other proteins and enable it to perform multiple cellular functions and interactions (Fearnhead et al., 2001). Some of its roles include mediation of cell-cell adhesion, stabilization of the microtubular cytoskeleton, the possible regulation of the cell cycle and apoptosis. It plays an essential role in signal transduction in the Wnt-signalling pathway (Fearnhead et al., 2001).

Two of the most important regions of the APC protein are the 15- and 20-amino acid repeats, as they mediate the binding and down-regulation of β-catenin, respectively. The 15-amino acid repeats are retained in the majority of mutant APC proteins as both wild type and mutant APC proteins can bind β-catenin (Fearnhead et al., 2001; Su et al., 1993). The majority of truncated APC proteins lack most or all of the 20-amino acid repeats, which suggests that this region may be a target for elimination during tumourigenesis (Polakis, 1997).

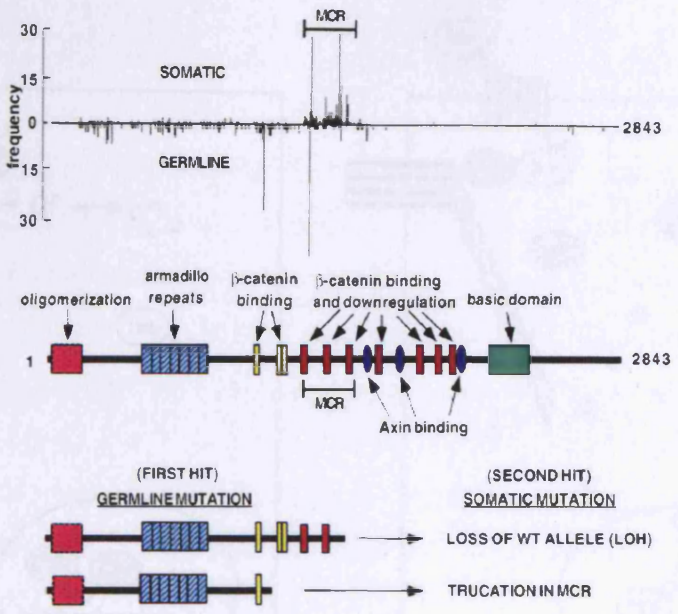


Figure 1.3.2.1. *APC* mutations in colorectal cancer. A collection of germline and somatic mutations in the *APC* gene illustrating selection for mutations in the mutation cluster region (MCR). MCR mutations result in truncated proteins retaining β-catenin binding but not regulatory activity. Somatic MCR mutations are more frequently selected for in FAP patients with germline mutations outside of the MCR. Adapted from (Polakis, 2000).

The initiation of cancer involving *APC* mutations is through the constitutively active Wnt signal transduction pathway (Bienz & Clevers, 2000). The APC protein in co-operation with other proteins, namely conductin and axin, regulates the free and cytoplasmic form of β-catenin by binding to it and directing it into a protein degradation pathway. In the presence of a Wnt signal or an *Apc* gene mutation, the phosphorylation of glycogen synthase kinase 3 beta (GSK3 β) occurs and leads to an increase in the amount of stabilized cytoplasmic β-catenin. β-catenin then migrates to the nucleus and binds to LEF1/TCF-4 transcription factor. This interaction with the transcription factor results in the activation of a number of important target genes including c-myc (He et al., 1998) and cyclin D1 (Tetsu & McCormick, 1999), which in turn leads to uncontrollable abnormal cell growth (Figure 1.3.2.2).

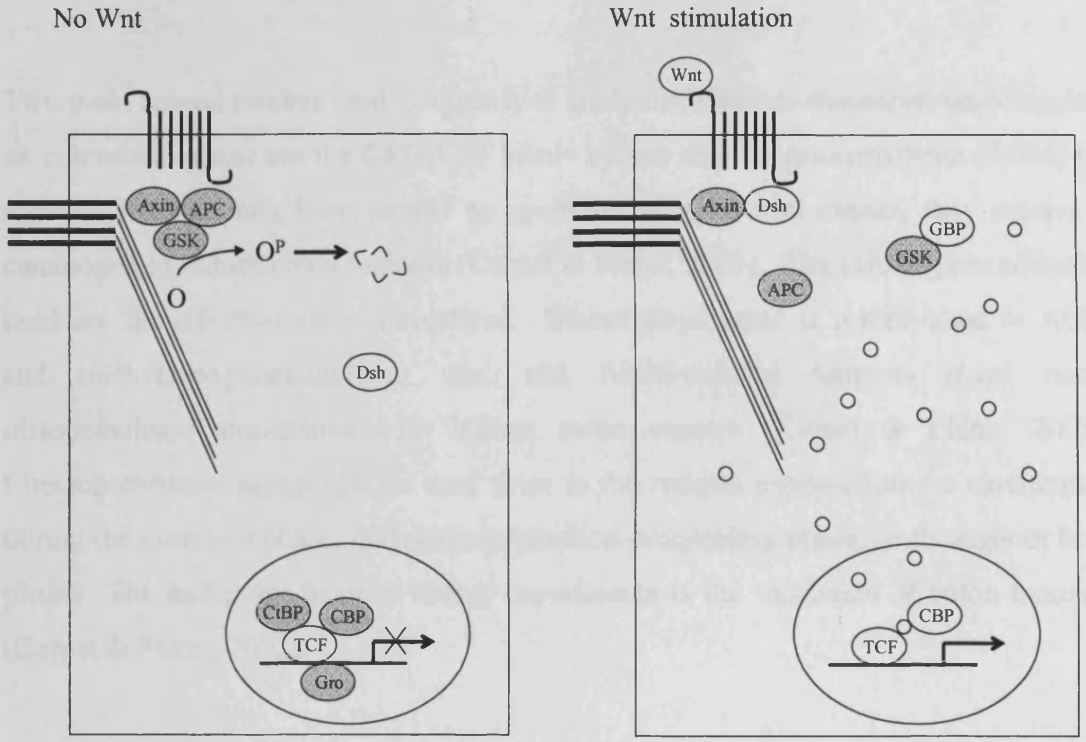


Figure 1.3.2.2. The Wnt signalling pathway. Activators of the pathway are shown in white; negative regulators are shown in grey. Left, in the absence of a wnt signal, the axin complex actively earmarks the β-catenin (white circles) for degradation by the proteasome. Cytoplasmic levels of β-catenin are low and the TCF is inactivated. Right, after stimulation of the Frizzled receptor by a wnt signal, Dsh, is recruited to the membrane where it binds to Axin to inhibit the Axin complex. β-catenin accumulates, translocates to the nucleus whereupon it binds to TCF to co-activate wnt target genes (c-myc, cyclin D etc.). CBP alternates from being a negative regulator to a coactivator, depending on the stimulation status of the cell. It is noteworthy that the Axin complex is anchored to the apicolateral adherens junctions, which are formed by the transmembrane protein E-cadherin (black bars). Adapted from (Bienz & Clevers, 2000).

1.3.3 Preclinical rodent models of colorectal cancer and FAP

Two main animal models used frequently to study the effect of chemopreventive agents on colorectal cancer are the C57BL/6J Min/+ mouse and the azoxymethane (AOM) rat model. Because rats have almost no spontaneous colorectal cancer, they receive a carcinogen to induce colon tumours (Corpet & Pierre, 2003). The carcinogens normally used are dimethylhydrazine derivatives. Dimethylhydrazine is metabolized to AOM and methylazoxymethanol in rats, and AOM-induced tumours share many histopathologic similarities with human colon cancers (Corpet & Pierre, 2003). Chemopreventive agents can be used prior to the rodents exposure to the carcinogen, during the initiation phase, during the promotion-progression phase, or throughout both phases. The end point in most rodent experiments is the incidence of colon tumours (Corpet & Pierre, 2003).

The main disadvantage of using chemically induced cancer models is that they are highly unlikely to mimic, at the genetic level, the observed human condition. However, genetically modified mouse models have been developed that produce preneoplastic and neoplastic lesions in the gastrointestinal tract without the need for chemical carcinogens. The use of these animals, with genetically modified or defective genes fundamental to the human condition, provides an exciting research tool to investigate chemopreventive agents. They allow insights into chemopreventive activity and mechanism of action relevant to the human condition. As the human condition, FAP, is caused by mutations in the *Apc* gene, mouse models have been developed reflecting these mutations. Table 1.3.3.1 provides a summary of the mouse models developed and the phenotypes associated with different *Apc* gene mutations.

There is a discrepancy between these mouse models for FAP and the human condition. The formation of adenomas in mice is predominantly in the small intestine rather than the colon. This difference could be explained, in part, by the faster rate of cellular turnover in the intestine of mice and also the fact that they have a different distribution of bacterial content making the environmental conditions of the gut between mouse and human completely different (Bertagnolli, 1999). Despite these apparent differences the murine FAP models have proven to be very valuable in the evaluation of chemopreventive agents. It has been shown that dietary factors and drugs can strongly modify the outcome of a highly penetrable disease gene (Alexander, 2000). The

adenoma-inhibiting effects of non-steroidal anti-inflammatory drugs (NSAIDs) in these animal models has also been reflected by a reduced risk effect of these agents in human FAP and sporadic colon cancers, highlighting the clinical relevance of these models (Smalley & DuBois, 1997).

Table 1.3.3.1. Mouse models for FAP and their phenotypes (Adapted from Heyer et al., 1999).

Name of allele	Location of mutation in APC protein	Heterozygous phenotype	Lifespan	References
Min	aa 850	Multiple adenomas in the GI tract	120-180 days	(Su et al., 1992)
Apc1638N	aa 1638	3-4 adenocarcinomas in the GI tract	> 1 year	(Fodde et al., 1994; Yang et al., 1997)
Apc1638T	aa 1638	Normal	Normal	(Smits et al., 1999)
Apc716	aa 716	Multiple adenomas in the GI tract	n.d.	(Oshima et al., 1995)
Apc580	aa 580 conditional	4 weeks after deletion Multiple adenomas		(Shibata et al., 1997)

The first mouse model developed to mimic the human condition FAP, was named the "multiple intestinal neoplasia" (Min/+) mouse which contains an induced germline mutation in codon 850 of the *Apc* gene resulting in a truncated protein of 850 amino acids (Su et al., 1992). These mice can develop more than 100 adenomas throughout the small intestine depending on their genetic background, and as a result of the high number of adenomas have a reduced lifespan of approximately 150 days (Moser et al., 1992).

Of the other FAP mouse models shown in Table 1.3.3.1 only two are commonly used, each bearing germline mutations in the Apc gene. The Apc716 mouse was generated by introducing a neomycin cassette into the *Apc* codon 716. The resulting APC protein is truncated and is approximately 80 kDa in size. These mice are similar to the Min/+ mouse as they develop multiple adenomas within the small intestine. In addition they have numerous extra-intestinal tumours (Oshima et al., 1995).

The Apc1638N mouse was developed by inserting a neomycin cassette into the *Apc* codon 1638, in the transcriptional orientation opposite to that of *Apc*. The resulting APC protein is truncated and 182 kDa in size. These mice develop between 3-4 adenocarcinomas in the small intestine and have a longer lifespan than the models discussed above due to the reduced number of adenomas (Fodde et al., 1994; Yang et al., 1997).

A further model, the Apc1638T mouse, was developed whereby the neomycin cassette was introduced into the *Apc* codon 1638 but in the same transcriptional orientation as the *Apc* gene. The resulting truncated APC protein is 182 kDa in size and is expressed in a 1:1 ratio with the wild-type protein which is much larger at 312 kDa. Although the truncated protein lacks the binding region for tubulin and EB1-like proteins, these mice are otherwise normal and do not develop small intestinal adenomas (Smits et al., 1999).

1.3.4 Diet and colorectal cancer

The genetic changes that occur during the development of colorectal cancer are well understood. It is also well documented that dietary involvement plays a pivotal role in colorectal cancer development (Cummings & Bingham, 1998). Epidemiological and other studies, into diet and cancer development are essential to indicate which dietary constituents may be effective as chemopreventive agents. Such studies suggest that a number of components found in fruit and vegetables might contribute to their ability to reduce the risk of cancer. Examples are dietary fibre, micronutrients and various phytochemicals (Greenwald, 2002). The results of epidemiological studies of colorectal cancer have indicated that dietary agents play an important role in the development of intestinal neoplasia (Ames et al., 1995). The regular consumption of fruit and vegetables is associated with a decrease in cancer incidence (Steinmetz & Potter, 1991). Colorectal cancer is also associated with diet quality. High consumption of meat, fat, and simple sugars have been associated with an increased risk of colorectal cancer (Potter, 1999) so lower consumption of these food groups could reduce this risk (Elmstahl et al., 1999; Osler & Heitman, 1997; Randall et al., 1991; Subar et al., 1994; Ursin et al., 1993).

Although epidemiological studies are important for identifying new chemopreventive agents, clinical trials cannot be carried out without additional information gained from

cell culture and animal experiments. This notion is particularly highlighted by the clinical trial of β-carotene, which was based almost entirely on epidemiological data showing that foods containing high levels of β-carotene reduced the risk of cancer. However, there was no data *in vitro* or in *vivo* to suggest β-carotene prevented cancer in the lung, prostrate or colon. The clinical trial ended up a failure (Sporn & Suh, 2002).

1.4 Cellular signalling pathways in carcinogenesis

Tumour cells show a number of features which differentiate them from normal cells. In normal cell growth there is a finely controlled balance between growth-promoting and growth-restraining signals, such that proliferation only occurs when required (Macdonald & Ford, 1997). In cancer cells, this process is disrupted, leading to uncontrolled cell proliferation and loss of differentiation. In addition, the normal process of apoptosis may also be disrupted (Macdonald & Ford, 1997). Cell proliferation, differentiation and survival are strictly controlled by extracellular signals via complicated, often cross-talking, signal transduction pathways (Figure 1.4.1). These pathways often start with ligand-dependent activation of membrane receptors (tyrosine kinase receptors, G-protein coupled receptors, or integrins) and then subsequent activation of downstream targets. The expression of these downstream cellular targets is frequently altered during cancer development and may therefore act as potential targets for chemopreventive agents.

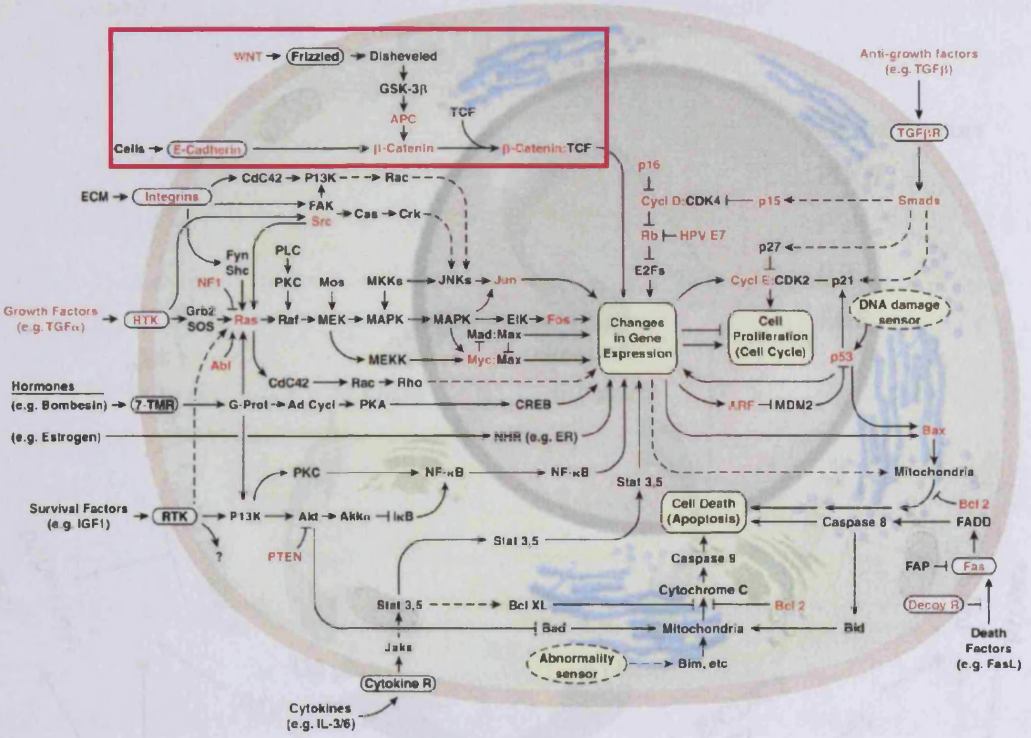


Figure 1.4.1. The signalling pathways involved in cancer. Highlighted in the red box is the Wnt signalling pathway, which has already been discussed, whereby a mutation in the *APC* gene is an early event in the development of colorectal cancer. Other major pathways shown include cell cycle, apoptosis and the NF-κB pathway which leads to the transcription of cyclooxygenases 1 and 2 are play an important role in the development and progression of colorectal cancer and will be discussed in section 1.4.1. Diagram taken from (Hanahan & Weinberg, 2000).

1.4.1 The role of cell cycle, apoptosis and the cyclooxygenase pathway in colorectal carcinogenesis

The cell cycle involves a series of events that lead to DNA replication and cell division (Macdonald & Ford, 1997). This procedure is stringently controlled in normal cells, however, in cancerous cells, mutations in the genes that control the cell cycle can result in the progression of cells with damaged DNA through this cycle (Macdonald & Ford, 1997). The cell cycle consists of 4 distinct phases (Figure 1.4.1.1).

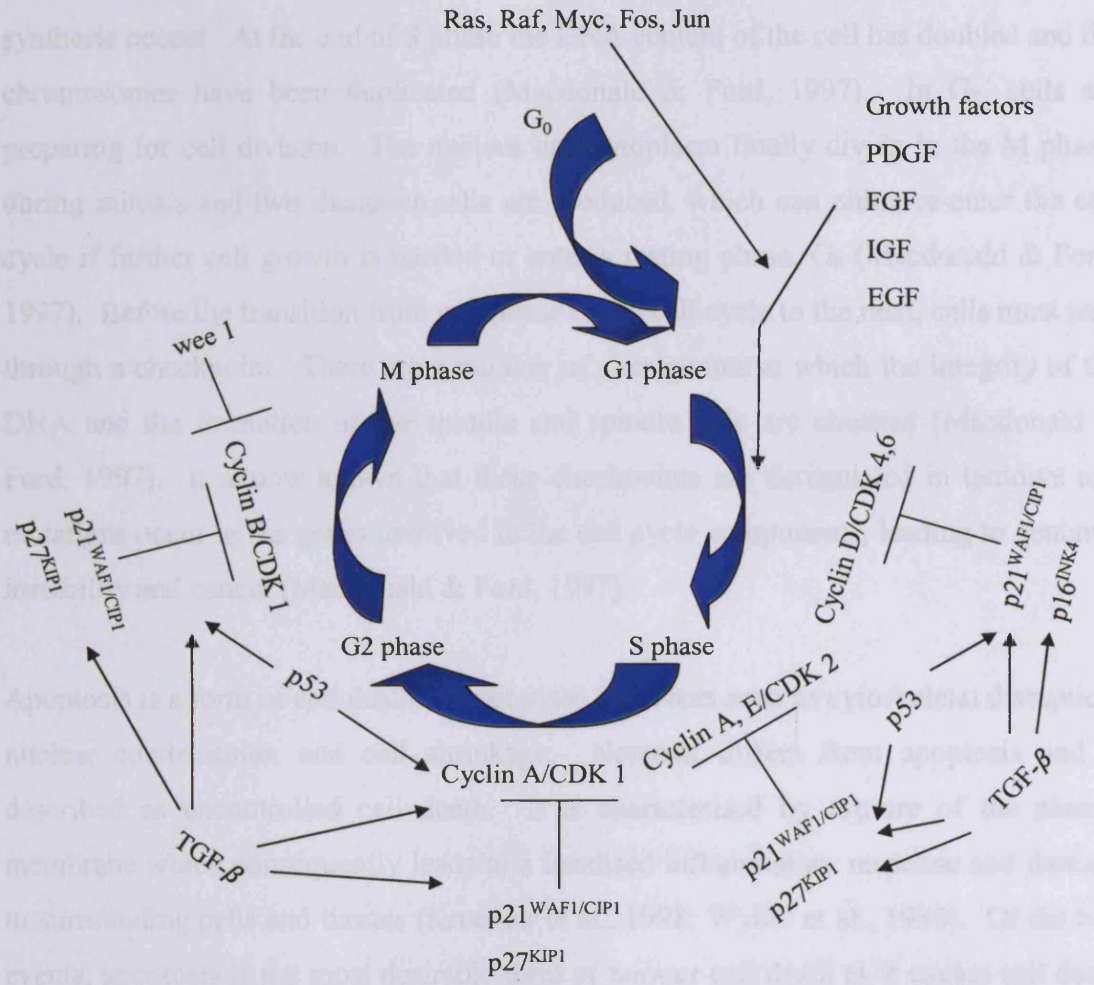


Figure 1.4.1.1. Schematic overview of cell cycle machinery. Following a mitogenic stimulus that promotes entry into early G_1 phase, progression through the cell cycle is strictly regulated by sequential activation of cell-phase specific cyclins and cyclin-dependent kinases. Activation of CDK 4 and CDK 6 by cyclin D propels the cell through G_1 phase. This is followed by the activation of CDK 2, a requirement for progression through the S phase into G_2 phase, where the CDK 1/cyclin B complex then assists its passage into M phase. The steps of the cell cycle are negatively controlled by endogenous cyclin-dependent kinase inhibitors, p21, p27, and p16. The p21 family of CDK inhibitors exerts a negative regulatory effect on all cyclins and CDKs, while a member of the p16 family interacts exclusively with CDK 4 and CDK 6. Adapted from (Elsayed & Sausville, 2001).

In G_1, cells are preparing to synthesize DNA and the biosynthesis of RNA and proteins occurs (Macdonald & Ford, 1997). During S phase, DNA is replicated and histone synthesis occurs. At the end of S phase the DNA content of the cell has doubled and the chromosomes have been duplicated (Macdonald & Ford, 1997). In G_2, cells are preparing for cell division. The nucleus and cytoplasm finally divide in the M phase, during mitosis and two daughter cells are produced, which can either re-enter the cell cycle if further cell growth is needed or enter a resting phase, G_0 (Macdonald & Ford, 1997). Before the transition from one phase of the cell cycle to the next, cells must pass through a checkpoint. There are a number of checkpoints at which the integrity of the DNA and the formation of the spindle and spindle pole are checked (Macdonald & Ford, 1997). It is now known that these checkpoints are deregulated in tumours and mutations occur in the genes involved in the cell cycle components, leading to genomic instability and cancer (Macdonald & Ford, 1997).

Apoptosis is a form of cell death characterised by events such as cytoskeletal disruption, nuclear condensation and cell shrinkage. Necrosis differs from apoptosis and is described as uncontrolled cell death. It is characterised by rupture of the plasma membrane which consequently leads to a localised inflammatory response and damage to surrounding cells and tissues (Kroemer et al., 1998; Wyllie et al., 1980). Of the two events, apoptosis is the most desirable form of tumour cell death as it causes cell death without inducing damage and inflammation in the surrounding tissues.

Two major pathways of apoptosis exist, the death receptor pathway and the mitochondrial pathway (reviewed by Zimmerman et al., 2001; Figure 1.4.1.2.). Both these pathways rely on the degradation of cellular proteins by a caspase cascade. The destruction of cells occurs due to the activation of the caspase cascade, whereby initiator caspases (caspase-8, -9, and 10) activate the effector caspases (caspase-3, -6 and 7) which in turn act on a wide range of substrates that destroy the unwanted cells.

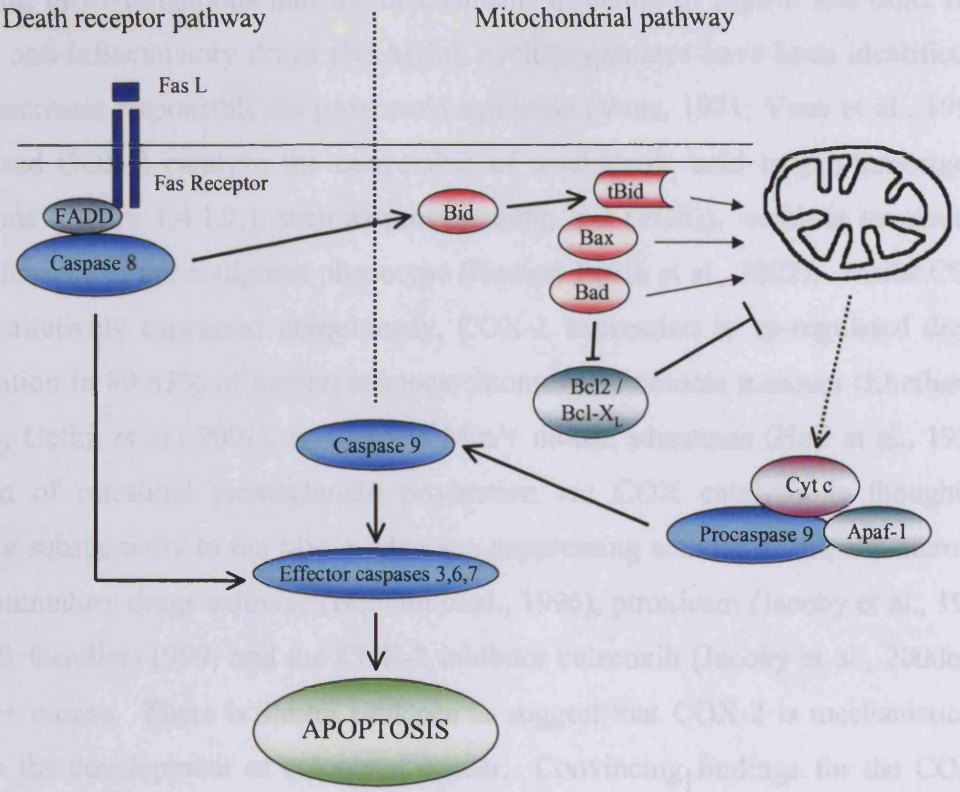


Figure 1.4.1.2. Induction of apoptosis by death receptor and mitochondrial pathways. Using the FAS receptor as an example for the death receptor pathway, Fas L recruits FADD to the intracellular region, which in turn recruits and then activates caspase 8. Caspase 8 can then induce apoptosis through the activation of effector caspases. Fas L also signals to the mitochondrion through the cleavage of Bid. Truncated bid (tBid) can then induce the release of cytochrome c (Cyt c). In the mitochondrial pathway, cellular defects, such as DNA damage cause the proapoptotic family proteins (red coloured) to translocate to the mitochondria, where they release Cyt c. Cyt c can then recruit Apaf-1 and procaspase 9 causing the promotion of caspase 9, which in turn leads to activation of the effector caspases and apoptosis follows.

As a result of investigations into the mechanisms of action of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs), cyclooxygenases have been identified as the key enzymes responsible for prostanoid synthesis (Vane, 1971; Vane et al., 1998). COX-1 and COX-2 catalyze the conversion of arachidonic acid to pro-tumorigenic eicosanoids (Figure 1.4.1.2.), such as prostaglandin E-2 (PGE_2), which is involved in the maintenance of the malignant phenotype (Hansen-Petrik et al., 2002). Whilst COX-1 is constitutively expressed ubiquitously, COX-2 expression is up-regulated during inflammation in 80-85% of human adenocarcinomas and colonic tumours (Eberhart et al., 1994; Uefuji et al., 2001), and also in Min/+ mouse adenomas (Hull et al., 1999). Inhibition of intestinal prostaglandin production *via* COX catalysis is thought to contribute substantially to the Min/+ adenoma-suppressing activity of the non-steroidal anti-inflammatory drugs sulindac (Boolbol et al., 1996), piroxicam (Jacoby et al., 1996; Ritland & Gendler, 1999) and the COX-2 inhibitor celecoxib (Jacoby et al., 2000b) in the Min/+ mouse. There is strong evidence to suggest that COX-2 is mechanistically linked to the development of colorectal cancer. Convincing findings for the COX-2 gene came from an *in vivo* study with knockout $Apc^{\Delta 716}$ mice (Chapter 1.6) (Oshima et al., 1996b). Knocking out the COX-2 gene caused a reduction in number and size of intestinal adenomas, a finding which was also shown to be gene-dose dependent (Oshima et al., 1996b). Mice that lacked both copies of the COX-2 gene had an 86% reduction in adenomas, whereas, those mice with only one gene lacking had a reduction of 66% (Oshima et al., 1996b). Knockout mice for the COX-1 gene also had a reduction in adenoma number, highlighting the importance of arachidonic acid metabolism in the development of colorectal cancer (Chulada et al., 2000) (Figure 1.4.1.3.).

These observations are supported by pharmacological evidence. The use of selective COX-2 inhibitors, for example celecoxib and refecoxib, reduced the formation of intestinal adenomas in the Min/+ mouse (Jacoby et al., 2000b; Kawamori et al., 1998; Oshima et al., 1996a; Oshima et al., 2001). Furthermore, selective COX-2 inhibitors can suppress the growth of established colorectal tumours in animals (Sheng et al., 1997). Subsequently, it has been confirmed by a clinical study that a COX-2 inhibitor can significantly reduce the number and size of colonic polyps in FAP patients (Steinbach et al., 2000).

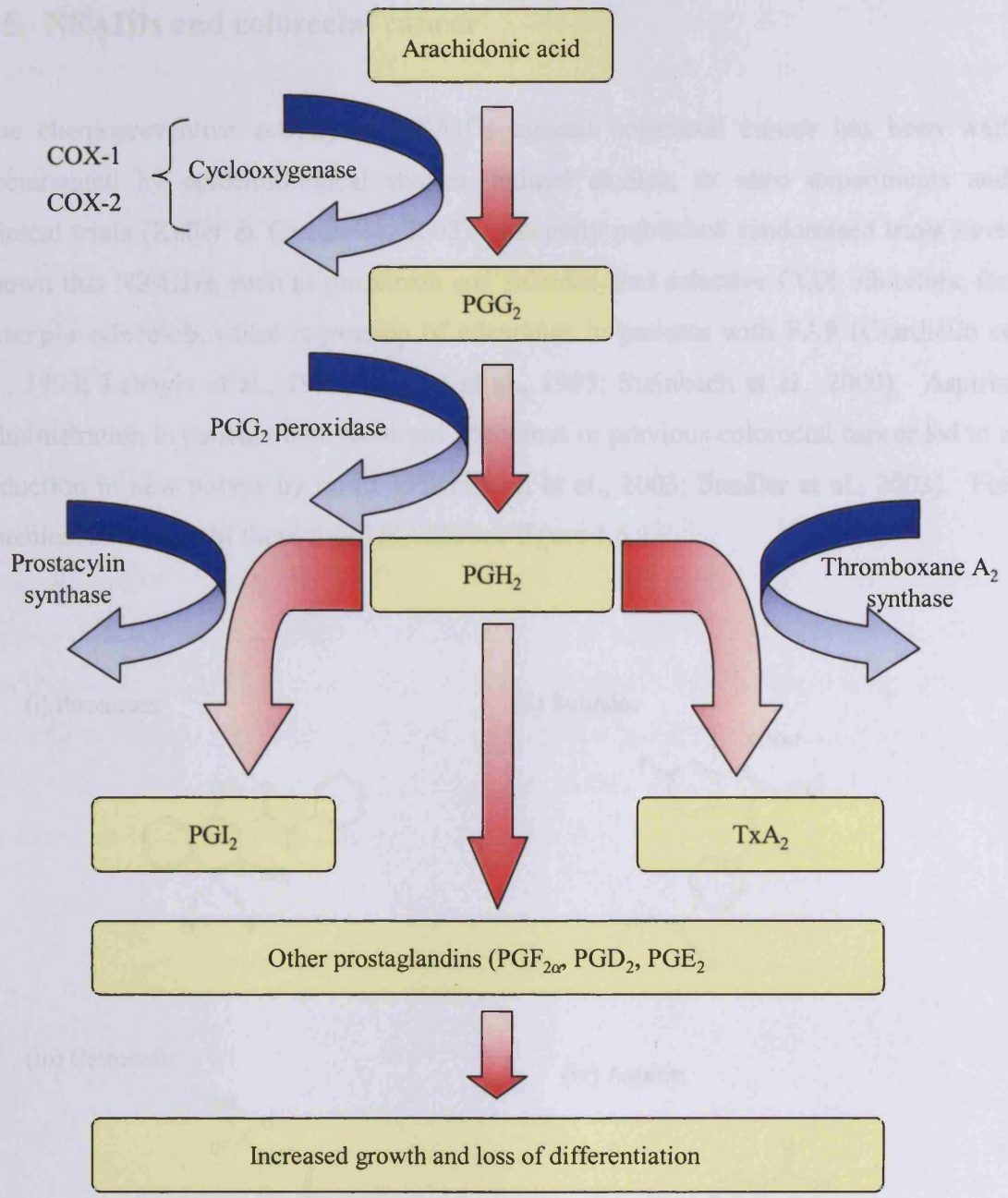
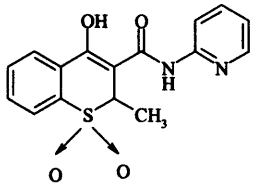


Figure 1.4.1.3. The biosynthesis of prostaglandins, prostacyclin and thromboxane from arachidonate. Adapted from (Page et al., 1997). Increased growth and loss of differentiation is brought about by the inhibition of apoptosis, induction of angiogenesis, increased cell growth and formation of free radicals. Blue arrows indicate the enzymes that initiate the conversion and red arrows indicate the progression of the pathway.

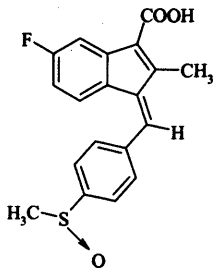
1.5. NSAIDs and colorectal cancer

The chemopreventive activity of NSAIDs against colorectal cancer has been well documented by epidemiological studies, animal studies, *in vitro* experiments and clinical trials (Keller & Giardiello, 2003). Recently published randomised trials have shown that NSAIDs, such as piroxicam and sulindac, and selective COX inhibitors, for example celecoxib, cause regression of adenomas in patients with FAP (Giardiello et al., 1993; Labayle et al., 1991; Nugent et al., 1993; Steinbach et al., 2000). Aspirin administration in patients with recurrent adenomas or previous colorectal cancer led to a reduction in new polyps by up to 35% (Baron et al., 2003; Sandler et al., 2003). For chemical structures of these four NSAIDs see figure 1.5.1.

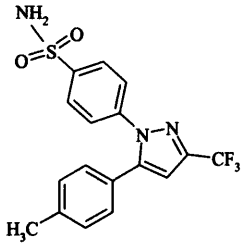
(i) Piroxicam



(ii) Sulindac



(iii) Celecoxib



(iv) Aspirin

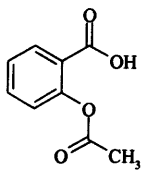


Figure 1.5.1. Chemical structures of (i) piroxicam, (ii) sulindac, (iii) celecoxib and (iv) aspirin.

Studies such as these indicate that chemoprevention could become a clinical reality. However, NSAIDs, including aspirin, have well-documented side-effects and complications associated with prolonged use such as gastric mucosal inflammation, bleeding dyscrasias, gastric ulceration and renal impairment. Another family of compounds was developed that is COX-2 inhibitor specific. These were known as the

"coxib" family. While NSAIDs inhibit both isozymes, coxibs inhibit COX-2 and are claimed to be as efficacious as NSAIDs, but less harmful to the gastrointestinal tract (MacRae et al., 2004). This is not to say that these compounds do not have there adverse effects. Coxibs, like NSAIDs, can cause or worsen hypertension, oedema, heart failure and renal or hepatic dysfunction (MacRae et al., 2004). For chemoprevention to be effective patients would be required to take these drugs for many years and the inevitable morbidity associated with these unwanted effects may obfuscate any benefit from reduction of colorectal cancer risk.

The mechanism of chemoprevention by NSAIDs can be explained in part by their ability to inhibit COX, however, other mechanisms of action are likely to exist. Other proposed targets include RAS, NF-κB, PPARδ, Bcl-X_L, FAK, NAG-1 and β-catenin (Baek et al., 2001; Hawcroft et al., 2002; He et al., 1999; Weyant et al., 2000; Yamamoto et al., 1999). Modulation of the β-catenin/TCF4 pathway via induction of p21 expression has also been postulated as a possible mechanism of action for the anti-cancer effect of sulindac (van de Wetering et al., 2002). Figure 1.5.2 outlines proposed mechanisms of chemoprevention by NSAIDs.

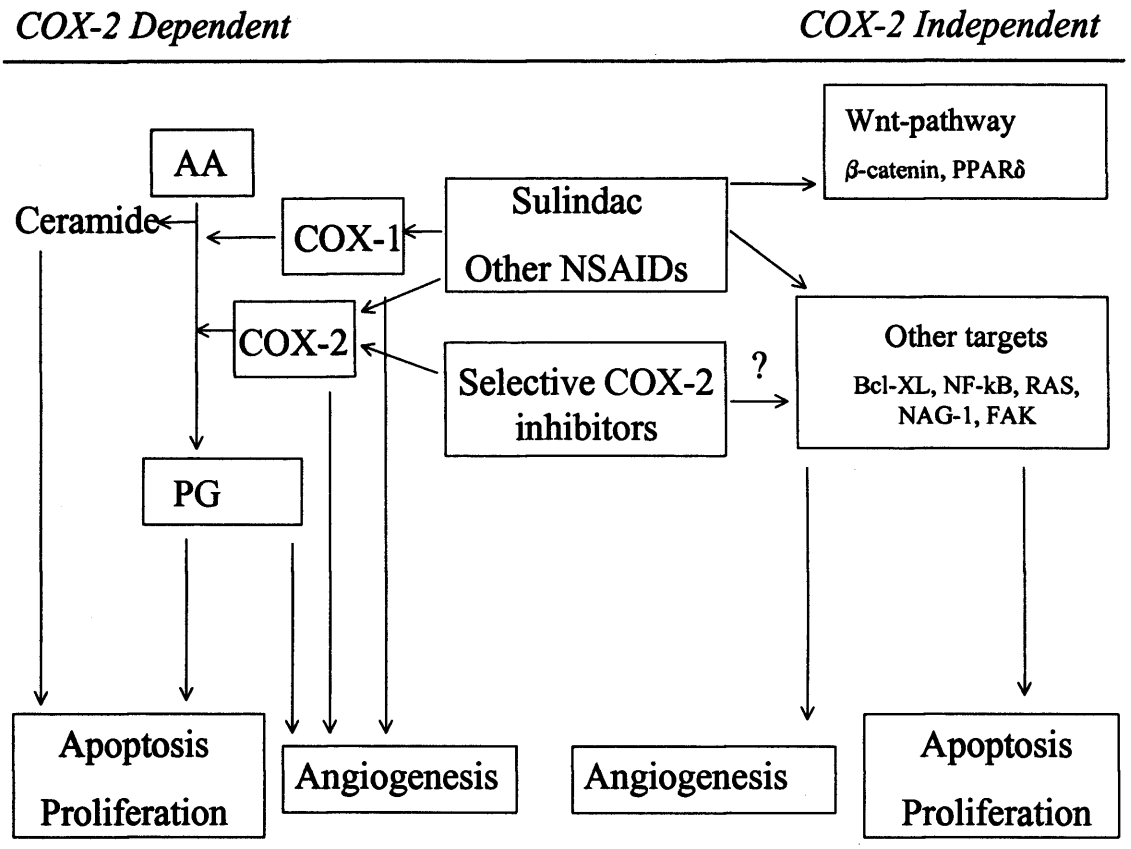


Figure 1.5.2. Proposed mechanism of chemoprevention by NSAIDs. Their chemopreventive activity can, in part, be explained by their ability to inhibit COX, however, additional mechanisms may be involved. Figure adapted from (Keller & Giardiello, 2003)

1.6. Polyphenols and colorectal cancer

Polyphenols are the most abundant antioxidants in the human diet. Polyphenolic phytochemicals occur abundantly in food sources such as fruits; beverages such as tea, coffee and wine; chocolate; vegetables; cereals and legumes (Scalbert et al., 2002). The concept that polyphenolic phytochemicals may possess colorectal cancer chemopreventive properties is based on epidemiological findings that intimate that the intake of foods rich in polyphenols may delay the onset of cancer. Observational and case-control studies indicate that intake of fruit and vegetables is associated with a lower risk of colorectal cancer (Deneo-Pellegrini et al., 1996; Franceschi, 1999; Franceschi et al., 1997; Sandler, 1996; Slattery et al., 1997; Terry et al., 2001; Voorrips et al., 2000). The protective effect of high fruit intake is evident amongst cohorts of individuals who are at high risk of colorectal cancer, such as those with previous cancer history, colonic polyps and ulcerative colitis (Matthew et al., 1997). Food components other than fruits and vegetables rich in polyphenols also confer a reduction in colorectal cancer risk. Regular ingestion of green tea has been found to be protective in several retrospective epidemiological trials (Ji et al., 1997; Kohlmeier et al., 1997; Zhang et al, 2002; Zhang et al., 2000). One large Japanese study followed up 8552 patients over a nine-year period and observed a delayed onset of cancer, in all sites, in those individuals who drank 10 cups of green tea daily (Imai et al., 1997). A considerable range of polyphenols have been pinpointed through epidemiological studies and are currently under evaluation using *in vitro* and *in vivo* models.

Curcumin is a bright yellow pigment derived from the rhizome *Curcuma* longa. It is found in the spice turmeric, which is widely used in Indian cuisine as a colouring and flavouring agent. Like some other polyphenols curcumin has been shown to inhibit COX-2 expression in human colorectal tumour cell lines (Plummer et al., 1999). Other mechanistic actions of curcumin include inhibition of oxidative DNA adduct formation (measured by, for example, levels of the pyrimidopurinone M_1G) (Sharma et al., 2001b), decreased expression of the onco-protein beta-catenin (Mahmoud et al., 2000) and induction of apoptosis (Mori et al., 2001; Samaha et al., 1997). Curcumin may also modulate, immune system-mediated tumour killing by increasing CD4+ T cells and B cells within the intestinal system (Churchill et al., 2000).

Cell work using colorectal tumour cell lines has shown that the most abundant catechin found in green tea, epicatechin gallate, induces apoptosis in tumour cell lines and interferes with the cell cycle (Chen et al., 1998; Lambert & Yang, 2003; Salucci et al., 2002; Uesato et al., 2001) and that this effect is specific to tumour cells only. Other effects observed include inhibition of DNA adduct formation (Xu et al., 1996), preservation of the colonic microflora (Kan et al., 1996) and electrophile scavenging (Dashwood et al., 1999). EGCG has been shown to inhibit topoisomerase I in multiple human colon carcinoma cell lines (Berger et al., 2001). DNA topoisomerase I is essential for cell survival and plays a critical role in DNA metabolism and structure. Tea polyphenols have also been shown to induce Phase II detoxification of dietary carcinogens (Santana-Rios et al., 2001).

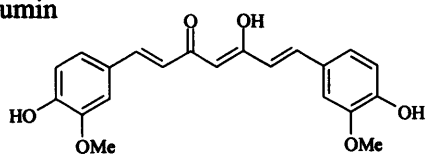
The chemopreventive action of polyphenols is thought to be exerted via pathways similar to those observed with NSAIDs (section 1.5). Studies in the Min/+ mouse suggest that the polyphenolic phytochemicals curcumin and resveratrol may be effective at inhibiting the formation of adenomas, even though it is conceivable that NSAIDs are more efficacious (Table 1.6.1). Resveratrol inhibited adenoma formation in the small intestine by up to 70 % and completely inhibited their formation in the colon (Schneider et al., 2001). Curcumin in two separate studies showed adenoma inhibition by up to 64 % (Mahmoud et al., 2000; Perkins et al., 2002). NSAIDs inhibited up to 99 %, however, the range of inhibition did vary between studies depending on their duration (Table 1.6.1).

Table 1.6.1. Summary of the effects of NSAIDs and common polyphenols on adenoma number in the Min/+ mouse.

Compound	Dietary dose (ppm)	Duration (days)	Treatment Effect (Inhibition) %	Reference
Piroxicam	25-220	7-180	34-95	Hansen-Petrik et al., 2002; Jacoby et al., 2000a; Jacoby et al., 1996; Jacoby et al., 2000b; Ritland & Gendler, 1999
Sulindac	30-300	7-80	32-99	Boolbol et al., 1996; Chiu et al., 1997; Hansen-Petrik et al., 2002; Huerta et al., 2002; Jacoby et al., 2002; Ritland & Gendler, 1999; Suganuma et al., 2001; Torrance et al., 2000
Celecoxib	150-1500	25-55	27-71	Jacoby et al., 2000b
Curcumin	1000-2000	70-75	6-64	Collett et al., 2001; Mahmoud et al., 2000; Perkins et al., 2002
Resveratrol	100 (in water)	49	70	Schneider et al., 2001
Tea extract	1000	70	22	Suganuma et al., 2001

Chemopreventive activity with these compounds were also seen in inhibiting aberrant crypt foci in the AOM-induced adenocarcinoma rat model. Curcumin and green tea extracts inhibited ACF formation to an extent similar to that shown by NSAIDs (Jia & Han, 2001; Kwon et al., 2002; Metz et al., 2000; Rao et al., 1999; Rao et al., 1993; Steele et al., 1999). NSAIDs were superior at reducing the incidence of tumour incidence in this rodent model.

(i) Curcumin



(ii) Epigallocatechin gallate (EGCG)

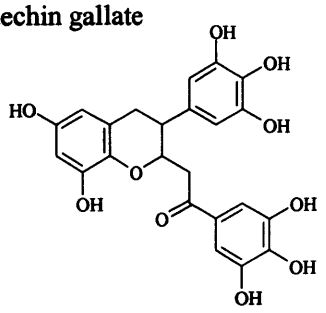


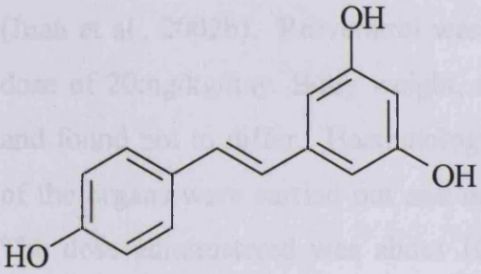
Figure 1.6.1. Chemical structures of two commonly studied polyphenols (i) curcumin and (ii) EGCG

1.7 Resveratrol

Resveratrol (*trans*-3,5,4'-trihydroxystilbene, for structure see figure 1.7.1) was originally identified as a phytoalexin by Langcake and Pryce (Langcake & Pryce, 1976), and since then has generated extensive attention due to its abundance in grapes, mulberries, cranberries, peanuts and grape products such as wine, an age-old component of the diet (Soleas et al., 1997). An internet search for "resveratrol" reveals numerous websites that offer a tablet supplementation of this compound (figure 1.7.2). However, resveratrol is not the only compound present in these products. These formulations also contain many other polyphenols. For example, quercetin is added to enhance the bioavailability of the resveratrol (Life Extension Website). *Trans*-resveratrol in isolation is stable for several months (except in high pH buffers, pH >10) when completely protected from light (Trela & Waterhouse, 1996). The health benefits of resveratrol first became apparent when a correlation was observed between red wine consumption and a reduction in the incidence of coronary heart disease (Siemann & Creasy, 1992). Resveratrol was then identified as one of the active ingredients found in

red wine. Epidemiological studies have since shown that moderate red wine intake may decrease coronary heart mortality (Gronbaek et al., 2000; Renaud et al., 1999) and hence explain the "French paradox", i.e. the finding that the incidence of coronary heart disease among people in the South of France is relatively low regardless of their high dietary consumption of fats.

(i) *trans*-resveratrol



(ii) *cis*-resveratrol

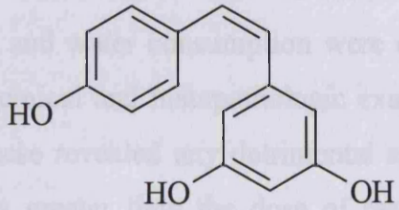


Figure 1.7.1. The chemical structure of (i), *trans*- and (ii), *cis*-isomers of resveratrol (3,5,4'-trihydroxystilbene).

Figure 1.7.2. Commercially available resveratrol tablets which are presently being sold over the internet.

1.7.1 Toxicity of resveratrol

Chemoprevention requires individuals to receive a compound for prolonged periods of time, possibly life. Therefore, it is imperative that any chemopreventive agents exerts negligible adverse effects. In both human and animal studies, administration of resveratrol has yet to show any significant toxicity. A study, in Sprague-Dawley rats, was set up to evaluate whether high doses of *trans*-resveratrol had any harmful effects (Juan et al., 2002b). Resveratrol was administered orally to male rats for 28 days at a dose of 20mg/kg/day. Body weight, and food and water consumption were monitored and found not to differ. Haematologic, biochemical and histopathologic examinations of the organs were carried out and none of these revealed any detrimental alterations. The dose administered was about 1000 times greater than the dose of resveratrol a human would receive through drinking a glass of red wine (Juan et al., 2002b). In humans there is very limited literature with regard to resveratrol toxicity. However, studies by Soleas *et al* and Goldberg *et al* investigating the absorption of resveratrol and other polyphenols following oral consumption did not report any adverse affects (Goldberg et al., 2003; Soleas et al., 2001b; Soleas et al., 2001c). In addition, resveratrol has been used for centuries as the active ingredient in many traditional medicines in India, China and Japan, suggesting the potential medicinal value and safety of this compound (Bhat & Pezzuto, 2002; Paul et al., 1999)

1.7.2. The cancer chemopreventive activity of resveratrol

Resveratrol inhibits various cellular events associated with the three major stages of carcinogenesis; initiation, promotion and progression (Jang et al., 1997). Since this discovery, resveratrol has been the subject of a large number of preclinical and mechanistic studies. The cancer chemopreventive potential of resveratrol has been demonstrated in models of carcinogenesis in cells *in vitro* and *in vivo* (for review see Gescher & Steward, 2003). It inhibits proliferation of a variety of cancer cell lines (for review see (Gusman et al., 2001)), formation of preneoplastic lesions in the 7,12-dimethylbenz(*a*)anthracene- (DMBA) induced mouse mammary organ culture model (Bhat et al., 2001) and benzo(*a*)pyrene-induced transformation of rat tracheal epithelial cells (Jang et al., 1997). In *in vivo* studies, resveratrol attenuated oesophageal carcinoma formation in rats which received *N*-nitrosomethylbenzylamine (NMBA) (Li

et al., 2002), and reduced mammary tumour formation in *N*-methyl-*N*-nitrosourea-(NMU) treated rats (Bhat et al., 2001).

Evidence for the colorectal chemopreventive efficacy of resveratrol *in vivo* is still limited. One study has investigated the efficacy of resveratrol in the AOM rat model (Tessitore et al., 2000) and only three have been undertaken in the Min/+ mouse (Gignac & Bourquin, 2001; Schneider et al., 2001; Ziegler et al., 2004). Tessitore *et al* (Tessitore et al., 2000) investigated the efficacy of resveratrol on azoxymethane-induced colon carcinogenesis in male F344 rats. Resveratrol, dissolved in drinking water to give the very low dose of 200μg/kg/day, decreased the number of colonic aberrant crypt foci by 40%, and their multiplicity was reduced by 50% (Tessitore et al., 2000). The multiplicity can be defined as the number of aberrant crypts per foci (see section 1.2 for explanation of ACF). In resveratrol-treated animals, Bax expression was enhanced in ACF but not in the surrounding mucosa. In both controls and resveratrol treated animals, proliferation was higher in ACF than normal mucosa (Tessitore et al., 2000). Expression of the cell cycle inhibitory protein $p21^{WAF1}$ was measured and found to be expressed in the aberrant crypts of control and treated rats as well as in the normal mucosa of the control animals. Expression, however, was lost in normal mucosa of the resveratrol treated rats (Tessitore et al., 2000). The protective role of resveratrol shown in this study suggests its mechanism of action for chemopreventive efficacy is via changes in the expression of Bax and p21 (Tessitore et al., 2000).

In the Min/+ mouse model, 0.01% resveratrol in the drinking water (containing 0.4 % ethanol) was administered for seven weeks to mice starting at five weeks age (Schneider et al., 2001). Control mice received the same diet and drinking water containing just the ethanol vehicle at a dose of 0.4 %. Resveratrol was found to reduce adenoma number in the small intestine by 70%, and completely inhibited colon adenoma formation (Schneider et al., 2001). Additionally, in this study, the expression of 588 genes in the small intestinal mucosa were compared. Resveratrol down-regulated genes directly involved in cell cycle progression or cell proliferation (cyclin D1 and D2, DP-1 transcription factor, and Y-box binding protein) (Schneider et al., 2001). Resveratrol also up-regulated genes involved in the recruitment and activation of immune cells (cytotoxic T lymphocyte Ag-4, leukaemia inhibitory factor receptor, and monocyte chemotactic protein 3) and in the inhibition of the carcinogenic process and tumour expansion (tumour susceptibility protein TSG101, TGF-β, inhibin-β A subunit, and

desmocollon 2) (Schneider et al., 2001). In the light of subsequent contradictory studies (Gignac & Bourquin, 2001; Ziegler et al., 2004) the findings in the Schneider *et al* (Schneider et al., 2001) study need to be interpreted with caution. These contradictory studies suggest that in the same mouse model, dietary doses comparable to, or considerably higher than, those used by Schneider *et al* (Schneider et al., 2001) were either completely ineffective at reducing adenoma numbers (Ziegler et al., 2004); or reduced adenoma number, by up to 50% when given at the much higher dose of 500 mg/kg for 14 days, and then only in female mice, with no effect in male mice (Gignac & Bourquin, 2001).

1.7.3. The mechanism of actions of resveratrol

Resveratrol has been reported to possess a variety of anti-inflammatory, anti-platelet, and both pro- and anti-oestrogenic effects (Bertelli et al., 1996; Gehm et al., 1997; Jang et al., 1997; Uenobe et al., 1997). It exerts a wide variety of biological effects associated with cancer chemoprevention, including inhibition of cytochrome P450 enzyme expression activity (Chun et al., 1999; Guengerich et al., 2003), induction of apoptosis (Mahyar-Roemer et al., 2001; Mahyar-Roemer et al., 2002), modulation of components of the cell cycle machinery (Schneider et al., 2000; Wolter et al., 2001), decrease in cyclooxygenase 1 (COX-1) activity and COX-2 expression (Li et al., 2002; Mutoh et al., 2000b; Subbaramaiah et al., 1998), antioxidation (Sgambato et al., 2001), inhibition of activities of protein kinase C and D (Haworth & Avkiran, 2001; Slater et al., 2003) and decrease in activity of transcription factors NFκB and AP-1 (Banerjee et al., 2002; Surh et al., 2001).

Resveratrol has been shown to exert anti-proliferative activity in colorectal cells *in vitro* (Delmas et al., 2002; Wolter & Stein, 2002). Resveratrol, at a concentration of 25 μM in CaCo-2 human colon cancer cells, caused a 70 % inhibition of cell growth (Schneider et al., 2000). The inhibition in cell growth was due to accumulation of cells in the S/G_2 phase transition of the cell cycle. Furthermore, this study highlighted that polyamines might represent one of the targets involved in the anti-proliferative effects shown by resveratrol as the activity of ornithine decarboxylase (ODC) was significantly reduced by resveratrol, a vital enzyme of polyamine biosynthesis, that is up-regulated in cancer cell growth (Schneider et al., 2000).

Additional studies in the same cell line by Wolter *et al* (Wolter et al., 2001) showed that the inhibitory effects on cell proliferation by resveratrol was dose dependent, with concentrations ranging from 12.5-200 μM. At the highest concentration of 200 μM, resveratrol caused caspase-3 activity activation, as well as preventing cell cycle progression from the S to G_2 phase at 50 μM. Reversal of this S phase arrest was seen, however, with higher concentrations (200 μM) of resveratrol (Wolter et al., 2001), as was the down-regulation of cyclin D1 and cyclin dependent kinase (CDK) 4 proteins. HCT-116 colon cancer cells were also investigated as part of this study and similar results were obtained (Wolter et al., 2001). Because both of these cell lines express or possess little or no cyclooxygenase-1 or 2 protein or activity (Kamitani et al., 1998; Sheng et al., 1997), it was concluded that the effects of resveratrol on cell cycle inhibition were not mediated by COX inhibition (Wolter et al., 2001). Cell cycle inhibition was still considered the major pathway by which resveratrol exerted its cancer chemopreventive activity in these colonic cancer cells but the underlying mechanism remains unknown.

A study in colon carcinoma HT29 cells treated with resveratrol concluded that resveratrol induced cell cycle arrest at the G_2 phase through inhibition of CDK7 kinase activity, suggesting that its anti-tumour activity might occur through the disruption of cell division at the G_2/M phase (Liang et al., 2003). Another study has linked the effects of resveratrol to the activation of the p53 tumour suppressor (Mahyar-Roemer et al., 2001). Using both, wild-type p53-expressing HCT116 colon carcinoma cells and HCT116 cells with both p53 alleles inactivated by homologous recombination, it was shown that at concentrations of resveratrol, found in some foods, apoptosis could be induced independently of p53 (Mahyar-Roemer et al., 2001). Furthermore, this cell death was shown to be mitochondria-mediated and not receptor-mediated. Resveratrol also activated the upregulation of Bax regardless of p53 status, even though the kinetics of Bax expression were still influenced by p53. Surprisingly, apoptosis was preceded by mitochondrial proliferation and indications of epithelial differentiation. This finding suggests that resveratrol activates a p53-independent apoptotic pathway in HCT116 cells that may be linked to cell differentiation (Mahyar-Roemer et al., 2001). In an additional study with HCT116 colon carcinoma cells it was proposed that at physiological concentrations of resveratrol, both Bax-mediated and Bax-independent mitochondrial apoptosis could be induced (Mahyar-Roemer et al., 2002).

Resveratrol's inhibitory effect on COX-2 has been proposed as one of the major mechanisms for its anti-cancer effects (Jang et al., 1997). *In vivo*, in the NMBA-induced rat oesophageal tumorigenesis model, resveratrol significantly reduced the number and mean volume of tumours compared to NMBA- treated only groups (Li et al., 2002). Treatment with resveratrol also suppressed the NMBA-induced expression of COX-2 and production of PGE_2 (Li et al., 2002). In another study resveratrol suppressed DMBA-induced mammary carcinogenesis in female rats and this suppression was associated with an inhibition of COX-2 and NF-κB activation (Banerjee et al., 2002).

Resveratrol can non-competitively inhibit the cyclooxygenase activity of COX-1 in a dose dependent manner (Shin et al., 1998). Resveratrol is able to inhibit the hydroperoxidase activity of COX-1, and to a much lesser extent COX-2, this is in contrast with most NSAIDs like aspirin and piroxicam (Jang et al., 1997). The cyclooxygenase activity of COX-2 is unaltered by resveratrol, or at most is slightly enhanced (Jang et al., 1997; Johnson & Maddipati, 1998). *In vitro* co-treatment with resveratrol (2.5-20 μM) inhibits a PMA-mediated increase in the production of PGE_2 by a direct dose-dependent inhibition of COX-2 activity and by the suppression of the increase in *COX-2* transcription without an alteration in the amount of COX-1 (Subbaramaiah et al., 1998). Resveratrol suppresses the PMA-mediated activation of *COX-2* transcription by inhibiting the protein kinase C (PKC) signal transduction pathway at multiple levels, namely by inhibiting the translocation of PKC from the cytosol to the membrane and the suppression of the activator protein-1 (AP-1)-mediated gene expression (Subbaramaiah et al., 1998).

Resveratrol has been reported to reduce iNOS expression and subsequent NO production in cultured cells (Chan et al., 2000; Tsai et al., 1999; Wadsworth & Koop, 1999) stimulated with LPS and/or IFN. This inhibition of iNOS by resveratrol is mediated by the down regulation of NF-κB binding activity via the blockade of IκB (Tsai et al., 1999). Resveratrol has also been shown to be a potent inhibitor of NF-κB nuclear translocation and IκB degradation (Holmes-McNary & Baldwin Jr, 2000). The effects of resveratrol were mediated through the inhibition of IKK, however, not through direct inhibition (Holmes-McNary & Baldwin Jr, 2000).

Resveratrol is also involved in the modulation of cytochrome P450 enzymes. Many carcinogens require metabolism in the body to acquire their detrimental activity. One of the most important enzymes in the formation of carcinogens is the cytochrome P450 isoenzyme 1A1 (CYP1A1). This enzyme is responsible for a pivotal step in the biological activation of many aryl hydrocarbon (AH) carcinogens, such as benzo(*a*)pyrene (B(*a*)P). AHs bind to the cytosolic AH receptor which then translocates to the nucleus, and in conjunction with the AH nuclear translocator, binds to the *CYP 1A1* promoter, resulting in increased transcription of *CYP 1A1*. Resveratrol has been shown to inhibit the activity of CYP 1A1 and also to inhibit the transcription of *CYP 1A1* in hepatocytes (Ciolino & Yeh, 1999), by preventing the binding of the AH receptor to the promoter region of the gene.

The enzyme CYP1B1 is thought to be overexpressed in some human tumours, it catalyses aromatic hydroxylation reactions. Potter *et al* showed that resveratrol undergoes metabolism by the cytochrome P450 enzyme CYP1B1 to generate a metabolite which has been identified as piceatannol (Potter et al., 2002b). This observation was interpreted as an explanation for the anti-tumour properties of resveratrol (Potter et al., 2002b). The anti-tumour properties of piceatannol will be discussed further in chapter 1.9.

1.7.4. Pharmacokinetics of resveratrol

The response of living organisms to resveratrol depends on its bioavailability (Fremont, 2000). The health benefits obtained by orally administering resveratrol were shown over twenty years ago by the ability of the gastrointestinal tract to absorb resveratrol (Fremont, 2000). This study showed that when rats were fed a diet which induced hyperlipidaemia, resveratrol was able to inhibit the hepatic accumulation of triacylglycerol and cholesterol (Arichi et al., 1982). Bertelli *et al* (Bertelli et al., 1996) demonstrated that resveratrol present in red wine was absorbed by rats. Resveratrol was administered as either an acute intragastric dose of 26 μg or as a repeated daily dose of 13 μg over 15 days, and was shown to rapidly enter the systemic circulation where it could be detected in the plasma (Bertelli et al., 1996). The same group, showed in two further studies, that intragastric dosing of 28 μg per rat produced significant cardiac bioavailability and high concentrations were also found in the liver and kidneys (Bertelli et al., 1998b). The results of their study on platelet aggregation confirmed that

even with modest doses of resveratrol, similar to those consumed daily by average drinkers of red wine, a pharmacological effect could be observed, and that these doses are comparable with the resveratrol concentrations obtained after oral administration in rats (Bertelli et al., 1998a).

Since these early studies, which primarily investigated resveratrol found in red wine, numerous studies have been conducted to further understand the bioavailability and metabolism of resveratrol. The resveratrol content of wine is approximately 5-10 mg/L (Fremont, 2000). Assuming moderate consumption of 250ml per 70kg person, intake of resveratrol through wine in humans is approximately 18 μg/kg per day (Gescher & Steward, 2003). This figure is a lot lower than the dose employed by Goldberg *et al* (Goldberg et al., 2003), where a dose of 360 μg/kg was used, 20 times greater than the dose considered normal consumption in humans. The resveratrol was dissolved in three different dietary matrices, grape juice, vegetable juice or white wine and a sensitive gas chromatography-mass spectrometry method was employed to measure resveratrol levels (Goldberg et al., 2003). Peak plasma concentrations of resveratrol were found to be 20nM authentic resveratrol and 2 μM "total" resveratrol 30 minutes post ingestion, and this concentration was regardless of the dietary matrix in which the resveratrol was dissolved (Goldberg et al., 2003). Preclinical studies performed in rats, using HPLC methods, provide consistent evidence that peak plasma levels are achieved 5-10 minutes post dosing and that rapid elimination of resveratrol from the plasma occurs with the half-life ranging from 12-15 minutes (Gescher & Steward, 2003). Although the peak plasma levels were all seen at similar time points post dosing, the actual concentrations of resveratrol in these studies differ. The three studies employed doses of 2 mg/kg (Juan et al., 2002a), 20 mg/kg (Asensi et al., 2002), and 50 mg/kg (Marier et al., 2002) resveratrol, each administered intragastrically. The peak plasma levels of resveratrol were found to be 2.0, 1.2 and 6.6 μM respectively. Marier et al (Marier et al., 2002) reported that the peak levels of the resveratrol glucuronide (~105 μM) were much higher than that of the parent compound and this study provided evidence to suggest that extensive enterohepatic recirculation was taking place. Using radiolabelled resveratrol administered via the oral route, relatively low plasma levels were found (peak level reached 1.5 μM) in mice (Vitrac et al., 2003). This finding is consistent with other results following orally administered resveratrol (Juan et al., 1999; Soleas et al., 2001a). Interestingly, during the duration of the experiment the concentration of radioactivity in the intestinal tract was higher in the proximal region than in the distal

end, suggesting that resveratrol was completely absorbed in the small intestine (Vitrac et al., 2003). This finding is comparable to that seen in the rat in which, 50-75% of the dose, was absorbed (Soleas et al., 2001a). Radioactivity was also recovered from the stomach, liver, kidney, intestine, bile and urine (Vitrac et al., 2003). A very low concentration of radioactivity was recorded in the colon suggesting that faecal excretion is a minor route of elimination. In contrast, decreasing levels of radioactivity in the kidney over time indicates that renal excretion of resveratrol might be one of the major routes of elimination, especially in view of the fact that high concentrations of radioactivity were found in the urine (Vitrac et al., 2003).

All of these findings suggest that resveratrol is absorbed in the intestinal tract, and is effectively metabolised in the liver and gut via conjugation. However, peak plasma concentrations of resveratrol, from all of these studies described above, are below 5 μM, even after high oral dosing and elimination from the body is exceedingly rapid due to conjugation. For this reason, analogues of resveratrol have been investigated, which comprise the same stilbene framework but with modified substituents that potentially possess increased bioavailability and are more potent with regard to their chemopreventive activity.

1.8. Resveratrol analogues

In the wake of the discovery of the interesting pharmacological properties of resveratrol, the trihydroxystilbene scaffold has become the subject of imaginative synthetic manipulations by medicinal chemists with the aim of generating novel analogues of pharmacological interest and to characterise structural features, which impart activity to the molecule. These structural alterations have been aimed at the optimisation of a number of properties of the molecule. These include its cytochrome P450 enzyme-inhibitory and antimutagenic potency (Chun et al., 2001a; Kim et al., 2002), its antioxidant activity (Lu et al., 2001), its apoptosis-inducing and growth-inhibitory activity (Kim et al., 2002; Lu et al., 2001; Nam et al., 2001) and its ability to inhibit cell transformation (She et al., 2003). These chemical modifications of the basic structure have predominantly been concerned with the introduction into the trihydroxystilbene framework of additional hydroxy moieties and with various degrees of methylation of the phenol groups. The structures and potential chemopreventive activities of some of these resveratrol analogues can be seen in table 1.8.1.

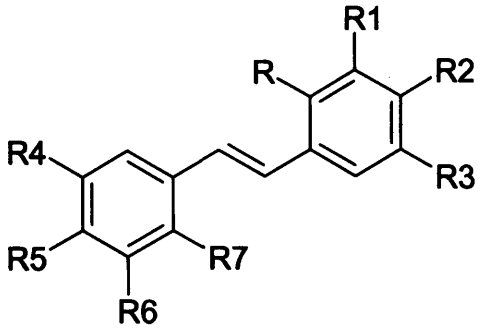


Table 1.8.1. Chemical structures and potential chemopreventive effects of some of the most interesting and more active resveratrol analogues investigated to date.

R	R1	R2	R3	R4	R5	R6	R7	Effects	Reference
-H	-OH	-H	-OH	-OH	-OH	-H	-H	Potent antiarrhythmic agent with cardioprotective activity	(Hung et al., 2001)
-H	-OH	-H	-OH	-H	$-OCH_3$	-H	-H	IC_{50} – 30.3μM on nitrite production	(Cho et al., 2002)
-H	$-OCH_3$	-H	$-OCH_3$	-H	$-OCH_3$	-H	-H	IC_{50} – 23.5μM on nitrite production	(Cho et al., 2002)
-H	-OH	-H	-OH	-OH	$-OCH_3$	-H	-H	IC_{50} – 24.6μM on nitrite production	(Cho et al., 2002)
-H	-OH	-H	-OH	-H	-OH	-H	-OH	IC_{50} – 63.5μM on nitrite production	(Cho et al., 2002)
-H	-OH	-OH	-OH	-H	-OH	-H	-H	Induces pro-apoptotic p53/Bax gene expression and inhibits growth only in transformed cells	(Lu et al., 2001)
-H	$-OCH_3$	$-OCH_3$	$-OCH_3$	-H	$-OCH_3$	-H	-H	Induces pro-apoptotic p53/Bax gene expression and inhibits growth on ly in transformed cells	(Lu et al., 2001)
-H	-OH	-H	-OH	-OH	-OH	-OH	-H	Potent inhibitory effect on EGF-induced cell	(She et al., 2003)

								transformation but less cytotoxic effects on normal untransformed cells	
-H	$-OCH_3$	-H	$-OCH_3$	-OH	$-OCH_3$	-H	-H	Antiproliferative (IC_{50}) and apoptotic-inducing activity (AC_{50}) of 0.7 and 0.9μM, respectively	(Roberti et al., 2003)
-H	$-OCH_3$	-H	$-OCH_3$	-OH	-OH	-H	-H	Antiproliferative (IC_{50}) and apoptotic-inducing activity (AC_{50}) of 0.7 and 0.9μM, respectively	(Roberti et al., 2003)
-H	-OH	-OH	-H	-OH	-H	-OH	-H	Antioxidant and free radical scavenging properties more active than resveratrol	(Wang et al., 1999)
-H	-OH	-OH	-OH	-OH	-H	-OH	-H	Antioxidant and free radical scavenging properties more active than resveratrol	(Wang et al., 1999)
-H	-OH	-OH	-OH	-H	-OH	-H	-H	Antioxidant and free radical scavenging properties more active than resveratrol	(Wang et al., 1999)
-H	-H	-H	-H	-H	-OH	-H	-H	Antioxidant and free radical scavenging properties similar to that of resveratrol	(Stojanovic et al., 2001)
-H	-OH	-H	-OH	-H	-OH	-OH	-OH	Potent inhibitory effect on tyrosinase activity	(Kim et al., 2002)
-H	$-OCH_3$	-H	$-OCH_3$	-OH	-OH	-H	-H	Peroxyl-radical scavenging potential similar to resveratrol but much less effective on the	(Rimando et al., 2002)

								inhibition of both isoforms of cyclooxygenases	
-H	-OH	-H	-OH	-H	$-OCH_3$	-OH	-H	Potent mechanism-based inactivator of human P450 1A1	(Chun et al., 2001b)
-H	$-OCH_3$	$-OCH_3$	$-OCH_3$	-H	-H	-H	-H	Potent cytotoxic effects greater than resveratrol in cultured human lung and colon cancer cells	(Lee et al., 2003)
-H	$-OCH_3$	-H	$-OCH_3$	-H	$-OCH_3$	-H	$-OCH_3$	Potent cytotoxic effects greater than resveratrol in cultured human lung and colon cancer cells	(Lee et al., 2003)

Another study did not strictly focus on resveratrol anologues, but rather investigated a common resorcin moiety found in five chemopreventive agents, including resveratrol (Mutoh et al., 2000b). The authors showed that these agents with this resorcin-type structure exhibited inhibitory effects on COX-2 promoter activity (Mutoh et al., 2000b). Another compound, daidzein, an analogue of genistein which had shown an inhibitory effect, was lacking in this resorcin-type structure and did not inhibit COX-2 promoter activity (Mutoh et al., 2000b). It was therefore concluded that the resorcin moiety might play a critical role in the inhibition of the COX-2 promoter activity, although other physiochemical factors may also be involved (Mutoh et al., 2000b) (Chemical structure of resorcin structure is shown in figure 1.8.1.).

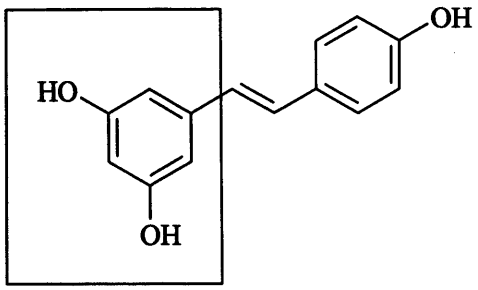


Figure 1.8.1. Chemical structure of resveratrol, with the resorcin moiety indicated by the box.

1.9. Mechanisms of action of resveratrol analogues

Wolter *et al* (Wolter et al., 2002) studied the effects of a naturally occurring resveratrol analogue, piceatannol on growth, proliferation, differentiation and cell cycle in the human cancer cell lines, CaCo-2 and HCT-116 (Wolter et al., 2002). The treatment of CaCo-2 cells with piceatannol, at a dose range between 12.5–200μM (for structure see figure 1.9.1.), caused inhibition of cellular proliferation; accumulation of cells in the S phase; and down-regulation of cyclin D1, cyclin B1 and cdk 4 (Wolter et al., 2002).

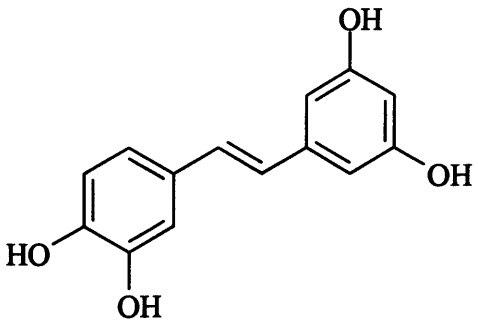


Figure 1.9.1. Chemical structure of piceatannol.

Piceatannol has been shown to have antileukaemic activity and is able act as a tyrosine kinase inhibitor (Palmieri et al., 1999). Piceatannol has also been shown to suppress NF-κB dependent reporter gene expression, IκB phosphorylation, p65 phosphorylation and IKK activation (Ashikawa et al., 2002). Stilbene which lacks functional hydroxyl groups, have been investigated and showed no effect on NF-κB activation. This finding

would suggest the hydroxyl groups are an essential feature for activity. Rhaponticin, a dihydroxystilbene (for chemical structure see figure 1.9.2), was also shown not to effect NF-κB activation (Ashikawa et al., 2002). This result highlighted that it is not only the presence of the hydroxyl groups that is important but also their position.

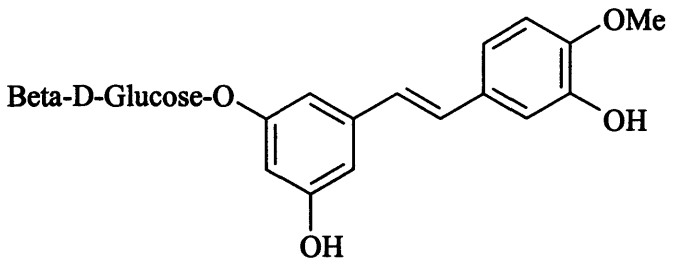


Figure 1.9.2. Chemical structure of rhaponticin.

An especially interesting finding concerning these resveratrol analogues is the fact that 3,4,5,4'-tetrahydroxystilbene, resveratrol with an additional hydroxy moiety, and its *O*-methylated analogue 3,4,5,4'-tetramethoxystilbene (DMU 212, for structure see figure. 1.9.3), were capable of preferentially interfering with proliferation and survival of transformed human lung-derived cells, with much lower growth–inhibitory and apoptotic properties than their untransformed counterparts (Lu et al., 2001). In contrast, resveratrol did not possess this discriminatory potential. DMU 212 is currently under preclinical evaluation as a potential antitumour prodrug which undergoes metabolic activation by certain cytochrome P450 enzymes (Potter et al., 2002a).

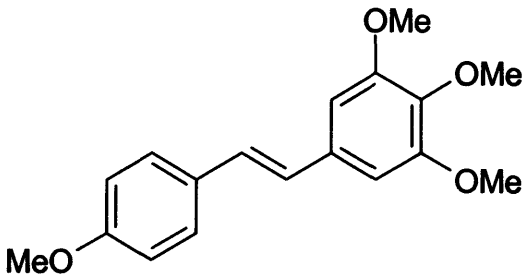


Figure 1.9.3. The chemical structure of 3,4,5,4'-tetramethoxystilbene (DMU212)

1.10 Project Aims

The main aim of the work as described in this thesis was to investigate resveratrol *vis-à-vis* DMU212, a resveratrol analogue, and compile data that will help contribute to decisions on the choice of resveratrol analogues for clinical development as colorectal cancer chemopreventive agents.

To achieve this main aim the following specific objectives were investigated:

i. comparison of the bioavailability of resveratrol and DMU212 to test the hypothesis that DMU212 may be more bioavailable due to its multiple methoxy groups.

ii. compare both agents in terms of their ability to interfere with colorectal cancer cell proliferation.

iii. to explore both agents ability to modulate COX-2 expression.

iv. to compare the ability of both agents to interfere with COX activity

v. to compare both agents efficacy in the Min/+ mouse model.

vi. to explore both agents ability to modulate pharmacodynamic biomarkers *in vivo*.

CHAPTER 2

MATERIAL AND METHODS

2.1 Materials

2.1.1. Chemicals and kits

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Company Limited (Poole, Dorset, UK), and solvents from Fisher Scientific (Loughborough, Leicestershire, UK).

Acrylamide (30% acrylamide: bis acrylamide)	Anachem
Agarose	Gibco BRL
AIN93G Animal Diet	Dyets Inc
Alpha Tubulin antibody	Oncogene Research Products
Annexin V kit	Bender MedSystems
Anti-goat HRP linked secondary antibody	Santa Cruz Biotechnology
Anti-mouse HRP linked secondary antibody	Oncogene Research Products
BioRad protein assay reagent	Bio-Rad
Broad range protein molecular markers	Bio-Rad
C57BL/6J Min/+ breeding pairs	Jackson Laboratories
C_{18} reverse phase columns	Varian
COX-1 purified protein	Alexis Biosciences
COX-2 purified protein	Alexis Biosciences
COX activity kit	Assay Designs
COX-2 antibody	Santa Cruz Biotechnology
DNA molecular weight markers	Gibco BRL
DMU212 and metabolite standards	Gift from Prof. Gerry Potter
ECL detection kit	Amersham
ECL-hyperfilm	Amersham
Foetal calf serum	Gibco BRL
HindIII restriction enzyme	Roche Diagnostics
Hybond nitrocellulose	Amersham
Isoton	Beckman Coulter
Male C57BL/6J mice	Charles River
Marvel (dried milk powder)	Premier Brands
Mouse plasma	Charles River
Prostaglandin E2 kit	R & D Systems

Reddy Load PCR mix	Advanced Biotechnologies
Resveratrol	Changchun Kingherb
RM3 animal diet	SDS
SuRE/Cut Buffer B	Boehringer Mannheim
TBE 10x buffer	Invitrogen
Trypsin/EDTA	Gibco BRL

2.1.2. Suppliers addresses

Advanced Biotechnologies, Surrey, UK.

Alexis Biosciences, UK

Amersham Pharmacia Biotech, Buckinghamshire, UK.

Anachem, Bedfordshire, UK.

Assay Designs, Ann Harbor, Michigan, USA.

ATCC, Manassas, V.A., USA.

Beckman Coulter, High Wycombe, UK.

Bender MedSystems, Vienna, Austria.

Bio-Rad, Hertfordshire, UK.

Boehringer Mannheim, Germany

Charles River, Margate, UK.

Changchun Kingherb International co., Ltd. Changchun, China.

Dyets Inc, Bethlehem, PA, USA.

Gibco-BRL (Invitrogen Life Technologies), Paisley, UK.

Jackson Laboratory, Bar Harbor, ME, USA.

Micromass, Manchester, UK.

Oncogene research products, California, USA.

Phenomenex, Cheshire, UK.

Premier Brands, Wirral, UK.

R & D Systems, Abingdon, UK.

Richardsons, Leicester, UK.

Roche Diagnostics, East Sussex, UK.

Santa Cruz Biotechnology, California, USA.

Special Dietary Services (SDS), Witham, UK.

Varian Inc, Surrey, UK.

2.2. Buffers

Annexin Buffer

Annexin buffer was supplied as a 4x concentrated stock that was stored at 4°C and diluted to a 1x solution in distilled water prior to use. The working concentration was 10 mM HEPES (pH 7.4), 140 mM NaCl and 2.5 mM $CaCl_2$.

COX-2 cell lysis buffer

COX-2 lysis buffer was prepared as a 10x stock and stored at 4°C. Immediately prior to use the 10x stock solution was diluted in distilled water to a 1x stock and complete protease inhibitor cocktail (1:100 dilution) was added. The 10x stock solution consisted of 0.15 M NaCl, 0.1 M Tris base (pH 8.0), 1 % Tween-20, 50 mM diethyl dithio carbamic acid (DDC) and 1 mM EDTA (pH 8.0) made up in distilled water.

HEPES buffer

HEPES buffer was used for the homogenisation of animal tissue and consisted of 50 mM HEPES (pH 7.5), prepared in HPLC grade water. The solution was filtered prior to use.

Neutral buffered formalin (10%)

The solution of 10 % neutral buffered formalin consisted of 10 % v/v formalin, 29 mM NaH_2PO_4 and 46 mM Na_2HPO_4 made up to the correct volume with distilled water.

PBND buffer

PBND buffer consists of 50 mM KCl, 10 mM Tris-HCl, 0.1 mg/ml gelatin, 0.45 % v/v IPEGAL CA 630 and 0.45 % v/v Tween-20 made up to appropriate volume in distilled water. The PBND buffer was stored at -20°C prior to use in 20 ml aliquots.

Polyacrylamide stacking gel (recipe for two gels)

To prepare two gels, 1.25 ml of 30 % acrylamide solution, 1.7 ml of 0.5 M Tris (pH 6.8), 6.8 ml distilled water and 0.1 ml 10 % SDS were mixed. To initiate polymerisation 0.1 ml 10 % ammonium persulphate and 0.01ml TEMED were added.

Polyacrlyamide resolving gel (recipe for two gels)

To prepare two gels, 6.8 ml of 30 % acrylamide solution, 5 ml of 1.5 M Tris (pH 8.8), 8 ml distilled water and 0.2 ml 10 % SDS were mixed. To initiate polymerisation 0.2 ml 10 % ammonium persulphate and 0.01ml TEMED were added.

Sample buffer (3x)

Sample buffer was prepared with 187.5 mM Tris-HCl (pH 6.8), 6% SDS, 30% v/v glycerol, 0.03 % bromophenol blue and 0.3 M dithiothreitol. Distilled water was used to make up the solution which were then stored as aliquots at -20°C.

Tris buffered saline tween (TBST)

TBST was prepared as a 1x stock and consisted of 20 mM (pH 7.5), 150 mM NaCl and 0.1 % Tween-20. The solution was diluted to the appropriate volume with distilled water.

Transfer buffer

Transfer buffer consisted of 47 mM Tris base, 37 mM glycine, 20 % methanol and 0.4 % SDS and diluted to the appropriate volume with distilled water.

Western running buffer

The Western running buffer was prepared as a 10x stock solution and diluted to a 1x stock with distilled water prior to use. The initial 10x stock solution consisted of 0.25 M Tris base, 1.9 M glycine, and 1 % SDS, pH 8.3.

Western stripping buffer

The stripping buffer consisted of 62 mM Tris base and 2 % SDS in distilled water. Immediately prior to use, 0.8 % v/v 2-mercaptoethanol was added to an aliquot of the stock solution.

Tris-phenol buffer

Tris-phenol buffer consists of 100 mM Tris, 0.5 mM phenol buffer. The buffer is prepared to pH 7.3 at 37°C to ensure correct pH measurement.

2.3. Methods

2.3.1. Methods and analysis of plasma and tissue samples

2.3.1.1. HPLC apparatus and conditions

HPLC analysis was carried out using a Varian Prostar HPLC system (Varian, UK) with a Pro-Star 230 solvent delivery system, a Pro-Star 310 UV-Vis detector, a Pro-Star 363 fluorescence detector, a 410 Varian autosampler and an Ultracarb C_{18} column (4.6 mm x 250 mm, 5 μm, Phenomenex, UK). The mobile phase consisted of the three components: aqueous ammonium acetate (pH 6.5, 50 mM), propan-2-ol and acetonitrile. The gradient system which determined the composition of the eluent for the three components was as follows: For resveratrol 80:10:10 at the start, 75:10:15 at 10 min, 70:10:20 at 15 min, 60:10:30 at 17 min, 50:10:40 at 20 min and 20:10:70 at 25 min; for DMU 212 45:10:45 at the start for up to 10 min, 20:10:70 at 15 min and 10:10:80 at 20 min. The flow rate for both methods was 1 ml/min.

Internal standards were carbamazepine and 4'-methoxy-4-methyl-*trans*-stilbene for resveratrol and DMU 212, respectively. Detection of resveratrol and its metabolites was achieved using UV detection (325 nm). DMU 212 and its metabolites were detected using fluorescence detection (335 nm excitation, 395 nm emission). The injection volume of samples reconstituted in mobile phase was 50 μl.

2.3.1.2. HPLC standards

Resveratrol, DMU 212 and its metabolites referred to in this thesis are exclusively the *trans*-isomers. Resveratrol was purchased from Changchun Kingherb International co., Ltd. (Changchun, China). Authentic resveratrol-3-sulfate was a gift from Dr. Tristan Booth (Mount Royal Pharma, Montreal, Canada), and its identity was corroborated by mass spectrometry. DMU 212 (3,4,5,4'-tetramethoxystilbene) was synthesized by Wittig olefination involving reaction of 4-methoxybenzyl-tri-phosphonium chloride with 3,4,5-trimethoxybenzaldehyde (Potter et al., 1999). This reaction yielded the *–cis* and *–trans* geometric isomers, which were separated by preparative column chromatography. The *–trans* isomer was purified by recrystallisation from ethanol. The DMU 212 analogues to be used for metabolite identification 4,4'-dihydroxy-3,5-dimethoxystilbene (4,4'-di-desmethyl-DMU 212, DMU 295), 4'-hydroxy-3,4,5-trimethoxystilbene (4'-desmethyl-DMU 212, DMU 281), 4-hydroxy-3,5,4'-trimethoxystilbene (4-desmethyl-DMU 212, DMU 291) and 3-hydroxy-4,5,4'-trimethoxystilbene (3-desmethyl-DMU 212, DMU 807) were synthesized in a similar fashion using the tert-butylmethylsilyl-protected Wittig precursors, and final deprotection with tetrabutylammonium fluoride (Potter et al., 1999). 3'-Hydroxy-3,4,5,4'-tetramethoxystilbene (3'-hydroxy-DMU 212, DMU 214, *trans* isomer of combretastatin A4) was synthesised according to the method of Pettit *et al* (Pettit et al., 1995). For chemical structures of DMU212 metabolites see figure 3.3.1.2.

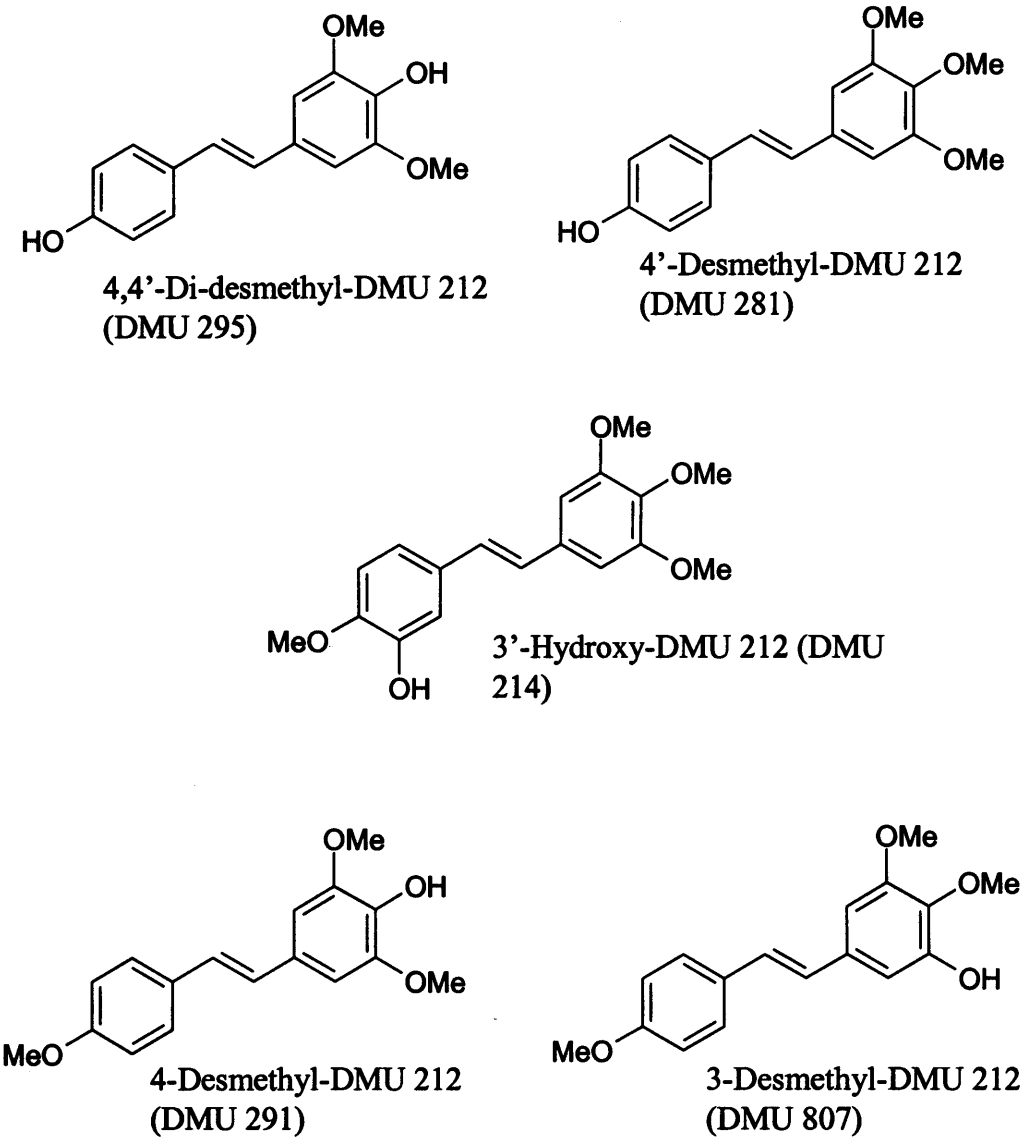


Figure 2.3.1.2. Chemical structures of DMU212 metabolites.

Resveratrol, carbamazepine, DMU216, DMU212 and all its metabolite standards were > 98 % pure. Stock solutions of each compound were prepared separately in DMSO. All stocks were prepared at a concentration of 1 mg/ml and serial dilutions were then prepared in mobile phase for each HPLC method to construct calibration curves (section 2.3.1.4.). Stock solutions were stored at -80°C and stable for a minimum of 3 months.

2.3.1.3. Plasma sample preparation for HPLC

Aliquots of 250µl of mouse plasma were spiked with an internal standard. For resveratrol samples 20 µg of carbamazepine was added to each sample and 2 µg of DMU216 for DMU212 samples. A volume of 1 ml of acetonitrile was added to each sample and vortexed for 1 minute, and placed on ice for 5-10 min before being vortexed again for a further minute. The samples were then centrifuged at 4000 rpm for 10 min at 4°C (Hereaus Megafuge EBA 12) and the supernatants were removed and pipetted into fresh sterile tubes. After centrifugation, the extractions were then evaporated to dryness under a stream of nitrogen before reconstitution in 100 µl of mobile phase prior to HPLC analysis.

2.3.1.4. Tissue sample preparation for HPLC

Tissue samples were obtained as described in chapter 3.6.1.1. Each tissue was thawed, blotted dry, weighed and homogenised (1:1 w/v) in 50 mM HEPES buffer at pH 7.5. Aliquots of 250µl of tissues were spiked with an internal standard. For resveratrol samples 20 µg of carbamazepine was added to each sample and 2 µg of DMU216 for DMU212 samples. A volume of 1 ml of acetonitrile was added to each sample and vortexed for 1 minute, and placed on ice for 5-10 min before being vortexed again for a further minute. The samples were then centrifuged at 4000 rpm for 10 min at 4°C (Hereaus Megafuge EBA 12) and the supernatants were removed and pipetted into fresh sterile tubes. After centrifugation, the extractions were then evaporated to dryness under a stream of nitrogen before reconstitution in 100 µl of mobile phase prior to HPLC analysis.

2.3.1.5. Calibration curves

Calibration curves were constructed by spiking blank mouse plasma or tissues with a known quantity of stock solution. Aliquots of 250µl of plasma or tissues were spiked with resveratrol or DMU212, to give a range of concentrations (6 concentration points) that ranged from 50 ng/ml-5000 ng/ml for resveratrol and 25 ng/ml-1000 ng/ml, for DMU212. The quantity of internal standard added to each sample was 20 µg of carbamazepine for resveratrol samples and 2 µg of DMU216 for DMU212 samples. Since calibration standards and samples were homogensised 1:1 (w:v) in HEPES buffer,

on the basis that 1 g tissue occupies a volume of 1 ml, then the concentration of analyte per g of tissue was twice that per ml of homogenate.

Calibration curves were prepared for each compound in each respective tissue by plotting the peak area ratio (PAR), which is the peak area of compound divided by the peak area generated by the internal standard, against theoretical compound concentrations. The calibration curves for both compounds in plasma and all tissues showed outstanding linearity, characterised by regression co-efficients of $R^2 = 0.99$ or above.

2.3.1.6. Stability

Since samples could be stored up to a month, at -80°C, before analysis and samples could sit for up to 12 h before analysis in the autosampler vial tray the stability of both compounds in these conditions was assessed. The stability of both compounds was investigated in mouse plasma at three QC concentrations (LoQC, MeQC and HiQC, three replicates) following a freeze/thaw cycle and storage for 24 h at room temperature. These samples were compared to QC samples that underwent immediate analysis after preparation.

2.3.1.7. Recovery of analytes from plasma and tissues

The recovery of both compounds from mouse plasma and tissues was assessed by comparing the gradient of the calibration curve prepared in mobile phase to that of the calibration curves produced with both compounds at the same concentrations extracted from plasma and tissues (% extracted = gradient extracted sample / gradient unextracted x 100 %). The extraction efficiencies (in percent) for resveratrol and DMU 212 were as follows: from plasma 102 ± 17 and 67 ± 2, respectively. Using the extraction method for tissues, the recovery of resveratrol and DMU212 was 86 ± 7 and 78 ± 15, respectively (mean ± SD, n=5-7).

2.3.1.8. Limits of detection and quantification in plasma

The limit of detection (LOD) is defined as three times the signal-to-noise ratio and determined in mouse plasma by the injection of extracted samples of both compounds.

The lower limit of quantification (LLOQ) is often referred to as ten times the signal-to-noise ratio and was determined for each compound according to the lowest concentration at which the CV value was less than 20 %. The LLOQ serves as the lowest concentration on the calibration curve.

2.4. Cell Culture.

2.4.1. Cell lines

HCA-7 cells, derived from a mucinous adenocarcinoma of the colon (Marsh et al., 1993) were provided by Dr. S. Kirkland (Imperial College, London, U.K.). Human colon epithelial cells (HCEC) were kindly provided by Dr A. Pfeifer (Nestec Ltd. Research Centre, Lausanne, Switzerland). HT29 cells were kindly provided by Prof Paraskeva (University of Bristol, UK) and are derived from an adenocarcinoma of the colon.

2.4.2. Maintenance of cell lines

All cell lines were cultured as adherent monolayers at 37°C, 5 % CO_2 and 100 % humidity in an incubator. All three cell lines were routinely maintained in DMEM containing Glutamax I and glucose (4500 mg/L) and 10 % foetal calf serum (Gibco, Paisley, UK).

2.4.3. Passaging of cell

Cells were passaged at approximately 80-90 % confluence and, following restoration from frozen stocks, were not sub-cultured more than 30 times.

2.4.4. Treatment of cells

Stock solutions of resveratrol and DMU212 were prepared in DMSO at a concentration of 20mM and fresh stocks were prepared for each experiment. Cells were treated so that all control and treated cells received the same percentage volume of DMSO, and this volume did not exceed a final concentration of 0.1 %. For all experiments an untreated control and an equivalent percentage DMSO only control were included.

2.4.5. Assessment of cell proliferation

Cells were seeded at a density of 1 x 10^4 per well, in a 24 well plate (Nunc, Fisher Scientific, Loughborough, UK), in DMEM containing Glutamax I and glucose (4500mg/L) and 10% foetal calf serum. Cells were treated 24 hours post plating with either resveratrol or DMU212. Resveratrol and DMU212 concentrations used were 1, 5, 10, 25, 50 and 100μM. Both compounds were dissolved in DMSO (final concentration 0.1%) and then counted 24, 96, 120, 144, and 168 hours post treatment. For cell counts, media was removed, cells washed twice in PBS buffer and 0.5ml trypsin added (5x trypsin HT-29 and 10x trypsin HCA-7). Plates were incubated for 5 minutes, after which 0.5ml media was added and the cells gently scraped. 200μl of the resultant solution was mixed with 9.8ml of Isoton and the cells counted using the Z2 Coulter Particle Count and Size Analyzer. Growth curves were repeated in triplicate and IC_{50} values were calculated from a plot of cell number as a percentage of DMSO control versus drug concentration at 168 h using the linear phase of the curve. IC_{50} values were calculated from each of three separate growth curves to provide a mean ± SD for each cell line.

2.4.6. Annexin V-FITC (Fluorescein isothiocyanate) assay for measurement of apoptosis

The method used to measure apoptosis distinguishes between live, apoptotic and necrotic cells according to the method described by Vermes *et al.* (Vermes et al., 1995). When cells enter apoptosis the phospholipids asymmetry of their plasma membrane is disrupted exposing phosphatidylserine (PS) to the outer layer of the membrane. Annexin V binds to PS in apoptotic cells as well as necrotic cells. Concurrent addition of propidium iodide (PI) discriminates between these apoptotic and necrotic cells, as the PI stain is only taken up by the necrotic cells. Neither Annexin V or PI is taken up by the live cells present. Thus the concurrent staining of cells with FITC-annexin V and PI allows one to distinguish between live cells (FITC and PI negative), early apoptotic (FITC positive, PI negative) and late or necrotic cells (FITC and PI positive).

Cells were seeded onto 6 well plates at a density that was dependent on treatment time; $2.5x10^5$ (24 h) and $1x10^5$ (48 h). The cells were allowed to adhere overnight before the

medium was removed and replaced with medium containing resveratrol or DMU212 (1-50μM) for 24 and 48 h.

Cells that had detached in the medium during the incubation time were removed and put to one side. Adherent cells were washed with PBS, trypsinised and then pooled with the detached cell media. Cells were centrifuged at 350 x *g* for 5 min at 4°C and the pellet was resuspended in 10ml fresh supplemented medium. Cells were incubated for 30 min at 37°C before being recentrifuged and the pellet formed was then resuspeded in 1 ml annexin buffer. Annexin V-FITC conjugate (5 μl of a 20 μg/ml stock) was added to the cell suspension and incubated for 8 min at room temperature, after which PI stain was added (15 μl of a 20 μg/ml stock) and the cells were incubated for 1 min at room temperature. All samples were then transferred onto ice until final analysis by flow cytometry using a FACScan flow cytometer.

2.4.7. Cell Cycle Distribution

Cells were seeded onto 6 well plates at a density that was dependent on treatment time; $1x10^5$ (24 h) and $5x10^4$ (48 h). The cells were allowed to adhere overnight before the medium was removed and replaced with medium containing resveratrol or DMU212 (1-50μM) for 24 and 48 h.

Cells were harvested by trypsinisation and then washed with PBS. Cells were pelleted by centrifugation (100 rpm; 2 mins). Cells were re-suspended in 200 μl PBS then 2 mls ice cold 70 % v/v EtOH/PBS was added while mixing vigourously. Cells were incubated overnight in the refrigerator to disrupt the cell membrane as to allow the PI stain into the cells. The cells were then pelleted once more by centrifugation (600 x g; 10 mins). The cells were then re-suspended in 800 μl PBS then 5 μl RNase A and 100 μl PI are added. Finally the cells were incubated at 37°C for 30-60 mins before being analysed using a FACScan flow cytometer.

2.5. Protein isolation and analysis

2.5.1. Preparation of whole cell lysates

Cells or tissues were lysed in 0.3 ml of COX-2 lysis buffer. Samples were vortexed and left to stand for 20 min before each sample was sonicated for 20 s. Cell lysates were then centrifuged at 13,000 rpm for 5 min and the supernatent retained. A protein assay was performed prior to running the samples to allow equal protein loading (section 2.5.2.)

2.5.2. Protein assay

The protein assay was performed to ensure equal protein loading on gels within a particular experiment. Protein concentrations of all cell lysates were measured by the Bio-Rad protein assay kit based on a method described by Bradford (Bradford, 1976). Bio-Rad assay reagent (200 µl) was added to 800 µl of diluted sample (2 µl of sample diluted in 998 µl distilled water of which 800 µl was taken for protein assay). The mixture was vortexed and left at room temperature to incubate for 5 min. The absorbance of the mixture was measured using a UV/VIS spectrophotometer at 595 nm. Protein concentrations from each sample were determined by comparison with a standard curve that was prepared using the protein standard (1 –20 mg/ml), bovine serum albumin (BSA).

2.5.3. SDS-PAGE and western blotting

To measure the effect that these stilbenes had on COX-2 protein expression 25-100 µg (25 µg for HCA-7 and HCEC cell line and 100 µg for HT29 cell line) of whole cell lysates were combined with SDS loading buffer and all samples were then boiled at 100°C for 5 min, centrifuged to collect the sample and loaded onto a polyacrylamide gel (10 %). A protein marker ladder was also added to one of the gel lanes so that the molecular weight of protein could be detected. Proteins were separated by electrophoresis in Western running buffer at 100 V for 90 min using a Bio-Rad Mini Protean 3 kit.

The separated proteins were then transferred onto hybond-N nitrocellulose membrane in Western transfer buffer using a Bio-Rad wet blotting system and run overnight at 30 V. Once transferred the blots were washed in TBST then blocked in 5 % non fat milk for 2 h on a rocking machine. The membrane was re-washed in TBST and the primary antibody added for 2h at room temperature (Table 3.5.3.1 for antibody conditions). The blots were washed 5 times for 5 min in TBST before the appropriate secondary antibody was added for 1 h at room temperature. Following a further 5 washes for 5 min in TBST the blots were visualised via chemiluminescence using ECL reagent. The blots were developed in ECL reagent (1:1 ratio of reagent A and reagent B) for 1 min, and the excess liquid drained off. The blots were wrapped protein side up in Saran wrap and placed in an autoradiographic cassette. ECL-hyperfilm was then exposed to the blots and then developed in the dark using an automated developer.

To confirm equal loading, blots were stripped (50 ml stripping buffer at 60°C in a shaking water bath for 45 min) and washed in TBST before being re blocked in 5 % non fat milk and probed with α-tubulin primary antibody. The appropriate secondary antibody was used before the blot was re-developed. The density of the bands on the films were measured, using a Syngene imaging system, and the values adjusted for protein loading.

Table 2.5.3.1. Antibodies and their condition of use. All antibodies were made up in 5 % non fat milk. Primary antibodies were incubated with the blots for 2 h at room temperature whereas secondary antibodies were incubated for 1 h at room temperature.

Primary Antibody	**Secondary Antibody**	**Molecular Weight**
COX-2 (1:1000 dilution)	Donkey anti-goat IgG (1:2000 dilution)	74 kDa
α-tubulin (1:4000 dilution)	Anti-mouse IgG (1:2000 dilution)	54 kDa

2.5.4. COX-2 protein expression *in vitro*

HT29 and HCA-7 colon cancer cells were seeded at a density that was dependant on treatment time; 2 x 10^6 (24 h), 1 x 10^6 (48 h) and 5 x 10^5 (96 h) in 9-cm plates (Nunc, Fisher Scientific, Loughborough, UK), in DMEM containing Glutamax I and glucose

(4500mg/L) and 10% foetal calf serum. HCEC colon cells were seeded at a density of 2 x 10^6 cells. After overnight adherence, the medium was removed and replaced with medium containing resveratrol (1-50μM) or DMU212 (1-50 μM) for varying times from 24-96 h for HT29 and HCA-7 cells and 1 h for the HCEC cell line. As HCEC colon cells do not express endogenous COX-2 protein after the 1 h incubation PMA (50 ng/ml) was added to the treatments for 5 h to induce COX-2 protein. PMA was not added to the other two cell lines as they were known to over express COX-2 protein already. After incubation for the appropriate time whole cell lysates were prepared. Medium was removed and the cells washed in ice cold PBS. The cells were lysed in 300μl lysis buffer on ice for 10 min. Lysates were harvested by scraping into eppendorf tubes and were then sonicated for 10 s and cleared by centrifugation at 13000 g for 5 min, 4°C. Western blot analysis for COX-2 protein levels were carried out and equal protein loading was determined with α-tubulin (Oncogene Research Products). Respective levels of both COX-2 and α-tubulin were determined by densitometric analysis.

2.5.5. Prostaglandin E2 (PGE_2) Assay

Aliquots of medium were taken from all the treatments described above and stored at -80°C until analysis for the HT29 and HCA-7 cell line samples. HT29 and HCA-7 colon cancer cells are both able to generate the prostaglandin PGE_2 without the additionally supplementation of the media with arachidonic acid. However, to measure PGE_2 levels in HCEC cell media the conditions were repeated as described above except arachidonic acid was added (50 μM) to each sample at the same time as the stilbene treatment to act as a substrate for PGE_2 formation. The incubation time was 24 h, as this was the found to be the optimum incubation period (Sharma., data unpublished). PGE_2 levels were measured from all these samples using the method described with the PGE_2 immunoassay kit and quantities of PGE_2 calculated for each sample as PGE_2 pg/mg protein.

2.5.6. COX Activity Assay

Inhibition of COX activity by the stilbene derivatives was investigated using a chemiluminescent cyclooxygenase activity kit (R&D Systems). Purified COX-1 (95 % pure) and COX-2 protein (70 % pure) was obtained from the seminal vesicles and

placenta of sheep, respectively (Alexis Biosciences, UK). Enzyme activity was measured following the instructions of the manufacturer (Assay Designs, Ann Arbor, Michigan, USA). In this assay the peroxidative activity of COX enzymes is measured following addition of arachidonic acid and a co-substrate which generates a chemiluminescent product. Enzyme preparations were incubated for 2 h with stilbene derivatives (0.1 nM–50 μM), or ibuprofen (0.01 μM–27 mM) or the COX-2-selective inhibitor NS 398 (0.01–90 μM) for validatory purposes. Light emission, which was measured over 15 sec using a BMG Fluostar Optima luminometer (BMG Labtech Company, UK), was directly proportional to residual COX activity. Experiments were repeated in triplicate.

2.6. Animal experimentation

All experiments in mice were conducted as stipulated by the Animals (Scientific Procedures) Act 1986 Project Licence 40/2496 granted to Leicester University by the United Kingdom Home Office, and experimental design was vetted and approved by the Leicester University Ethical Committee for Animal Experimentation. The animals were fed on a standard diet, AIN93G unless otherwise stated and water *ad libitum*. The animals were reared under positive pressure isolator conditions and were routinely tested and shown to be pathogen free by bacteriological and serological testing.

2.6.1. Pharmacokinetic studies

2.6.1.1. Animals and treatment

C57BL/6J wild-type male mice aged 8 weeks (20-22 g) received resveratrol or DMU 212 (240 mg/kg body weight, equivalent to 1 mmol/kg resveratrol or 0.8 mmol/kg DMU 212) *via* the intragastric cannulation route (3 animals per time point). The vehicle was glycerol formal, and the dose volume approximately 10 ml/kg. In the case of DMU 212 the vehicle also contained 10 % DMSO. Mice were killed by terminal gaseous anaesthesia (halothane, Sigma, UK) at 10, 30, 60 or 120 min *post* dosing. Blood was collected into heparinized tubes by cardiac puncture, and plasma was obtained by centrifugation and stored at -80°C until analysis. Liver, kidney, lung, heart, brain and gut were excised and scrapings were obtained from small intestine and colon. All tissues were snap-frozen (liquid nitrogen) and stored at –80°C until analysis.

2.6.1.2. Metabolism studies using liver microsomes to identify stilbene metabolites

Microsomes were prepared by differential centrifugation of fresh mouse liver homogenate. One gram of liver was homogenised using a Potter-Elvehjem type Teflon glass homogeniser (clearance 0.125mm, 10 passes at 1100 rpm) in 5 ml ice cold Tris buffer (0.05 M Tris, 154 mM KCl, 0.25 M sucrose, pH 7.6). Tissues were kept a 4°C during the preparation of the microsomes. The homogenate was centrifuged first at $9x10^3g$ (20 min, 4° C), then at 10^5 g (1 h) in a Beckman L-8-60 ultracentrifuge (Beckman Coulter UK Ltd, High Wycombe, Buckinghamshire, UK). The microsomal pellet was suspended in Tris buffer, recentrifuged at 10^5g (1 h), and then the final pellet was resuspended in storage buffer (0.25 M potassium phosphate buffer containing 30 % glycerol), aliquoted and stored at -80°C until analysis.

Microsomes (0.5 mg protein/ml) were incubated at 37°C with NADPH (1 mM), $MgCl_2$ (1 mM) and resveratrol or DMU 212 (1 mM) for 20 min (final volume: 0.2 ml). Addition of one volume (0.2 ml) of ice-cold methanol terminated the reaction. The mixture was vortexed (30 sec), centrifuged (3 min, 13,400 g) and the supernatant was collected and analysed by HPLC. For the biosynthesis of resveratrol glucuronide for use as reference compound, microsomes were incubated with resveratrol (1 mM) as described above, except that NADPH was replaced by uridine-diphosphoglucuronic acid (1 mM).

2.6.1.3. Mass spectrometry to identify stilbene metabolites

Mass spectrometry was performed using a Quattro Bio-Q tandem quadrupole mass spectrometer upgraded to Quattro MK II specifications (Micromass, Manchester, UK) with a pneumatically assisted electrospray interface. Samples were analysed in positive ion mode. The temperature was maintained at 120°C, the operating voltage of the electrospray capillary was 3.88 kV and the cone voltage 32 V. HPLC conditions used for the on-line HPLC-mass spectrometric analyses were as described for DMU 212 above.

2.6.1.4. Determination of steady state levels of stilbene derivatives

To determine steady state levels of stilbene, C57BL/6J mice were fed 0.05, 0.2 and 0.5 % of either stilbene admixed in the diet (AIN93G) for three weeks. At the end of the study animals were culled by cardiac exsanguinations under halothane anaesthesia and samples of blood and tissues were taken from each animal and analysed for the presence of either resveratrol or DMU212 and their respective metabolites using reversed-phase HPLC methods described previously (Chapter 3.3.1.1.).

2.6.2. Maintenance of C57BL/6J $Apc^{Min/+}$ mouse colony

To establish a breeding colony, male C57BL/6J $Apc^{Min/+}$ mice (referred to in the following as "Min/+ mice") were purchased from the Jackson Laboratory (Bar Harbor, ME) and mated with female C57BL/6J mice (wild-type) also obtained from Jackson Laboratory (Bar Harbor, ME). The colony was maintained on a C57BL/6J background whereby Min/+ males were mated with wild type C57BL/6J females. The breeding animals were fed on a standard pelleted diet, RM3 and water *ad libitum.* Ear punching of offspring of batch matings were carried out at 3 weeks of age which permitted animal identification and the superfluous tissue was retained for genotyping. Biomedical Services, University of Leicester, performed general maintenance of the C57BL/6J Min/+ mice colony.

The experiments described in 2.6.2.5 were conducted with two different cohorts of mice and were performed at an 8 months interval. Control animals from the experiment exploring the effects of 0.2 % dietary resveratrol and DMU212 on adenoma number averaged 105.8 ± 8.5 adenomas in the small intestine and 8.7 ± 1.5 in the colon (n = 10), whereas the control animals from the experiment exploring adenoma number following the dietary administration of 0.05 and 0.5 % resveratrol and DMU212 averaged 55.0 ± 9.2 adenomas in the small intestine and 4.4 ± 0.9 in the colon (n = 12). For this reason, findings from these experiments are presented as percentage of control rather than crude adenoma count. Two different cohorts of mice were used for these experimentations because during the 8 months interval between experiments the number of intestinal adenomas in the animals varied considerably and had fallen drastically to under 10 per mouse in some instances. For this reason they were not considered suitable for experimentation. The new cohort of mice purchased from Jackson Laboratories

developed fewer adenomas than the initial cohort of mice however the numbers seen were consistent between animals and therefore used for the latter experiments.

2.6.2.1. Determination of Min/+ genotype

The presence of the mutant allele, in the Min/+ mice, was detected in DNA extracted from ear punch tissue obtained during litter identification of the mice at weaning using an allele specific PCR assay. Any amplified DNA possessing the Min/+ mutation, which converts codon 850 from a leucine (TTG) to a stop (TAG) codon does not contain a *Hind III* restriction endonuclease site, therefore the DNA strand remains uncut after incubation with the restriction enzyme. When run on a 3% electrophoresis gel, the uncut band appears at 150-160 base pairs while the wild type band shows at 125-130 base pairs. Ear punched tissue was digested overnight at 56°C in 200µl PBND buffer and 40µg of proteinase K. After digestion, the enzyme was inactivated by exposure at 95°C for 15 min. The PCR mixture contained 5 µl of DNA lysate, 41 µl of Reddy load PCR mix and 10pmol of both the forward and reverse primers (forward primer: 5`-TCTCGTTCTGAGAAAGACAGAAGCT-3`; reverse primer: 5`-TGATACTTCTTCCAAAGCTTTGGCTAT-3`). Optimisation studies revealed the following appropriate PCR conditions.

Table 2.6.2.1.1. Appropriate PCR conditions for genotyping of the Min/+ mice

Step	Temperature (°C)	Time (min)	Number of Cycles
1	94	2	1
2	94	1	}
3	60	1	} 40
4	72	1	}
5	72	10	1

PCR sample (17µl) was incubated at 37°C for 2-3 h with 40 units of *Hind III* and 2µl of SuRE/Cut Buffer B (Boehringer Mannheim, Germany). The samples were then run on 3 % agarose electrophoresis gel, prepared with 5µl of ethidium bromide, at 100V for 1-2 h. The gel was visualised using a dual intensity UV transilluminator and a Polaroid photograph taken.

2.6.2.2. Stability and solubility of stilbenes in drinking water

Resveratrol or DMU212 (0.01 % w:v) was dissolved in (0.4 % w:v) ethanol and then diluted to the required concentration in tap water. The solutions were left at room temperature and aliquots of each solution were analysed by HPLC everyday, using methods described in section 2.3.1.1., for a period of a week. An aliquot of each solution was analysed immediately after preparation and the peak area obtained was taken as 100 %. Peak areas of samples analysed over the week were all compared to this initial day 0 reading and expressed as a percentage of it. Daily samples were analysed in triplicate.

2.6.2.3. Effect of resveratrol in drinking water on tumour formation in C57BL/6J Min/+ mice

The study consisted of C57BL/6J Min/+ mice receiving study diet from 5 weeks to 12 weeks of age, a period of 7 weeks. Each group was comprised of 10 mice (5 male and 5 female). The control group received drinking water containing 0.4 % ethanol and the treatment group received drinking water containing 0.01 % resveratrol dissolved in 0.4 % ethanol. At the end of the experiment when Min/+ mice reached the age of 12 weeks, they were killed by cardiac exsanguinations under terminal anaesthesia (halothane). The entire gastrointestinal tract was removed for dissection and flushed with PBS (~10 ml) to remove all the intestinal content. Tissue was opened longitudinally and washed extensively with PBS. Stomach and caecum were omitted from the analysis. Small intestine and colon were examined under 3-fold magnification. The small intestine was divided visually into three segments of approximately equal lengths (referred to in the following as proximal, middle and distal segments). Multiplicity, and size of adenomas were recorded within each segment and the colon. Adenomas were differentiated by size (diameter) into <1mm, 1-3mm and >3mm. Blood samples were collected and drawn by capillary attraction into heparinized microhaematocrit tubes (75mm; Richardsons, Leicester, UK) and centrifuged at 11000 rpm for 15 minutes in a microhaematocrit centrifuge (Hawksley, UK). The haematocrit, which constitutes the proportion of the blood volume occupied by the erthryocytes, was determined as described previously (Strumia et al., 1954).

2.6.2.4. Levels of stilbenes when admixed in the diet

Resveratrol and DMU212 were blended at three different doses (0.05, 0.2 and 0.5 %) into AIN93G maintenance diet, using a mechanical tumbling mixer to ensure uniform distribution and fed to Min/+ mice from 4 to 18 weeks of age. AIN93G is the growth diet for rodents and has been recommended by the American Institute of Nutrition. It is formulated to substitute the previous version (AIN76A) to improve animal performance. The diets were stored at 4°C and changed weekly. Six batches of mix for each concentration dose were tested to ensure the correct dosage was administered to the animals and the stability of the compounds in the diet. Diets were analysed by HPLC using the methods described in section 2.3.1.1 and the samples were extracted using the method described for plasma and tissue extraction (section 2.3.1.3.)

2.6.2.5. Effect of lifetime administration of dietary stilbenes on tumour formation in C57BL/6J Min/+ mice

Offspring from batch mates were weaned at three weeks of age and genotyped to determine "Min/+" status. At 4 weeks male and female Min/+ littermates were divided following a randomised block design, into control (AIN93G) or treatment groups, which constituted either resveratrol or DMU212 at 0.05, 0.2 and 0.5 % admixed in with the AIN93G diet. Each experimental group comprised between 10 and 15 mice. At the end of the experiment when Min/+ mice reached the age of 18 weeks, they were killed by cardiac exsanguination under terminal anaesthesia (halothane). The entire gastrointestinal tract was removed for dissection and flushed with PBS (~10ml) to remove all the intestinal content. Tissue was opened longitudinally and washed extensively with PBS. Stomach and caecum were omitted from the analysis. Small intestine and colon were examined under 3-fold magnification. The small intestine was divided visually into three segments of approximately equal lengths (referred to in the following as proximal, middle and distal segments). Multiplicity and size of adenomas were recorded within each segment and the colon. Adenomas were differentiated by size (diameter) into <1mm, 1-3mm and >3mm. Blood samples were collected and drawn by capillary force into heparinized microhaematocrit tubes (75mm; Richardsons, Leicester, UK) and centrifuged at 11000 rpm for 15 minutes in a microhaematocrit centrifuge (Hawksley, UK). The haematocrit, which constitutes the proportion of the

blood volume occupied by the erthryocytes, was determined as described previously (Strumia et al., 1954).

2.6.2.6. Micro dissection of intestinal adenomas and mucosa from C57BL/6J Min/+ mice

Min/+ mice were culled under licence at 12 and 18 weeks of age. The entire gastrointestinal tract was removed by dissection and flushed with approximately 10 ml of phosphate buffered saline (PBS) to remove any intestinal content. The intestinal tissue was placed on blotting paper and opened longitudinally using iris scissors and then washed again with PBS. All intestinal adenomas were identified under 3 x magnification and collected by micro dissection using iris scissors. The intestinal epithelial mucosa was collected by gently scraping the epithelial layers with a metal spatula and frozen in liquid nitrogen and stored at -80°C.

2.6.2.7. COX-2 protein expression in C57BL/6J Min/+ mice

Control animals were fed AIN93G from 4 weeks to either 12 weeks or 18 weeks of age to assess the role of COX-2 on adenoma development and progression. Adenomas were pooled from Min/+ mice (n = 3) by size (<1mm, 1-3 mm and >3 mm). Intestinal adenomas and mucosa was micro dissected out of the intestinal tract as described above (3.6.2.6). Adenomas and mucosa were homogenised with a hand-held homogeniser in 500µl of COX-2 lysis buffer on ice. The homogenate was transferred to an eppendorf and left on ice for 20 min. The samples were then sonicated for 20 s each and centrifuged at 13,000 rpm for 5 min. The supernatent was collected and a protein assay performed on an aliquot. For each experiment 200 µg of protein was loaded onto the gel.

2.6.2.8. Effect of stilbene derivatives on intestinal PGE2 levels in C57BL/6J mice

To determine mucosal levels of PGE_2, intestinal mucosal scrapings were taken from each animal, stored on ice and homogenised in 6ml ice cold 0.1 M Tris buffer (pH 7.5). An aliquot of the homogenised tissue was taken to determine total protein levels in each sample. The homogenised tissue was then centrifuged at 3000 x *g* for 10 min, 4°C to remove any precipitate. C_{18} reverse phase columns were prepared by washing with 10

mL ethanol and then 10 mL distilled water. Each sample was applied to the column under a slight positive pressure then the columns were washed with 10 ml distilled water, 10 ml 15 % ethanol, and 10ml hexane. The samples were eluted with 10 ml ethyl acetate and evaporated under a stream of nitrogen. Samples were reconstituted in 50 µl ethanol and 200 µl of EIA buffer provided with the PGE_2 immunoassay kit. The levels of PGE_2 were measured from each sample using the PGE_2 immunoassay kit and a value of PGE_2 pg/mg total protein calculated for each sample.

2.7. Statistical Evaluation

All data values were subjected to ANOVA using Excel and SPSS (v11.0) software packages (Microsoft Windows 2000). Statistical significance ($p < 0.05$) was established by *post hoc* Bonferroni or Tukeys comparison or a Pearsons two-tailed correlation.

CHAPTER 3

PHARMACOKINETICS AND TISSUE DISPOSITION OF RESVERATROL AND DMU212 IN MICE

3.1 Introduction

As with many polyphenolic compounds that have shown cancer chemopreventive properties, the systemic bioavailability of resveratrol is poor. This observation is supported by studies in mice, rats and dogs which consistently propose that resveratrol is well absorbed but rapidly glucuronidated and sulphated both in the liver and intestinal epithelial cells (Asensi et al., 2002; Juan et al., 2002b; Marier et al., 2002). One study in humans also intimates at the poor bioavailability of resveratrol (Goldberg et al., 2003). Due to the interesting pharmacological properties of resveratrol, the trihydroxystilbene scaffold has become the subject of synthetic alterations by medicinal chemists with the aim of developing novel analogues of pharmacological interest and to characterise structural features, which impart activity to the molecule. These chemical syntheses have predominantly been concerned with the introduction of additional hydroxy moieties into the trihydroxystilbene framework and with various amounts of methylation of the phenol groups. A previous study highlighted the potential benefit of resveratrol analogues over that of resveratrol itself. In this study the 3,4,5,4'-tetrahydroxystilbene analogue (resveratrol with an additional hydroxy moiety) and its *O*-methylated analogue 3,4,5,4'-tetramethoxystilbene (DMU212), were capable of preferentially interfering with proliferation and survival of transformed human lung-derived cells, with a lower growth–inhibitory and apoptotic properties as compared to their untransformed counterparts (Lu et al., 2001). In contrast, resveratrol did not possess this discriminatory potential. DMU212 is currently under preclinical evaluation as a potential antitumour prodrug, which is thought to undergo metabolic activation by specific cytochrome P450 enzymes (Potter et al., 2002a). However, this compound may also have direct activity and not need metabolism to exert chemopreventive efficacy.

Before undertaking efficacy studies comparing resveratrol analogues to the parent compound, it is desirable to determine whether such analogues are adequately bioavailable in the tissues in which malignancies are to be prevented. Pharmacokinetic exploration of this type is a vital part of the chemopreventive drug discovery process. Therefore, DMU212 was chosen, as it is one of the most promising resveratrol analogues described so far (Lu et al., 2001; Potter et al., 2002a), and its levels in murine tissues following oral administration were compared with those of resveratrol. Thus, the hypothesis was tested that replacement of the hydroxyl groups in resveratrol by

methoxy moieties, and the addition of a further methoxy group, affects the pharmacokinetic properties of the parent molecule. Additionally, the metabolism of DMU212 was compared with that of resveratrol in both liver homogenate preparations *in vitro* and in C57BL/6J mice *in vivo*.

3.2 Plasma and tissue levels of resveratrol and DMU212 following intragastric administration.

C57BL/6J male mice received an intragastric bolus of resveratrol or DMU 212 (240mg/kg, vehicle glycerol formal for resveratrol, or glycerol formal/10% DMSO for DMU212), and drug levels were measured in the plasma, liver, kidney, lung, heart, brain, small intestinal mucosa and colonic mucosa. Representative HPLC chromatograms of extract of plasma and tissues samples are shown in figures 4.2.1 and 4.2.2.

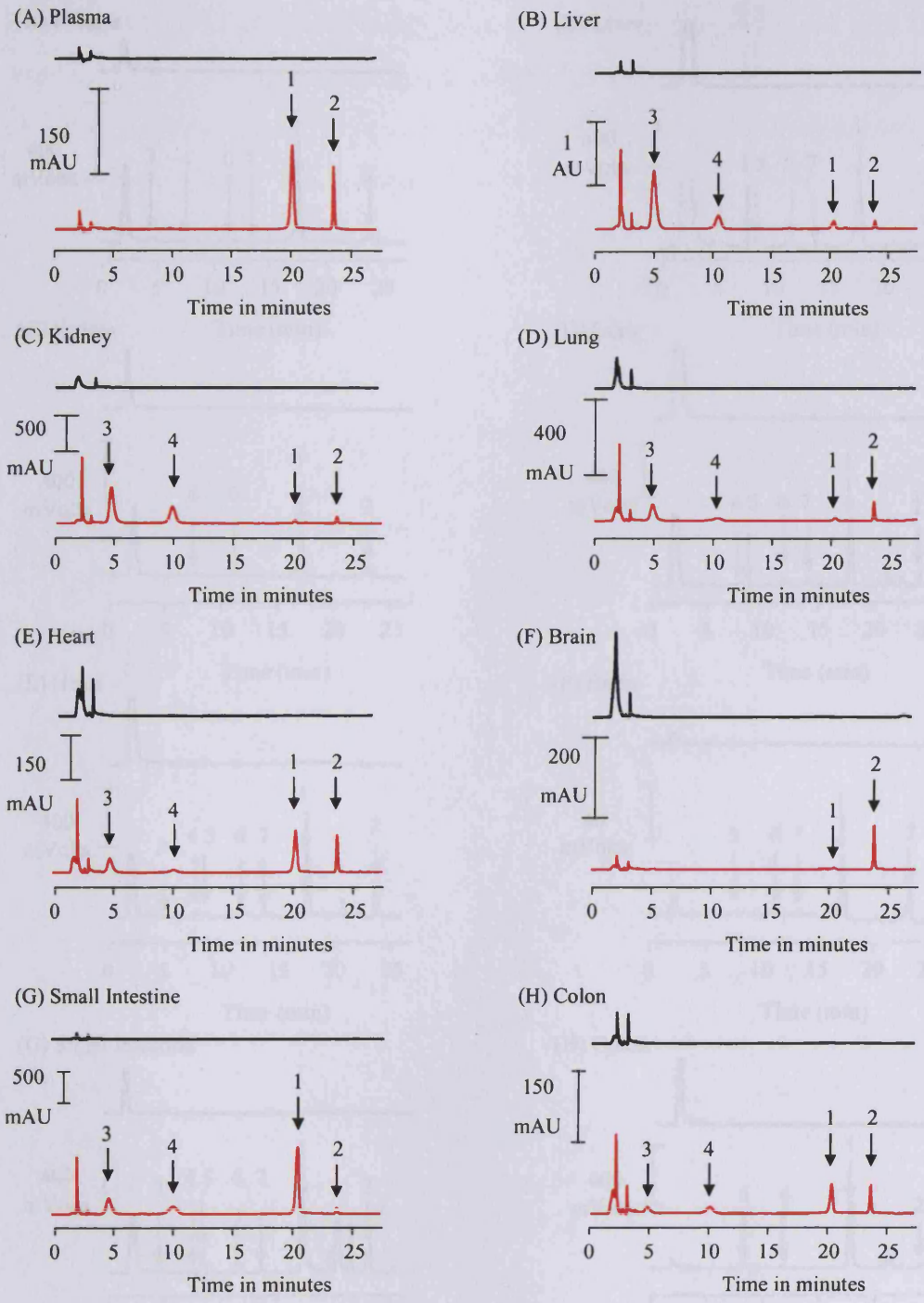


Figure 3.2.1. HPLC UV chromatograms of blank plasma or tissue (black solid line) and 30 min plasma or tissue samples from mice which received 240mg/kg resveratrol (red solid line). Peak 1 co-eluted with resveratrol and peak 2 with the internal standard (20μg) carbamazepine. Peaks 3 and 4 are potential metabolites.

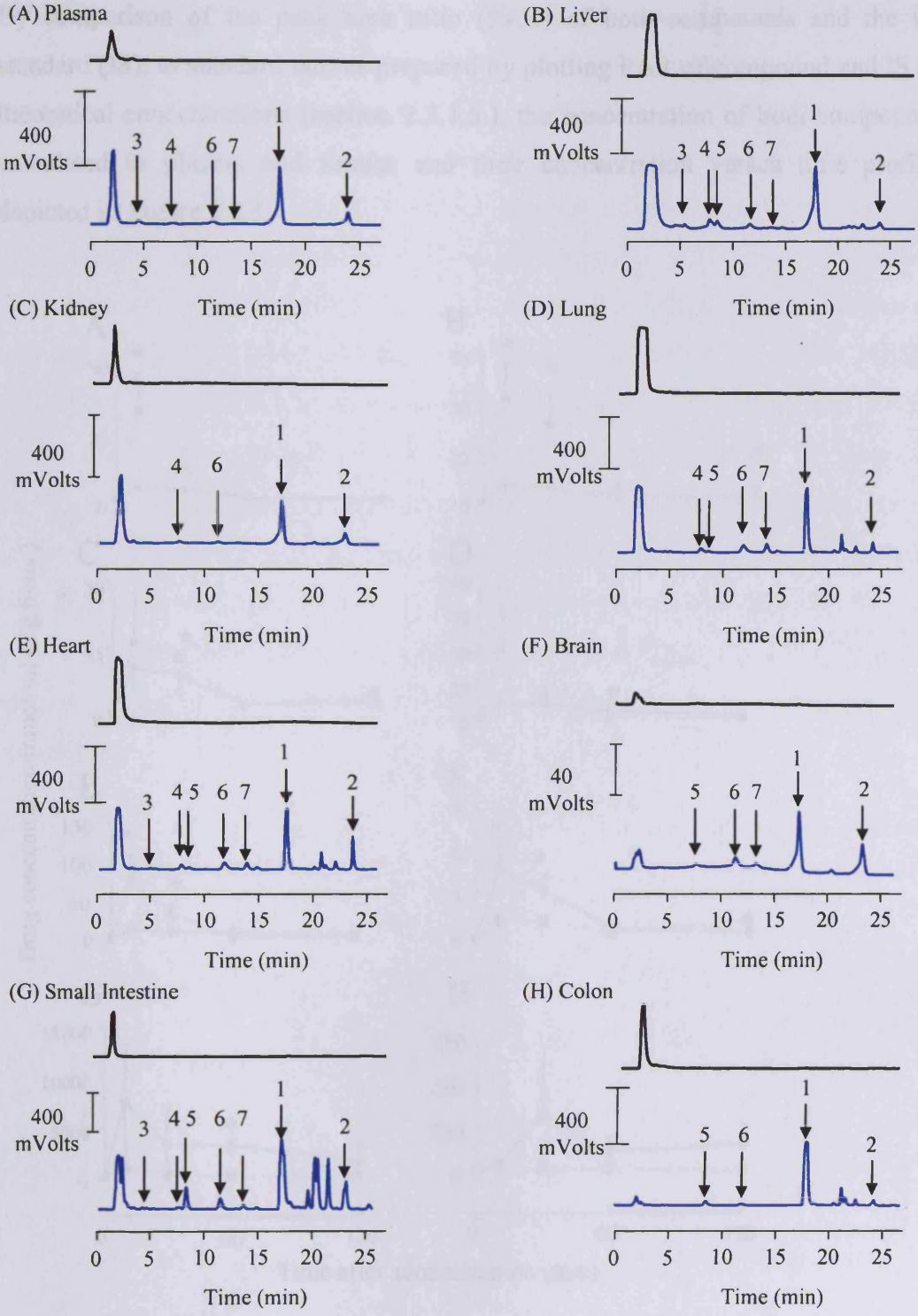


Figure 3.2.2. HPLC fluorescence chromatograms of blank plasma or tissue (black solid line) and 30 min plasma or tissue samples from mice which received 240mg/kg DMU212 (blue solid line). Peak 1 co-eluted with DMU212 and peak 2 with the internal standard (2μg) DMU216. Peaks 3-7 are potential metabolites.

By comparison of the peak area ratio (PAR) of both compounds and the internal standard (IS), to standard curves prepared by plotting PAR of compound and IS against theoretical concentrations (section 2.3.1.5.), the concentration of both compounds was calculated in plasma and tissues and their concentration versus time profiles are depicted in Figure 3.2.3.

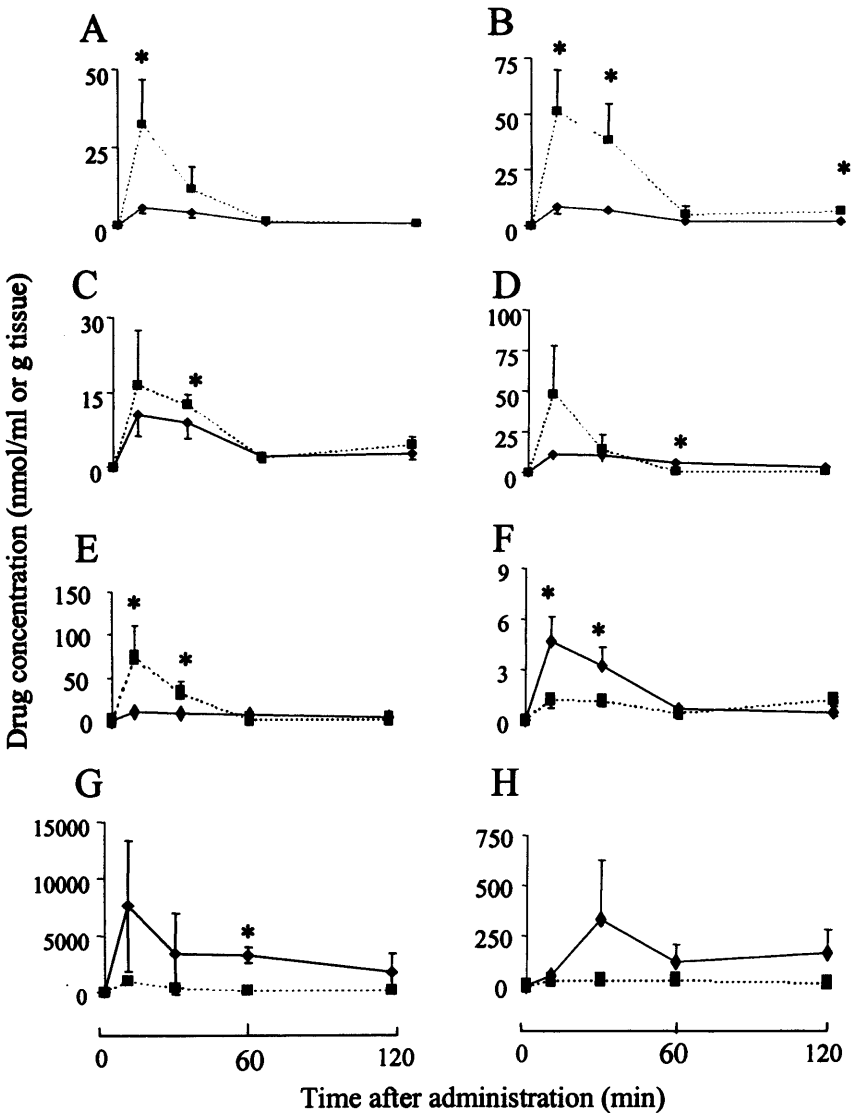


Figure 3.2.3. Concentrations of resveratrol (dotted line) and DMU 212 (solid line) in plasma (A), liver (B), kidney (C), lung (D), heart (E), brain (F), small intestinal (G) and colonic mucosa (H) following a single intragastric dose of drug (240 mg/kg). Values are the mean ± SD (n = 3). "*" indicates that the values differ significantly ($p < 0.05$, one-way ANOVA).

The plasma and tissue level data (shown above, figure 3.2.3) were used to calculate the respective mean $AUC_{0\text{-}120\ min}$ values (Table 3.2.1). Concentrations of resveratrol were consistently higher than those of DMU 212 in plasma, liver and heart. Levels were similar in the kidney and lung, whilst resveratrol concentrations were below those of DMU 212 in the brain, small intestinal and colonic mucosae. The most dramatic discrepancy in levels occurred in the liver, in which the AUC for resveratrol was five times higher than that for DMU 212, and in the small intestinal and colonic mucosae, in which the AUCs for DMU 212 exceeded those for resveratrol by factors of 10 and 7, respectively (Table 3.2.1.). Resveratrol levels peaked in all tissues after 10 min, the first of the time points chosen, and peak concentrations were 32 μM in plasma and 51, 16, 50, 1.2, 75, 960 and 30 nmoles/g tissue in liver, kidney, lung, brain, heart, small intestinal and colonic mucosa, respectively. DMU 212 levels reached a peak in all tissues except colon after 10 min. In the colon, peak levels occurred after 30 min. The peak levels for DMU 212 were 5 μM in plasma and 8, 11, 11, 5, 10, 7600 and 330 nmoles/g tissue in liver, kidney, lung, brain, heart, small intestinal and colonic mucosae, respectively.

Table 3.2.1. Area under the plasma or tissue concentration time curve (AUC) for resveratrol and DMU 212 in mice that received these agents at 240mg/kg *i.g.*

Tissue	AUC (nmoles ml or g/min)		AUC_{res}/AUC_{DMU}
	Resveratrol	DMU 212	
Plasma	863	246	3.5
Liver	2,150	432	5
Kidney	785	566	1.5
Lung	1,123	778	1.5
Heart	2,072	750	3
Brain	103	193	0.5
Intestinal mucosa	36,690	369,315	0.1
Colonic mucosa	2,869	19,256	0.15

AUC values were calculated from the curves shown in figure 3.1.3 using the mean plasma or tissue concentration values between 0 and 120 min *post* administration.

3.3. The metabolism of resveratrol and DMU212 *in vitro* and *in vivo*.

3.3.1 The metabolism of resveratrol

HPLC chromatograms of tissue samples from mice that received resveratrol contained two peaks with retention times of 4 and 9.5 min in addition to those of resveratrol and the IS. Resveratrol undergoes rapid conjugation and these peaks were thought to be resveratrol conjugates. Resveratrol glucuronide was synthesised using a microsomal preparation (section 2.6.1.2.) and authentic metabolite was used to spike a 30 min liver sample extract. Figure 4.3.1.1 shows the trace of the 30 min liver sample extract with and without added resveratrol glucuronide.

The synthesised resveratrol glucuronide eluted from the column with exactly the same retention time as the previously unidentified metabolite. This study confirmed that this

metabolite was a resveratrol glucuronide. The peak was also collected and analysed by mass spectrometry to further confirm it to be the resveratrol glucuronide.

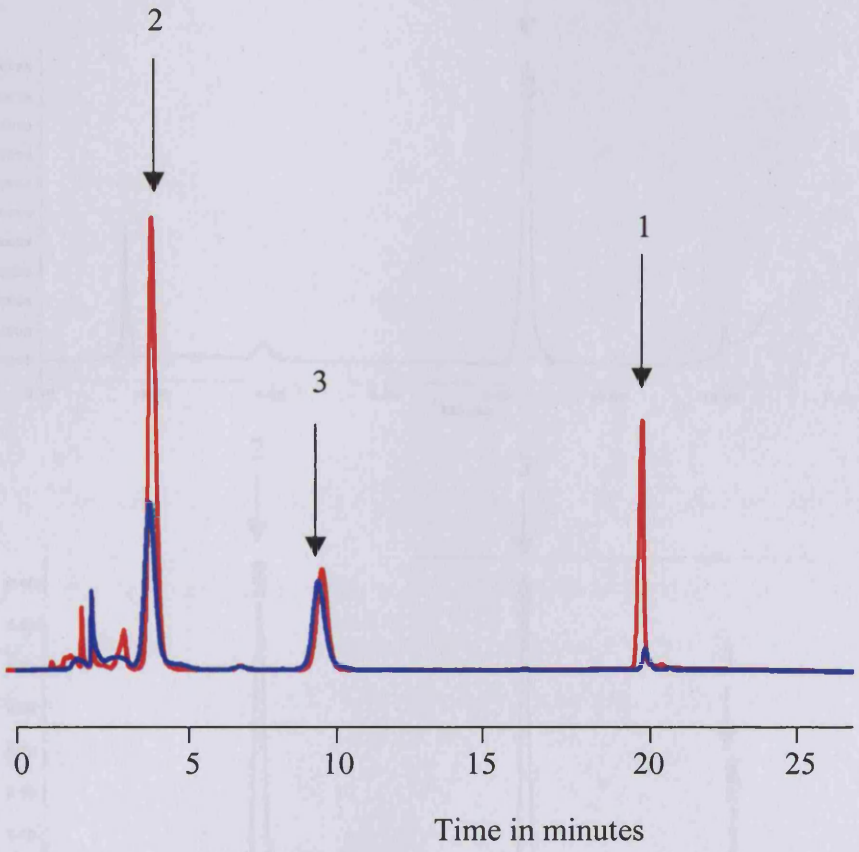


Figure 3.3.1.1. Chromatogram trace of a mouse liver extract (blue line), and the same sample spiked with resveratrol glucuronide (peak 2), synthesised *in vitro* (red line). Peak 1 is resveratrol and peak 3 is another metabolite.

The other unidentified peak (Peak 3, Figure 3.3.1.1) was thought to be resveratrol sulphate. As part of an ongoing clinical trial of resveratrol in our laboratory, resveratrol-3-sulphate standard had been provided by Dr T Booth (Mount Royal Pharma, Montreal, Canada). The liver sample extract, containing the unidentified peak, was analysed by a separate HPLC system. The sample analysis showed resveratrol-3-sulphate eluted at the same retention time as the unidentified peak in the liver sample

extract (Figure 3.3.1.2.). This was also confirmed by mass spectrometry (Figure 3.3.1.3.).

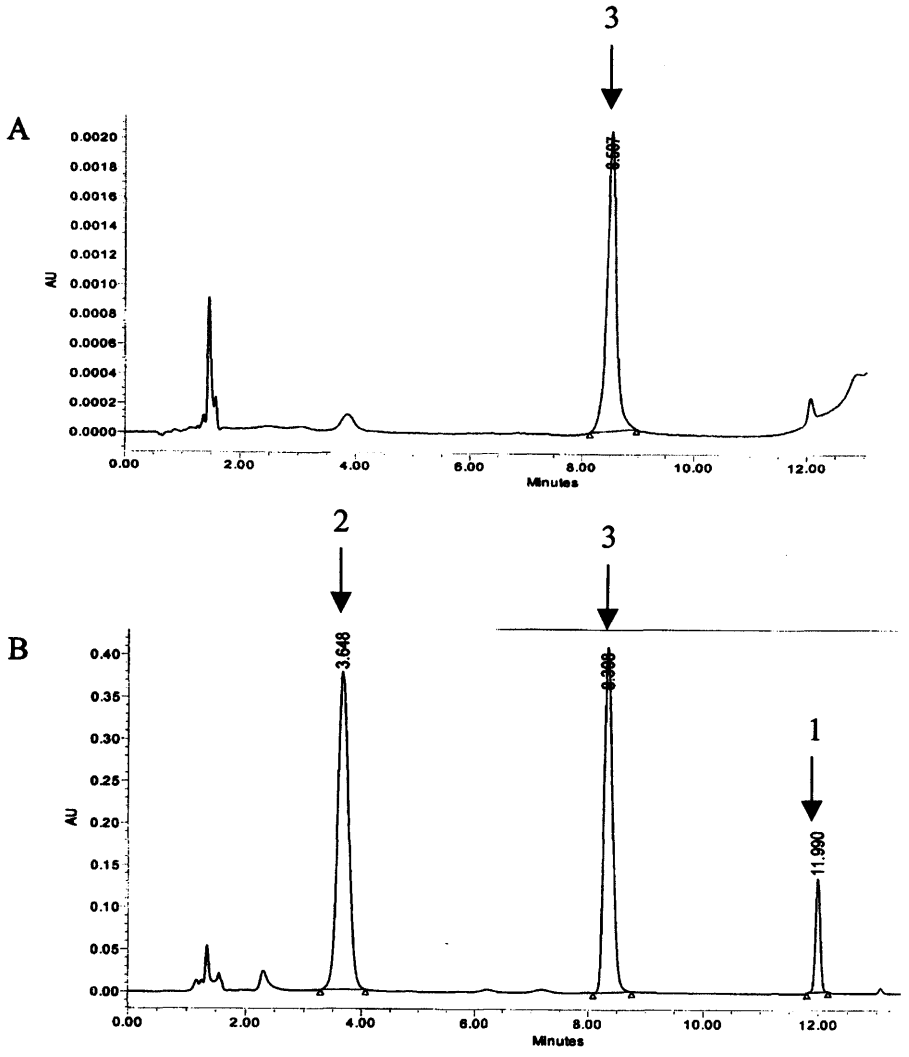


Figure 3.3.1.2. HPLC trace of (A) resveratrol sulphate standard (peak 3) and (B) a mouse liver extract 30 min post dosing with 240mg/kg resveratrol. Peak 1 is resveratrol, peak 2, a resveratrol glucuronide and peak 3, co-eluted with resveratrol-3-sulphate.

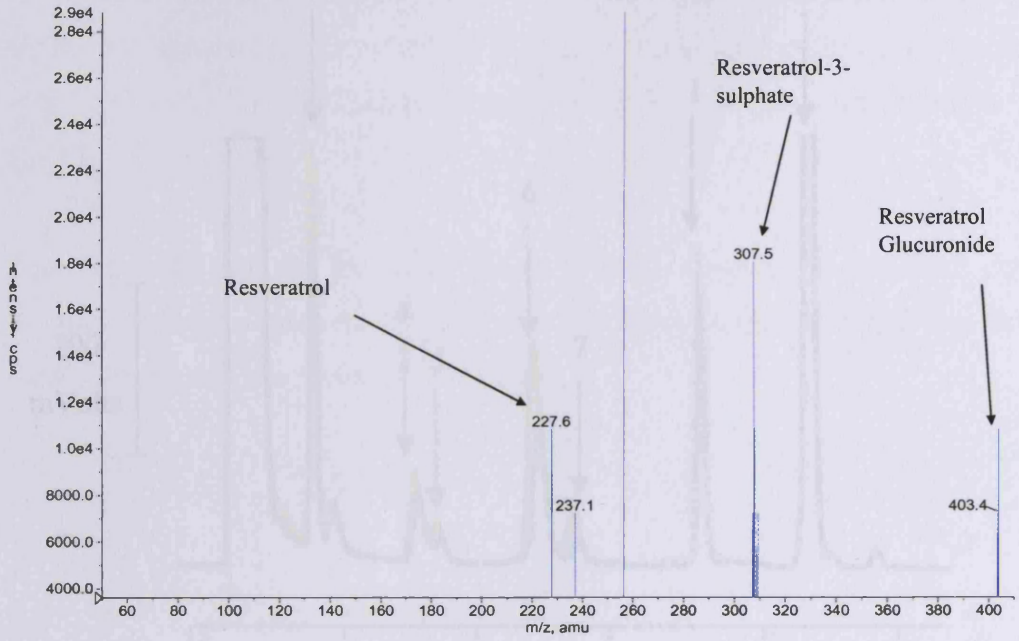


Figure 3.3.1.3. Mass spectrometric trace of a 30 min liver extract from a mouse treated with 240 mg/kg resveratrol *i.g.*. The spectrum shows a scan from 50 –410 m/z. Present in the sample is resveratrol [m/z 227], resveratrol sulphate [m/z 307] and resveratrol glucuronide [m/z 403].

3.3.2. The metabolism of DMU212

HPLC chromatograms of plasma and tissue samples from mice that received DMU212 contained up to five additional peaks with retention times of 4.5, 7.8, 8.2, 12 and 13.5 min. As DMU212 has four methoxyl moieties, it is unable to undergo conjugation unless it has first undergone oxidative metabolism. Using mouse liver microsomes, the oxidative metabolism of DMU212 was investigated. Co-chromatography studies using liver sample extracts from mice spiked with a number of potential DMU212 metabolites provided by Prof. Gerry Potter (DeMontfort University, Leicester, section 2.3.1.2.) were investigated. An HPLC chromatogram is shown in Figure 3.3.2.1.

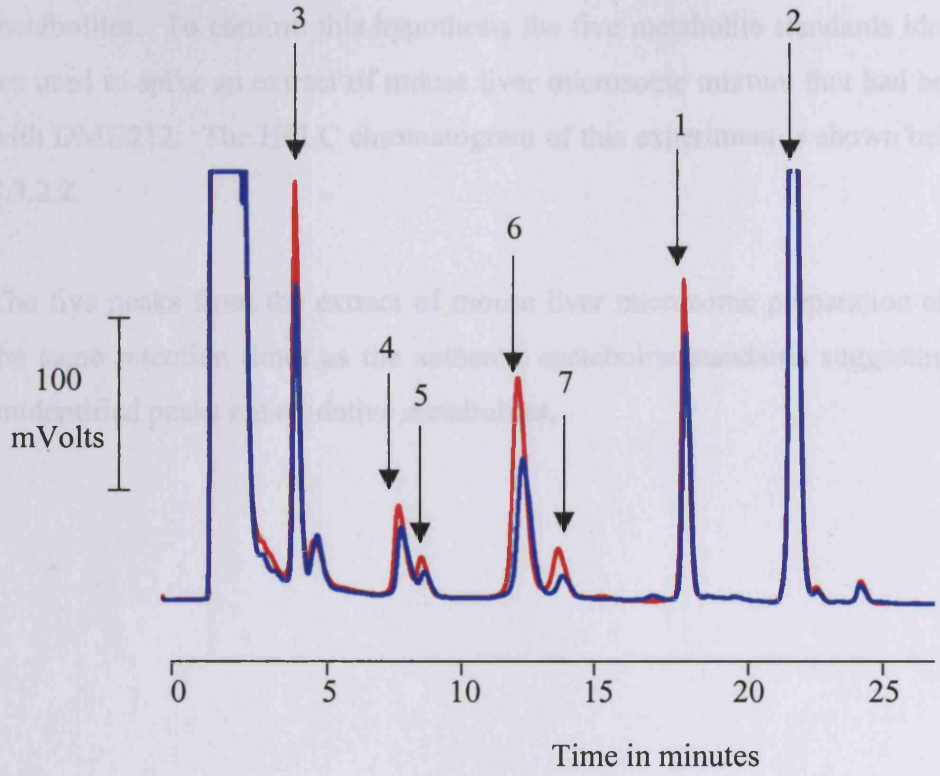


Figure 3.3.2.1. HPLC analysis of liver extracts of mice which received DMU 212 (240 mg/kg) *po* (blue line), and a mixture (red line) of this extract to which the following authentic standards were added 4,4'-di-desmethyl-DMU 212 (DMU 295), 4'-desmethyl-DMU 212 (DMU 281), 3'-hydroxy-DMU 212 (DMU 214), 4-desmethyl-DMU 212 (DMU 291) and 3-desmethyl-DMU 212 (DMU 807). Liver tissue was obtained 60 min post administration. Peak allocation is (3) DMU 295, (4) DMU 281, (5) DMU 214, (6) DMU 291, (7) DMU 807, (1) DMU 212 and (2) DMU216 internal standard (2μg).

The five peaks were correlated with five of the standards provided by Prof. Gerry Potter, but we were still unsure as to whether these metabolites were oxidative metabolites. To confirm this hypothesis the five metabolite standards identified above we used to spike an extract of mouse liver microsome mixture that had been incubated with DMU212. The HPLC chromatogram of this experiment is shown below in Figure 3.3.2.2.

The five peaks from the extract of mouse liver microsome preparation all eluted with the same retention times as the authentic metabolite standards suggesting that all the unidentified peaks are oxidative metabolites.

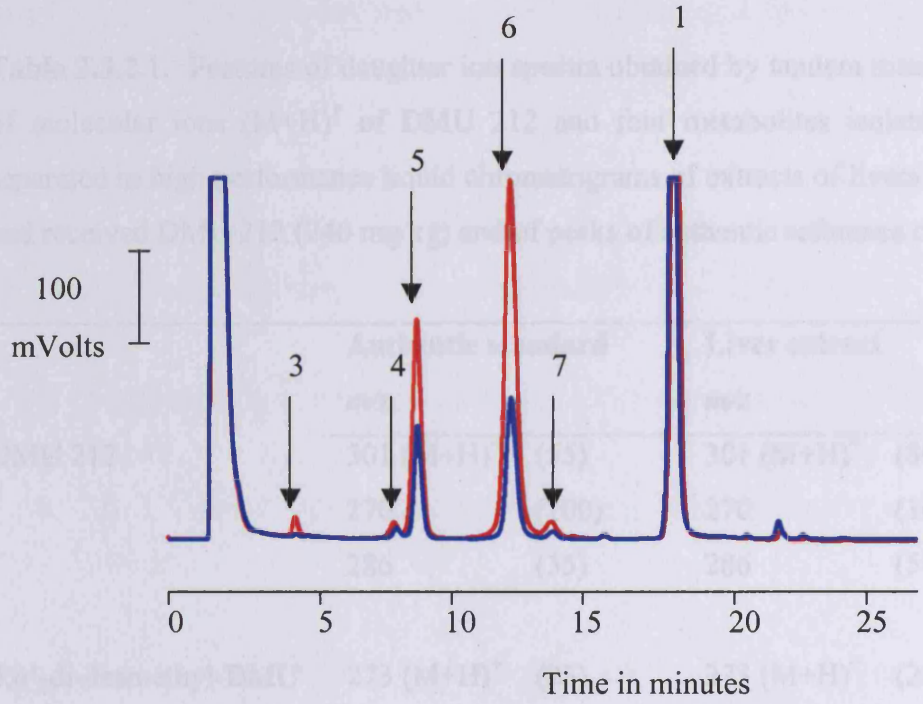


Figure 3.3.2.2. HPLC analysis of extracts of an incubate of mouse liver microsomes with DMU 212 (1 mM) (blue line) and of a mixture (red line) of this extract to which the following authentic standards had been added 4,4'-di-desmethyl-DMU 212 (DMU 295), 4'-desmethyl-DMU 212 (DMU 281), 3'-hydroxy-DMU 212 (DMU 214), 4-desmethyl-DMU 212 (DMU 291) and 3-desmethyl-DMU 212 (DMU 807). Incubations were terminated after 20 min. Peak allocation is (3) DMU 295, (4) DMU 281, (5) DMU 214, (6) DMU 291, (7) DMU 807 and (1) DMU 212.

To fully characterise these metabolites liver extracts were analysed by HPLC and the metabolite peaks subsequently collected and analysed by mass spectrometry (section 2.6.1.3.). The product ions formed were compared to those from the authentic standards (Table 3.3.2.1.)

Table 3.3.2.1. Features of daughter ion spectra obtained by tandem mass spectrometry of molecular ions $(M+H)^+$ of DMU 212 and four metabolites isolated from peaks separated in high performance liquid chromatograms of extracts of livers of mice which had received DMU 212 (240 mg/kg) and of peaks of authentic reference compounds.

	Authentic standard		**Liver extract**	
	m/z		***m/z***	
DMU 212	301 $(M+H)^+$	(55)	301 $(M+H)^+$	(80)
	270	(100)	270	(100)
	286	(35)	286	(50)
4,4'-di-desmethyl-DMU 212	273 $(M+H)^+$	(23)	273 $(M+H)^+$	(25)
	198	(100)	198	(50)
	181	(62)	181	(81)
3-desmethyl-DMU 212	287 $(M+H)^+$	(10)	287 $(M+H)^+$	(75)
	227	(72)	227	(20)
	195	(70)	195	(18)
4'-desmethyl-DMU 212	287 $(M+H)^+$	(10)	287 $(M+H)^+$	(60)
	256	(100)	256	(100)
	272	(80)	272	(47)
3'-hydroxy-DMU 212	317 $(M+H)^+$	(31)	317 $(M+H)^+$	(21)
	225	(55)	218	(100)
	286	(22)	286	(10)

Only three prominant product ions are shown. Relative abundance in percentage in brackets.

3.4. Semi-quantification of stilbene metabolites in plasma and target tissues.

Metabolites of both stilbenes were observed and have been characterised from plasma and tissue samples of mice. The plasma and target tissue concentrations of these metabolites were semi-quantified by making certain assumptions for the resveratrol metabolites, and by the use of authentic standards for DMU212 metabolites.

Due to the small amount of resveratrol metabolite standards available it was impossible to plot standard curves for these metabolites in plasma and each tissue. Therefore, the following assumptions were made to semi-quantify the concentrations:

1. Metabolites have the same extraction efficiency as resveratrol from plasma and tissues
2. The UV absorbance co-efficient for the metabolites and resveratrol were exactly the same

HPLC chromatograms from plasma samples only contained two peaks. One of the parent compound resveratrol and the other peak was the IS. Figure 3.4.1, therefore, only shows the concentrations of resveratrol metabolites in the intestinal and colonic mucosae.

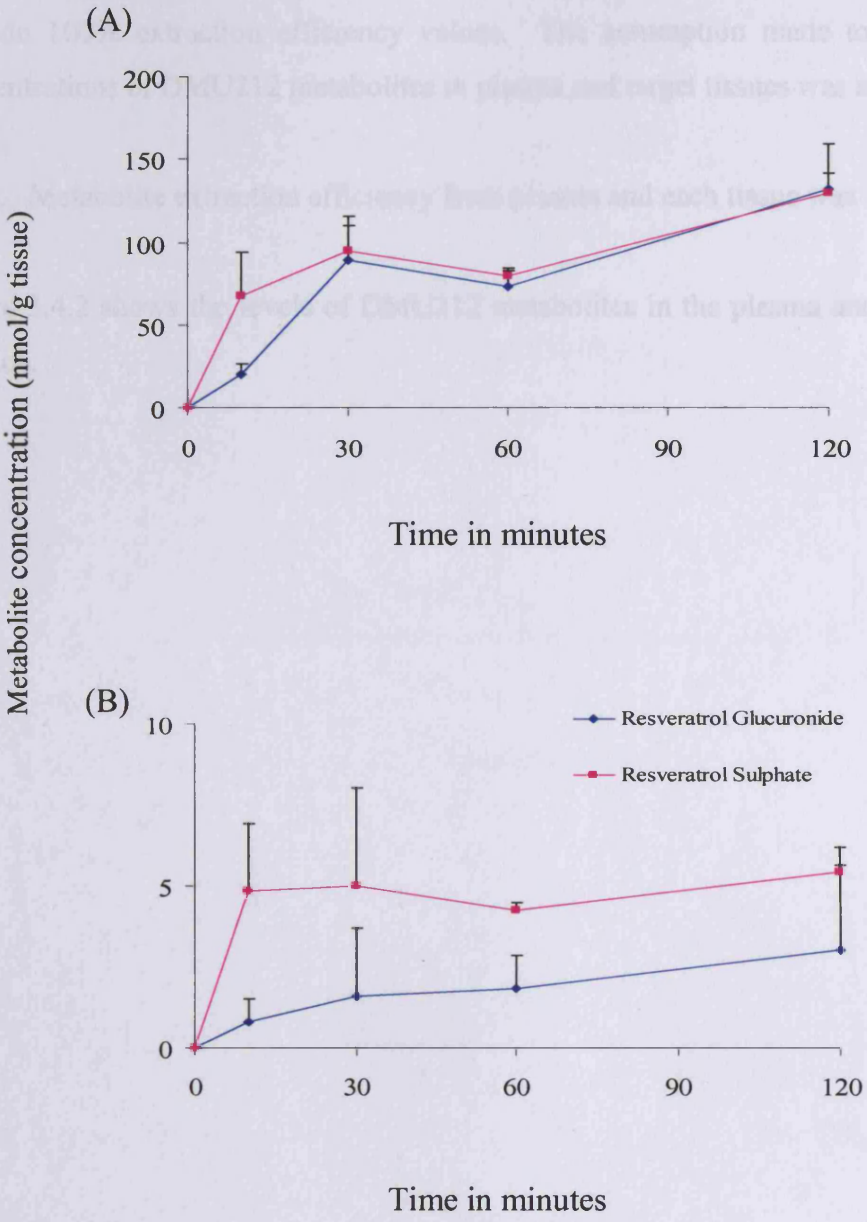


Figure 3.4.1. Resveratrol metabolite concentrations (nmol/g tissue) in (A) small intestinal mucosa and (B) colonic mucosa following a single dose of resveratrol (240 mg/kg) *ig*. Values are the mean ± SD (n = 3).

Although DMU212 metabolites could be more accurately calculated, certain assumptions were still made. Calibration curves were plotted for each metabolite from authentic standards prepared in mobile phase. These calibration curves would therefore provide 100% extraction efficiency values. The assumption made to calculate the concentrations of DMU212 metabolites in plasma and target tissues was as follows:

1. Metabolite extraction efficiency from plasma and each tissue was 100%

Figure 3.4.2 shows the levels of DMU212 metabolites in the plasma and target tissues of mice.

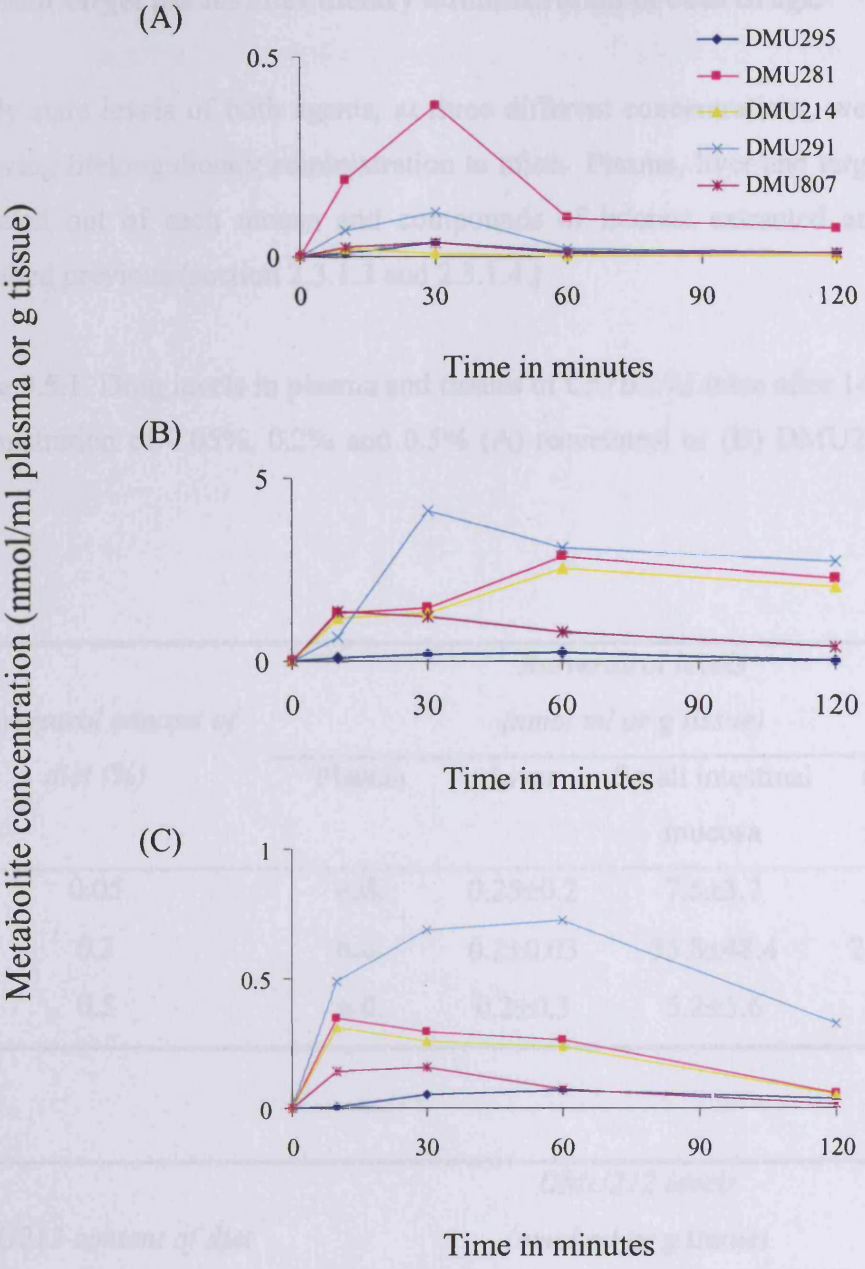


Figure 3.4.2. DMU212 metabolite levels (nmol/ml plasma or g tissue) were determined in the systemic circulation (A) and also in the target tissue, the small intestinal mucosa (B) and colonic mucosae (C) following a single dose of DMU212 (240 mg/kg) *ig*. Values are the mean of n = 3.

3.5. Determination of steady state levels of resveratrol and DMU212 in plasma, liver and target tissues after dietary administration of both drugs.

Steady state levels of both agents, at three different concentrations, were investigated following lifelong dietary administration to mice. Plasma, liver and target tissues were dissected out of each mouse and compounds of interest extracted and analysed as described previous (section 2.3.1.3 and 2.3.1.4.)

Table 3.5.1. Drug levels in plasma and tissues of C57BL/6J mice after 14 weeks dietary administration of 0.05%, 0.2% and 0.5% (A) resveratrol or (B) DMU212 in AIN93G diet.

A

Resveratrol content of diet (%)	*Resveratrol levels (nmol ml or g tissue)*			
	Plasma	Liver	Small intestinal mucosa	Colonic mucosa
0.05	n.d.	0.25±0.2	7.5±3.7	2.2±1.9
0.2	n.d.	0.2±0.03	35.8±48.4	28.3±16.2
0.5	n.d.	0.2±0.3	5.2±5.6	0.6±0.4

B

DMU212 content of diet (%)	*DMU212 levels (nmol ml or g tissue)*			
	Plasma	Liver	Small intestinal mucosa	Colonic mucosa
0.05	0.05±0.03	0.08±0.03	5.7±1.8	44.1±41.2
0.2	0.5±0.35	0.4±0.03	19.3±8.9	58.9±11.5
0.5	0.4±0.2	0.7±0.4	263.2±217.8	58.7±56.7

Values represent the mean ± SD (n = 4). n.d. = not detected

Levels of resveratrol in the small intestinal mucosa ranged from 5-35 nmol/g tissue equating to a concentration of approximately 5-35 μM and DMU212 levels ranged from 5-265 nmol/g tissue equating to concentrations of approximately 5-265 μM. Previous studies with resveratrol have revealed chemopreventive efficacy in the low micromolar concentration range. Therefore, based on these values achieved *in vivo,* potential chemopreventive effects from these compounds should be easily achieved with the levels seen.

3.6 Discussion

These results suggest that, compared to resveratrol, DMU212 with the addition of four methoxy groups onto the stilbene framework, fails to significantly increase the systemic availability of the molecule. For example, the concentration of DMU212 was lower to that of resveratrol in the plasma, liver and heart following oral administration. In contrast, the concentration of DMU212 was found to be higher than resveratrol in intestinal and colonic mucosa, and in the brain. It is difficult to theoretically predict how such a structural modification might alter the pharmacokinetic profile of the stilbene molecule. One of the physicochemical effects of this alteration, which certainly impacts on the pharmacokinetics of the molecule, is the increase in lipophilicity imparted by four methoxy functionalities when compared to resveratrol, which has three hydroxy groups instead. The difference in lipophilicity between DMU212 and resveratrol is highlighted by the fact that reverse phase HPLC analysis of a mixture of both agents using the gradient system (section 2.3.1.1.) for DMU212 gave a retention time of 17 min for DMU212, whilst resveratrol eluted with the solvent front.

An additional factor, which suggests differential pharmacokinetic properties between resveratrol and DMU212, is the differences in their metabolic profile. It has been shown that resveratrol undergoes metabolic phase II reactions involving conjugation with sulphate and glucuronic acid (Yu et al., 2002). Our findings are consistent with this study, as levels of resveratrol sulphate and resveratrol glucuronide in liver and other tissues of mice, which had received resveratrol, were detected. In contrast to resveratrol, DMU212 underwent hepatic metabolic oxidation, especially single or double *O*-demethylation reactions in the 3, 4 or 4' positions of the molecule. Furthermore, the identification of a hydroxylated metabolic molecule (DMU 214) was shown. The spectrum of *O*-demethylated and hydroxylated metabolites found *in vivo* was accurately reflected by the metabolic profile obtained on incubation of DMU212 with NADPH-fortified liver microsomes. These findings are consistent with results of recent *in vitro* experiments using cytochrome P450 isoenzyme preparations, in which DMU212 was found to undergo both aromatic hydroxylation and *O*-demethylation reactions primarily catalysed by isoenzymes of the CYP1 family (Wilsher et al., unpublished). In comparison, resveratrol was recently found to undergo metabolic oxidation *in vitro* to piceatannol (3,5,2',4'-tetra-hydroxystilbene), when incubated with CYP1B1 (Potter et al., 2002b). In the study described here piceatannol was not

identified as a metabolite of resveratrol in mice *in vivo* or in mouse liver microsomes *in vitro*. It is conceivable that piceatannol was present but at such low levels, that it might have been undetectable by the methods used here. The ability to convert resveratrol to piceatannol is thought to be a relatively specific property of CYP1B1 (Potter et al., 2002b). The lack of detectable levels of piceatannol in our study in the mouse is consistent with a report that CYP1B1 is not expressed at appreciable levels in mouse liver (Shimada et al., 2003). Taken together our results suggest that the hydroxylation of resveratrol to piceatannol is probably not a major metabolic route, in the mouse.

It is plausible that DMU295, DMU281, DMU214, DMU291 and DMU807, the metabolites of DMU212 identified here, undergo phase II metabolism to form sulphate and/or glucuronide metabolites. Despite no conjugates of metabolic derivatives from DMU212 being found in the mouse samples we cannot exclude the possibility that such conjugates were formed *in vivo* but were not detected.

The pharmacokinetic properties of DMU212 are to date unknown. On the contrary, resveratrol has been the subject of several pharmacokinetic studies, including those in the rat (Soleas et al., 2001a) and the mouse (Vitrac et al., 2003), in which resveratrol has been administered *via* the oral route. In terms of peak levels and systemic disappearance, the results outlined in this study in mice are consistent with the published studies. The increased drug levels in liver, kidney, lung and heart obtained after ingestion of resveratrol, in comparison to those after DMU212, reflect the difference in availability observed in the plasma. In contrast, levels of DMU212 in brain, small intestinal and colonic mucosa, after DMU212 administration, exceeded those of resveratrol. The higher concentration of DMU212 in the brain suggests that it is capable of crossing the blood-brain barrier more easily than resveratrol, which is probably a consequence of its higher lipophilicity. The differences between resveratrol and DMU212 concentration achieved in the small intestine and colon may be the consequence of their differential metabolic susceptibilities. The lower concentrations of resveratrol compared to DMU212 may be a consequence of the susceptibility of resveratrol to undergo conjugation reactions catalysed by enzymes (De Santi et al., 2000; Kuhnle et al., 2000; Marier et al., 2002), which are abundantly present in the gut (Eisenhofer et al., 1999). In contrast, on the basis that activities of oxidising enzymes in the gut are much lower than those in the liver (Doherty & Charman, 2002), the ability of the gut mucosa to *O*-demethylate or hydroxylate DMU212 is arguably much lower than

its ability to biotransform resveratrol by conjugation. Therefore, on the assumption that DMU212 and resveratrol share biochemical mechanisms important to cancer chemoprevention, and given that DMU212 is not inferior to resveratrol in intrinsic potency, this observation possibly suggests a potential advantage of DMU212 over resveratrol when applied as an experimental colorectal cancer chemopreventive agent. The superior growth-inhibitory and apoptotic potency of DMU212 in comparison to resveratrol in transformed human lung-derived cells (Lu et al., 2001) is consistent with this hypothesis. Peak levels of DMU212, achieved in the colonic and small intestinal mucosa in mice, exceeded those required to cause significant arrest of transformed lung cell growth *in vitro* (10 μM) by factors of 32 and 760, respectively. Steady state levels of both compounds in intestinal mucosa were also similar if not higher than those required for *in vitro* efficacy with concentrations ranging from 5-35 μM for resveratrol and 5-265 μM for DMU212.

In conclusion, the work described here provides an initial pharmacokinetic profile of the resveratrol analogue, DMU212. Such a pharmacokinetic study could aid the development of further resveratrol analogues highlighting their possible suitability for testing in pre-clinical models of carcinogenesis. This work has shown DMU212 to have more favorable pharmacokinetic properties than resveratrol, in that it formed higher levels of drug in the small intestinal and colonic mucosa. Corroborated by our recent finding that DMU212 is devoid of any toxicity in rats when administered at single doses of up to 40 mg/kg *via* the *iv* route or up to 400 mg/kg when administered *po* (Verschoyle et al., unpublished), the results presented here provide a strong argument to further explore DMU212 for chemopreventive efficacy both *in vitro* and *in vivo*.

CHAPTER 4

EFFECTS OF RESVERATROL AND DMU212 ON CELL GROWTH, APOPTOSIS AND CELL CYCLE AND THE CYCLOOXYGENASE PATHWAY

4.1 Introduction

Resveratrol possesses anti-oxidant, anti-inflammatory, anti-angiogenic, anti-mutagenic, kinase-inhibitory and both pro- and anti-oestrogenic properties (Atten et al., 2001; Gehm et al., 1997; Haworth & Avkiran, 2001; Jang et al., 1997; Uenobe et al., 1997). An important mechanistic feature of resveratrol thought to contribute to its cancer chemopreventive activity is its ability to induce apoptosis (Mahyar-Roemer et al., 2001; Mahyar-Roemer et al., 2002). Among the biochemical events engaged by resveratrol which may induce apoptosis is interference with the activity of cyclooxygenase (COX) by enzyme inhibition and/or downregulation (Li et al., 2002; Maccarrone et al., 1999; Martinez & Moreno, 2000; Mutoh et al., 2000a; Pace-Asciak et al., 1995; Subbaramaiah et al., 1998; Szewczuk et al., 2004; Zhang et al., 2004). COX-1 and COX-2 catalyze the conversion of arachidonic acid to pro-tumorigenic eicosanoids, such as prostaglandin E-2 (PGE_2), which are involved in the maintenance of the malignant phenotype (Hansen-Petrik et al., 2002). Whilst COX-1 is constitutively expressed ubiquitously, COX-2 expression is up-regulated in inflammation and in 80-85% of human adenocarcinomas and colonic tumors (Eberhart et al., 1994; Uefuji et al., 2001).

The main aims of the studies described in this chapter were to determine the growth inhibitory effects that resveratrol and DMU212 have on two human derived colorectal cancer cell lines, HT29 and HCA-7. These cell lines were also selected due to the fact that they both overexpress COX-2, a protein known to be involved in colorectal carcinogenesis. In addition, the ability of both compounds to induce apoptosis and/or cell cycle arrest was compared in the HCA-7 cell line. Another aim was to investigate the effects these agents have on the cyclooxygenase pathway. COX-2 protein expression in cell lysates was investigated following treatment with either compound and PGE_2 levels in culture media was measured as an indirect indicator of COX-2 activity. Finally, the effects of resveratrol and DMU212 on COX-1 and COX-2 activity were investigated in a cell free system using purified enzyme preparations.

4.2 Effect of resveratrol and DMU212 on colon cell proliferation

The growth inhibitory potential of resveratrol and DMU212 was investigated in two human colorectal cancer lines, HT29 and HCA-7. Cells were grown in the absence and presence of resveratrol or DMU212 (1 – 100 μM) for durations of up to 168 h and counted as described in section 2.4.5. Figures 4.2.1 and 4.2.2 show the effect of resveratrol and DMU212 on the growth of HT29 cells. Figures 4.2.3 and 4.2.4 show the effect of resveratrol and DMU212 on HCA-7 cell growth.

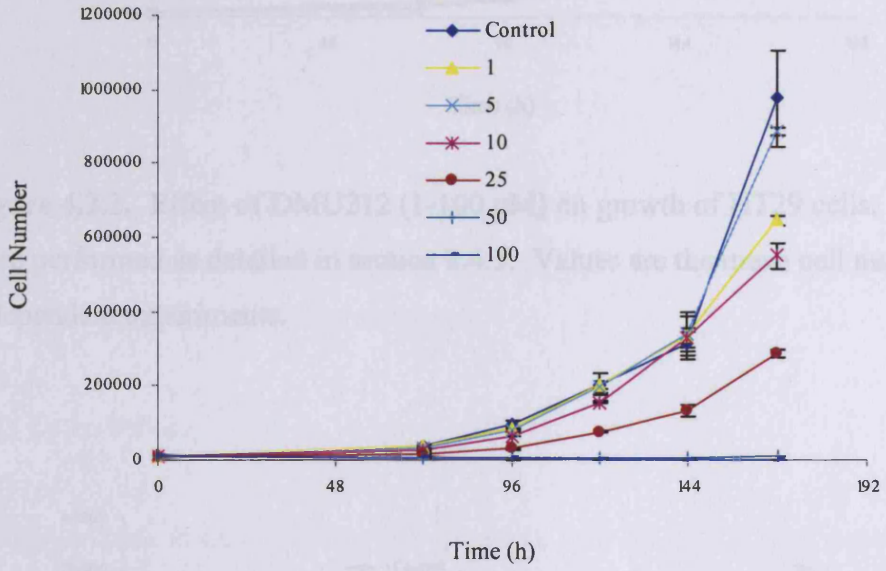


Figure 4.2.1. Effect of resveratrol (1-100 μM) on growth of HT29 cells. Growth curves were performed as detailed in section 2.4.5. Values are the mean cell number ± SD of 4 independent experiments.

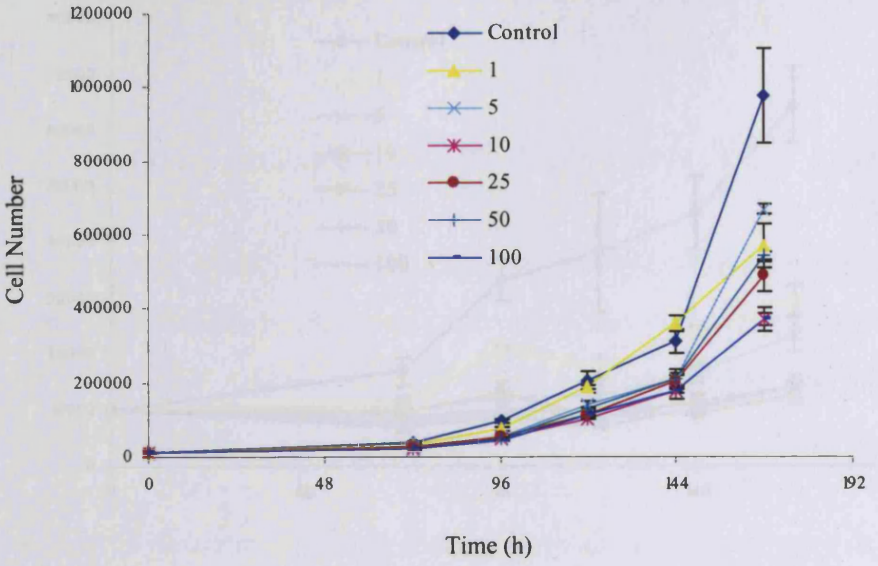


Figure 4.2.2. Effect of DMU212 (1-100 μM) on growth of HT29 cells. Growth curves were performed as detailed in section 2.4.5. Values are the mean cell number ± SD of 4 independent experiments.

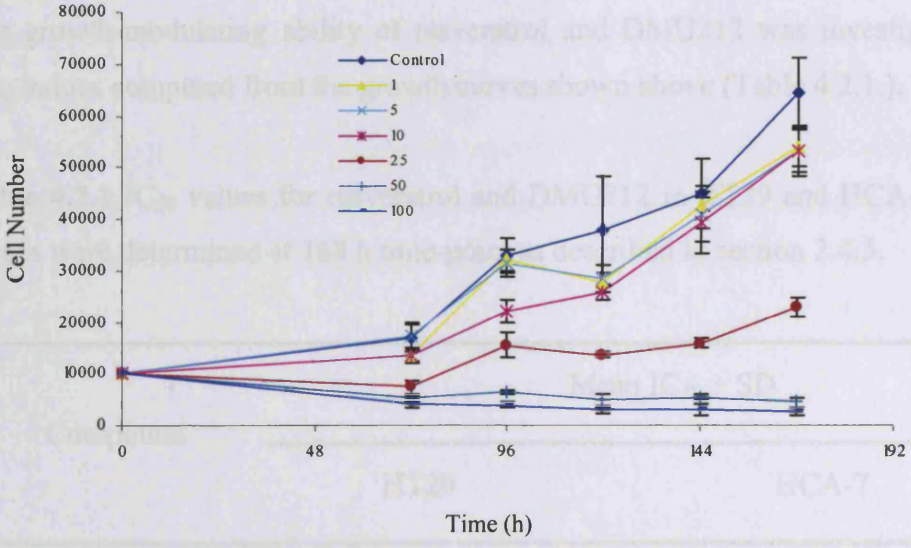


Figure 4.2.3. Effect of resveratrol (1-100 μM) on growth of HCA-7 cells. Growth curves were performed as detailed in section 2.4.5. Values are the mean cell number ± SD of 4 independent experiments.

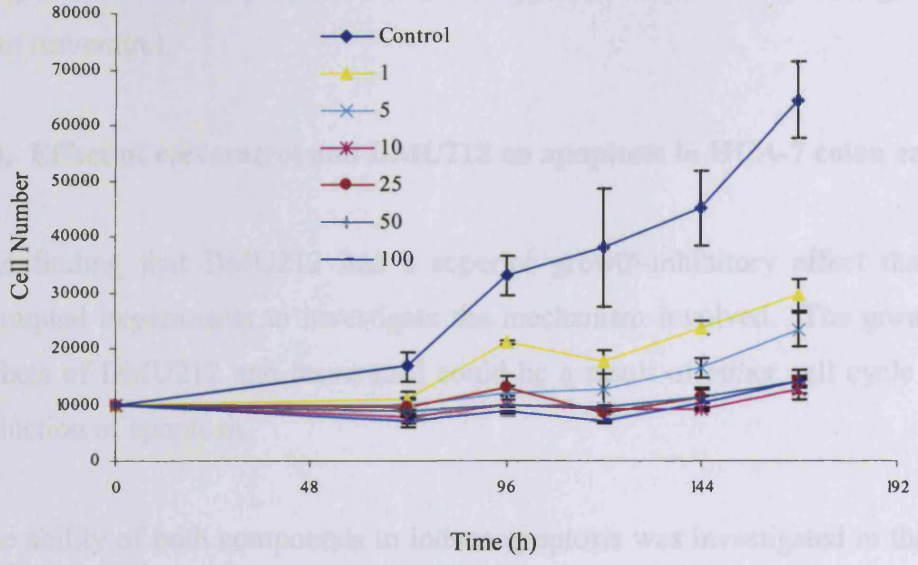


Figure 4.2.4. Effect of DMU212 (1-100 μM) on growth of HCA-7 cells. Growth curves were performed as detailed in section 2.4.5. Values are the mean cell number ± SD of 4 independent experiments.

IC_{50} Calculations

The growth-modulating ability of resveratrol and DMU212 was investigated and the IC_{50} values computed from the growth curves shown above (Table 4.2.1.).

Table 4.2.1 IC_{50} values for resveratrol and DMU212 in HT29 and HCA-7 cells. IC_{50} values were determined at 168 h time-point as described in section 2.4.5.

Compound	Mean IC_{50} ± SD	
	HT29	HCA-7
Resveratrol	19.9 ± 0.3 μM	26.2 ± 0.8 μM
DMU212	11.3 ± 1.1 μM	6.0 ± 0.2 μM

Data shown are the mean ± SD (n = 4).

The results indicate that both resveratrol and DMU212 induce a dose- and time-dependent inhibition of cell proliferation in the HT29 and HCA-7 cells. The IC_{50} values ranged from 6 to 26 μM, and DMU212 appears to be a more potent growth inhibitor than resveratrol.

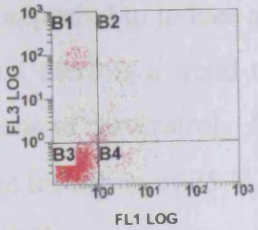
4.3. Effect of resveratrol and DMU212 on apoptosis in HCA-7 colon cancer cells.

The finding that DMU212 had a superior growth-inhibitory effect than resveratrol prompted experiments to investigate the mechanism involved. The growth-inhibitory effects of DMU212 and resveratrol could be a result of either cell cycle arrest and/or induction of apoptosis.

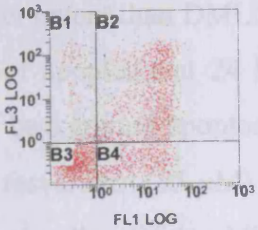
The ability of both compounds to induce apoptosis was investigated in the HCA-7 cell line, as this appeared to be more sensitive to both compounds. HCA-7 cells were cultured in the presence of resveratrol or DMU212 for 24 and 48 h, treated with annexin V and PI and analysed by flow cytometry (section 2.4.6). Representative fluorescence scattergrams from the analysis of the cells for the 48 h treatment are shown in Figure 4.3.1.

The scattergrams are divided into quadrants representing the proportion of cells that are either living (B3), or undergoing early apoptosis (B4), late apoptosis (B2) or necrosis (B1). Treatment of HCA-7 cells with resveratrol and DMU212 did not result in any significant time- or dose- dependent induction or inhibition of apoptosis. These findings suggest that induction of apoptosis is not a major mechanism by which these agents reduce cell number in this cell line.

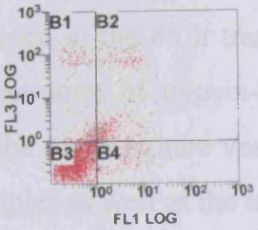
Control



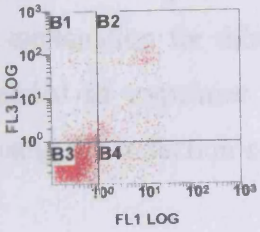
Positive Control



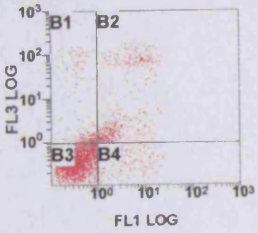
Resveratrol 1μM



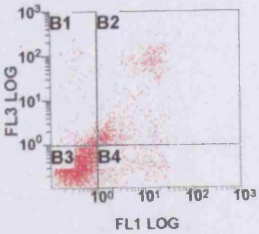
DMU212 1μM



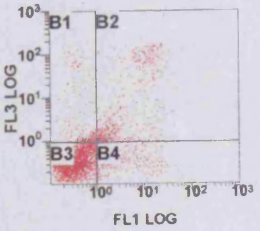
Resveratrol 10μM



DMU212 10μM



Resveratrol 50μM



DMU212 50μM

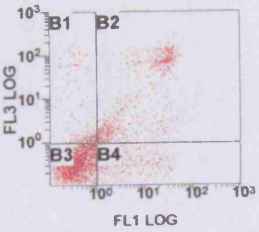


Figure 4.3.1. Representative fluorescence scattergrams of flow cytometric analyses of HCA-7 cells. Cells were treated with DMSO, etoposide (positive control, 25μM), resveratrol or DMU212 for 48 h. Cells were stained with annexin V and PI as described in Chapter 3.4.6. Live cells do not stain with either compound and therefore appear in the bottom left quandrant (B3). Apoptotic cells stain with annexin V and appear in the bottom right (early apoptosis, B4) and top right (late apoptosis, B2) quadrants. Necrotic cells stain with both annexin V and PI and appear in the top left quadrant (B1).

The difference in growth inhibitory potential between the compounds was not reflected in any differences in the induction of apoptosis. The results were inconclusive, but resveratrol appeared to induce apoptosis to a greater extent than DMU212. Figure 4.3.2 suggests that there is a trend towards induction of apoptosis at 24 h with increasing concentrations of resveratrol. At 24 h the level of background apoptosis appeared high, therefore the induction of 25 % apoptotic cells by resveratrol (25 μM) did not reach any significant difference between treated and untreated cells. With additional data points for each concentration, the trend may prove significant. High levels of background were also seen in the 48 h treatment group. An explanation for this finding is that a high concentration of trypsin-EDTA (10x) is needed to trypsinise the cells off the bottom of the tissue culture vessels, therefore, causing the induction of apoptosis in the control samples as well as the treatment samples.

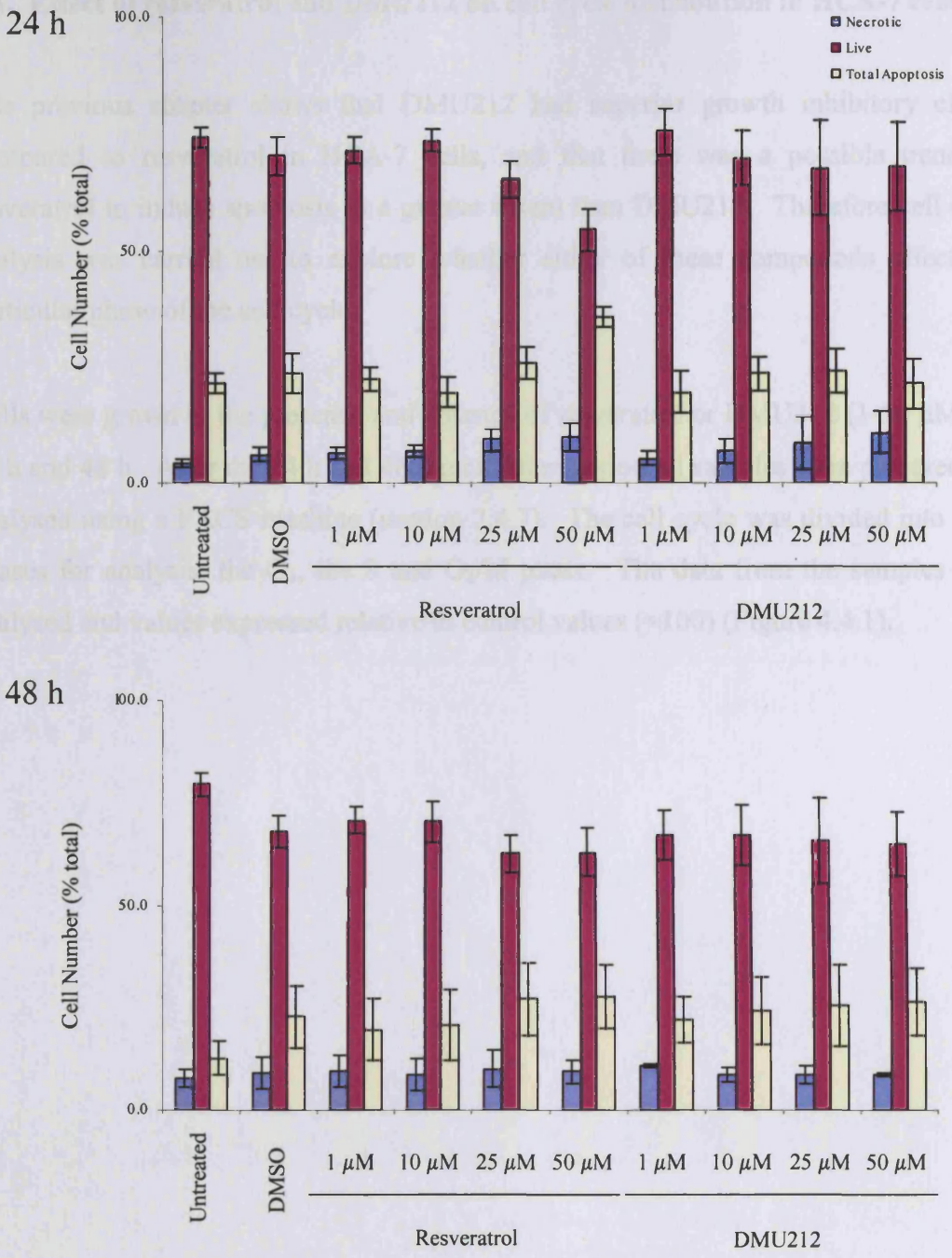


Figure 4.3.2. The proportion of live, apoptotic and necrotic HCA-7 cells following 24 and 48 h incubations with resveratrol or DMU212 (1 – 50 μM). Data shown are the mean ± SD (n= 3).

4.4. Effect of resveratrol and DMU212 on cell cycle distribution in HCA-7 cells

The previous chapter shows that DMU212 had superior growth inhibitory effects compared to resveratrol in HCA-7 cells, and that there was a possible trend for resveratrol to induce apoptosis to a greater extent than DMU212. Therefore cell cycle analysis was carried out to explore whether either of these compounds affected a particular phase of the cell cycle.

Cells were grown in the presence and absence of resveratrol or DMU212 (1-50 μM) for 24 h and 48 h. After the 24 h and 48 h incubation period all samples were prepared and analysed using a FACS machine (section 2.4.7). The cell cycle was divided into three phases for analysis; the G_1, the S and G_2/M phase. The data from the samples were analysed and values expressed relative to control values (=100) (Figure 4.4.1).

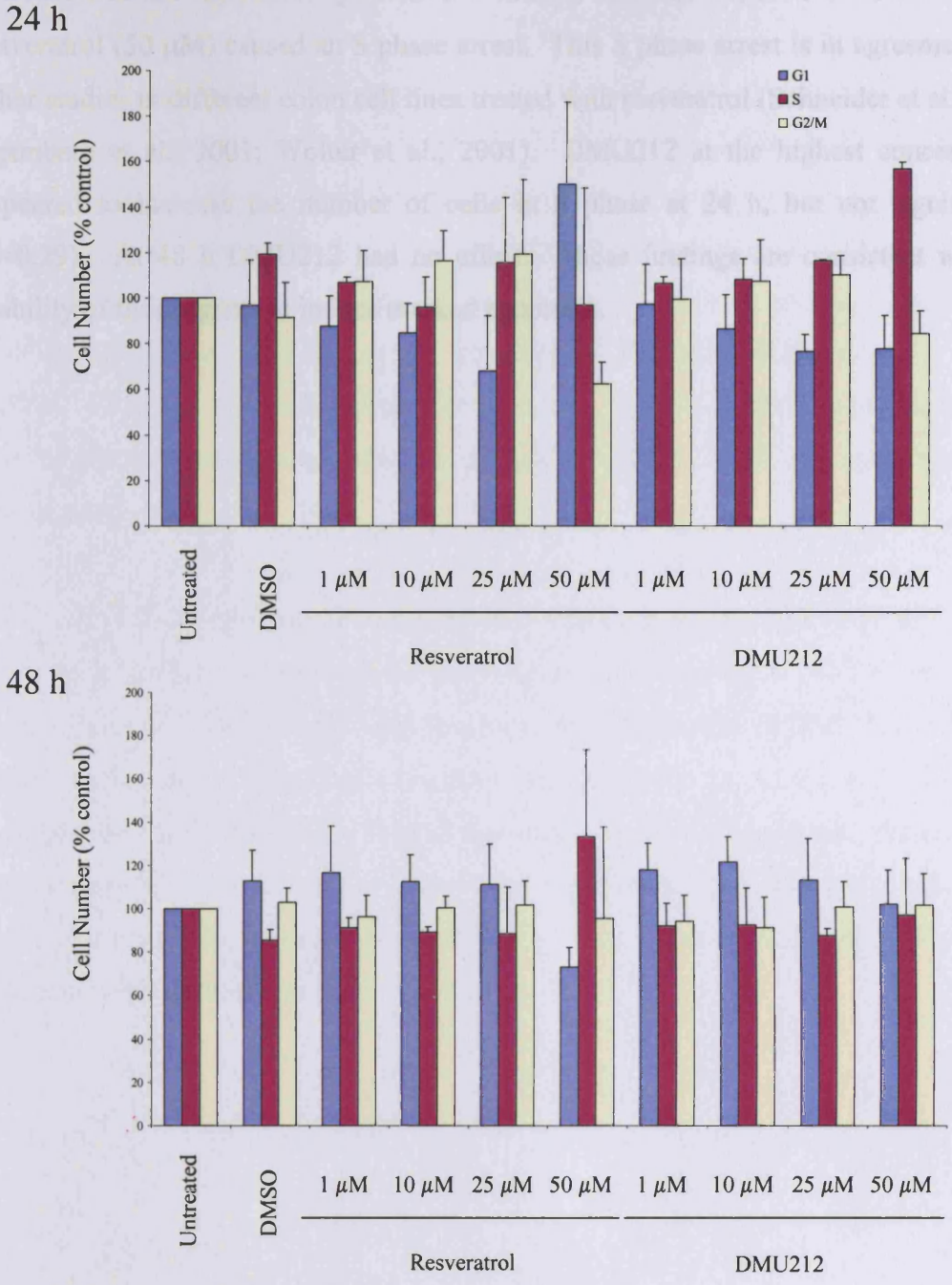


Figure 4.4.1. Analysis of HCA-7 cell cycle distribution following incubation with resveratrol and DMU212 (1 – 50 μM) for 24 and 48 h. Values are the mean ± SD for 3 separate experiments.

At 24 h, resveratrol (50 μM) caused an accumulation of cells in G_1, however the increase was not significant (p=0.127), a finding that was not seen at 48 h. At 48 h resveratrol (50 μM) caused an S phase arrest. This S phase arrest is in agreement with other studies in different colon cell lines treated with resveratrol (Schneider et al., 2000; Sgambato et al., 2001; Wolter et al., 2001). DMU212 at the highest concentration appeared to increase the number of cells in S phase at 24 h, but not significantly (p=0.29). At 48 h DMU212 had no effect. These findings are consistent with the inability of these agents to induce marked apoptosis.

4.5. Effect of resveratrol and DMU212 on Cyclooxygenase-2 (COX-2) expression in colon cells

The two colorectal cancer cell lines, HT29 and HCA-7, used in these experiments have been shown to over express the protein COX-2 (Sharma et al., 2001a). COX-2 has been shown to play an integral role in the development of colorectal cancer (Chapter 1.4.1). Therefore, COX-2 is a potential target for intervention for these compounds, and these cell lines are suitable models to test the hypothesis that these compounds may interfere with the expression of COX-2 protein. Another cell line used, HCEC, does not express COX-2. However, if stimulated with PMA for 5 h, COX-2 expression can be induced. The effects of these compounds on inducible COX-2 in HCEC cells were also investigated.

HT29 and HCA-7 cells were incubated with resveratrol or DMU212 (1-50 μM) for 24, 48 or 96 h, before cell lysates were prepared and analysed for COX-2 expression by Western analysis. HCEC cells were incubated with resveratrol or DMU212 (1-50 μM) for 1 h before being stimulated with PMA (50 ng/ml) for 5 h after which cell lysates were prepared and analysed for COX-2 expression by Western analysis. Protein assays were performed on all cell lysates before Western analysis but blots were striped and re-probed for α-tubulin to ensure equal protein loading. Representative blots for all cell lines and time points are shown in figures 4.5.1-4.5.7.

HT29 cells

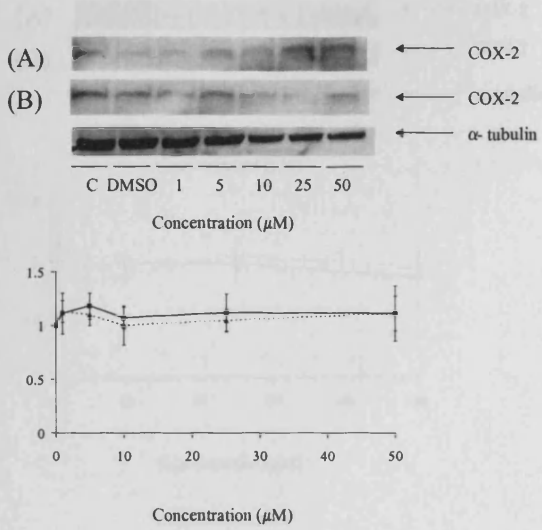


Figure 4.5.1. Effect of 24 h incubation with (A) resveratrol (dashed line) and (B) DMU212 (solid line) on COX-2 expression in HT29 cells. Densitometric COX-2 levels are expressed relative to control (=1) and corrected for protein loading using α-tubulin. Values are the mean ± SD (n = 3).

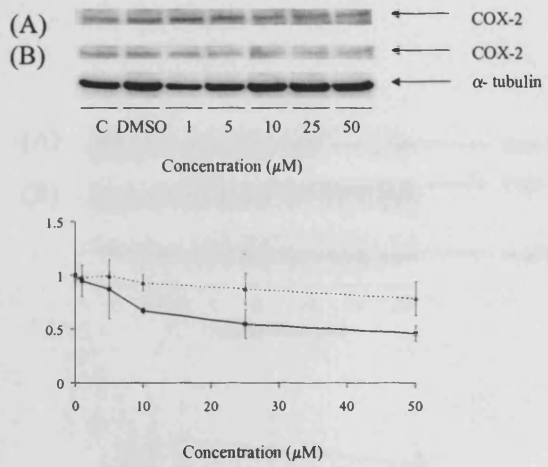


Figure 4.5.2. Effect of 48 h incubation with (A) resveratrol (dashed line) and (B) DMU212 (solid line) on COX-2 expression in HT29 cells. Densitometric COX-2 levels are expressed relative to control (=1) and corrected for protein loading using α-tubulin. Values are the mean ± SD (n = 3).

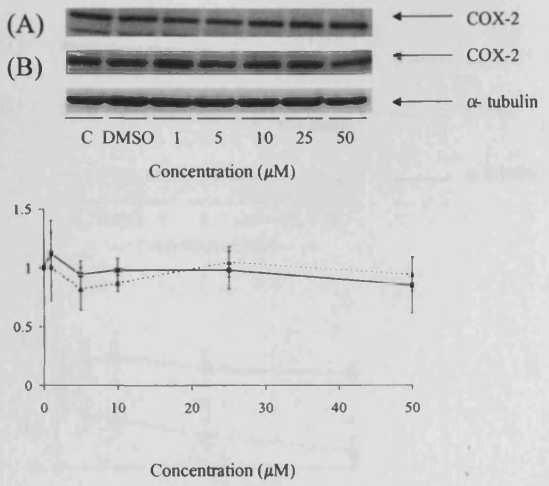


Figure 4.5.3. Effect of 96 h incubation with (A) resveratrol (dashed line) and (B) DMU212 (solid line) on COX-2 expression in HT29 cells. Densitometric COX-2 levels are expressed relative to control (=1) and corrected for protein loading using α-tubulin. Values are the mean ± SD (n = 3).

HCA-7 cells

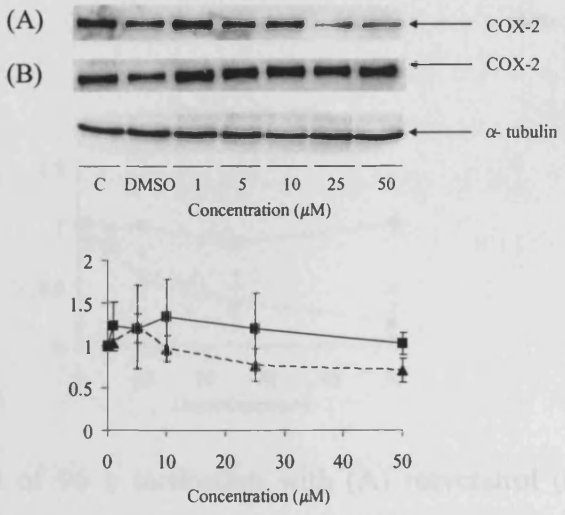


Figure 4.5.4. Effect of 24 h incubation with (A) resveratrol (dashed line) and (B) DMU212 (solid line) on COX-2 expression in HCA-7 cells. Densitometric COX-2 levels are expressed relative to control (=1) and corrected for protein loading using α-tubulin. Values are the mean ± SD (n = 3).

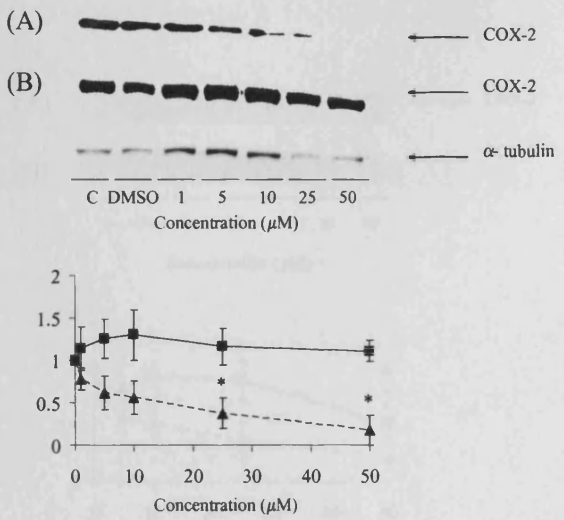


Figure 4.5.5. Effect of 48 h incubation with (A) resveratrol (dashed line) and (B) DMU212 (solid line) on COX-2 expression in HCA-7 cells. Densitometric COX-2 levels are expressed relative to control (=1) and corrected for protein loading using α-tubulin. Values are the mean ± SD (n = 3). Statistical significance from the control values are indicated by the "*" ($p< 0.05$).

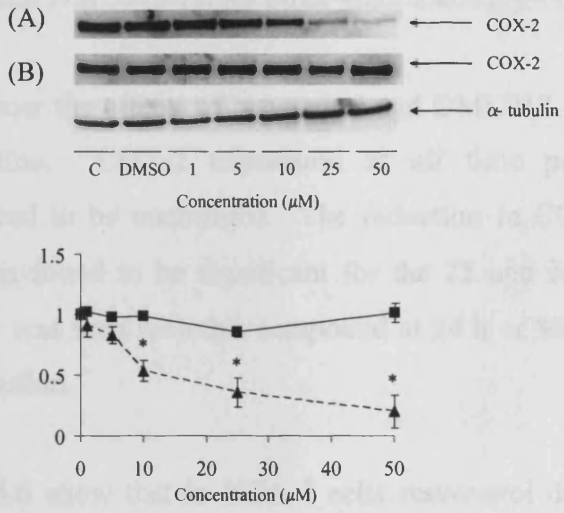


Figure 4.5.6. Effect of 96 h incubation with (A) resveratrol (dashed line) and (B) DMU212 (solid line) on COX-2 expression in HCA-7 cells. Densitometric COX-2 levels are expressed relative to control (=1) and corrected for protein loading using α-tubulin. Values are the mean ± SD (n = 3). Statistical significance from the control values are indicated by the "*" ($p< 0.05$).

HCEC cells

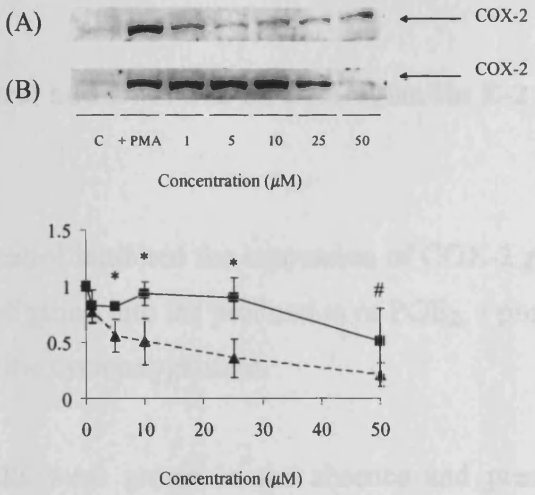


Figure 4.5.7. Effect on inducible COX-2 expression with (A) resveratrol (dashed line) and (B) DMU212 (solid line) in PMA stimulated HCEC cells. Densitometric COX-2 levels are expressed relative to control (=1) and corrected for protein loading using α-tubulin. Values are the mean ± SD (n = 3). Statistical significance from the control is indicated by either the "*" (only resveratrol values differ significantly, $p< 0.05$) or the "#" (both resveratrol and DMU212 values differ significantly, $p< 0.05$).

Figures 4.5.1-4.5.3 show the effects of resveratrol and DMU212 on COX-2 expression in the HT29 cell line. COX-2 expression at all time points and compound concentrations appeared to be unchanged. The reduction in COX-2 expression with DMU212 at 48 h was found to be significant for the 25 and 50 μM concentrations, however, as no effect was seen with this compound at 24 h or 96 h this finding should be interpreted with caution.

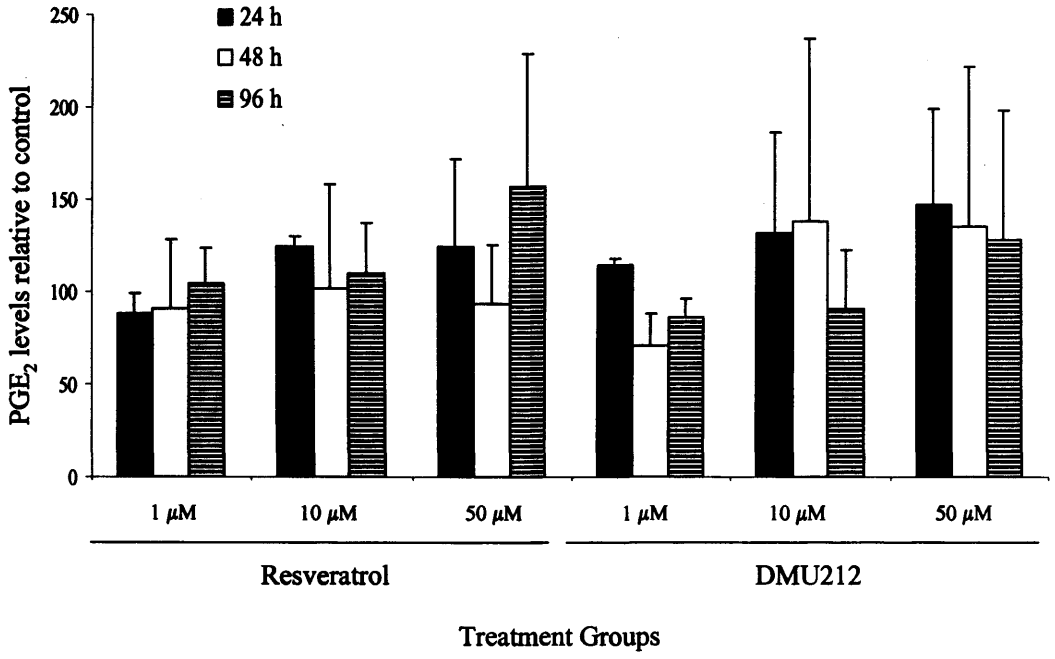
Figures 4.5.5 and 4.5.6 show that in HCA-7 cells resveratrol downregulated COX-2 expression after exposure for 48 or 96 h. Such a decrease in protein level was not seen after 24 h, suggesting that during this short time span the rate of degradation of pre-existing protein was too short to allow inhibition of COX-2 expression to be observed. DMU212 failed to affect COX-2 levels in HCA-7 cells at any time point.

In HCEC cells (figure 4.5.7) resveratrol inhibited PMA-mediated induction of COX-2 expression with an IC_{50} of between 5 and 10 μM, whilst DMU-212 did not affect COX-2 levels except at the highest concentration of 50 μM.

4.6 Effect of resveratrol and DMU212 on prostaglandin E-2 (PGE_2) production in colon cells

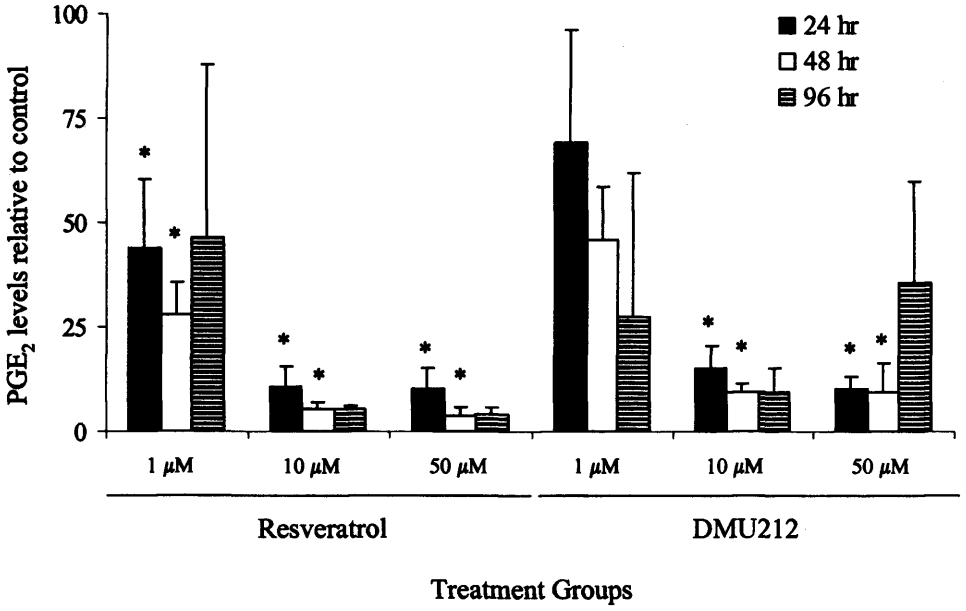
The finding that resveratrol inhibited the expression of COX-2 protein whilst DMU212 did not, led to an investigation into the production of PGE_2, a product of the metabolism of arachidonic acid by the cyclooxygenases.

HT29 and HCA-7 cells were grown in the absence and presence of resveratrol or DMU212 (1-50 μM) for 24 h, 48 h or 96 h respectively. HCEC cells were incubated with medium containing resveratrol or DMU212 (1–50 μM), arachidonic acid (25 μM) and the tumour promoter, PMA (50 ng/ml) for 24 h. PGE_2 levels in the cellular supernatant were determined using a PGE_2 Enzyme Immunoassay (EIA) Kit. PGE_2 levels were expressed as amount per mg cellular protein. Results from these experiments are shown in Figures 4.6.1 – 4.6.3.



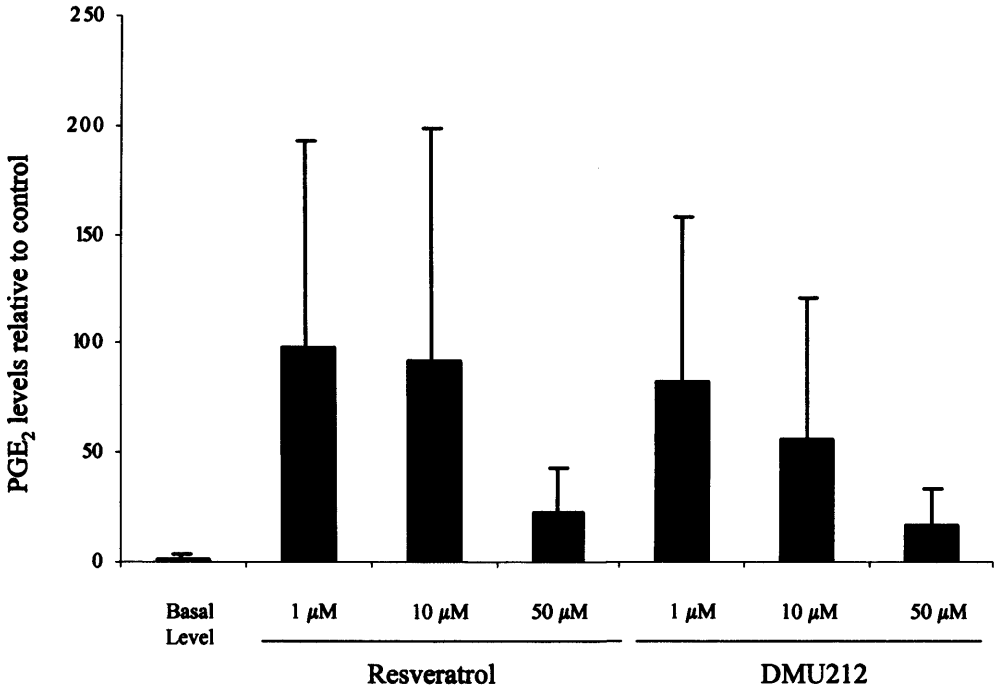
		PGE$_2$ Levels (pg/mg protein)		
		24 h	**48 h**	**96 h**
Control		171 ± 11	155.5 ± 52.5	69.6 ± 3.2
Resveratrol	**1 μM**	150.4 ± 19.6	141.5 ± 57.6	72.7 ± 13.3
	10 μM	212.3 ± 10.3	157.9 ± 88.5	76.4 ± 19.3
	50 μM	196.3 ± 81.5	145.8 ± 48.9	109.5 ± 49.6
DMU212	**1 μM**	225.7 ± 6.5	110.7 ± 26.5	59.9 ± 7.2
	10 μM	251.6 ± 92.8	215.3 ± 154.1	63.2 ± 22.5
	50 μM	255.5 ± 89	211.2 ± 134	89.3 ± 48.8

Figure 4.6.1. PGE_2 levels in supernatent of HT29 cells after 24 h, 48 h or 96 h incubation with resveratrol or DMU212. Values in the graph are expressed relative to control (=100) and represent the mean ± SD (n = 3). The table below represents the raw data of PGE_2 levels expressed as pg PGE_2 / mg protein.



		PGE_2 Levels (pg/mg protein)		
		24 h	**48 h**	**96 h**
Control		222824 ± 72242	279225 ± 148850	209936 ± 142580
Resveratrol	**1 μM**	97302 ± 36599 *	78162 ± 21362 *	96840 ± 86998
	10 μM	23848 ± 11026 *	14849 ± 4808 *	10835 ± 2320
	50 μM	23276 ± 10772 *	10383 ± 5385 *	8299 ± 3772
DMU212	**1 μM**	154509 ± 60470	127673 ± 36470	57617 ± 72062
	10 μM	33677 ± 12069 *	26531 ± 5455 *	19938 ± 11594
	50 μM	22948 ± 6439 *	26240 ± 19700 *	74978 ± 50596

Figure 4.6.2. PGE_2 levels in supernatent of HCA-7 cells after 24 h, 48 h or 96h incubation with resveratrol or DMU212. Values in the graph are expressed relative to control (=100) and represent the mean ± SD (n = 3). The table below represents the raw data of PGE_2 levels expressed as pg PGE_2 / mg protein. Statistical significance compared to control values are indicated by "*" ($p < 0.05$).



		PGE_2 Levels (pg/mg protein)
Control		4875008 ± 4850866
Basal (no AA added)		81099 ± 66155
Resveratrol	**1 μM**	4769327 ± 4640071
	10 μM	4510853 ± 5142454
	50 μM	1108025 ± 888763
DMU212	**1 μM**	4040736 ± 3635373
	10 μM	2742496 ± 3129676
	50 μM	826766 ± 830772

Figure 4.6.3. PGE_2 levels in supernatent of HCEC cells stimulated with PMA and arachidonic acid addition after 24 h, incubation with resveratrol or DMU212. Values in the graph are expressed relative to control (=100) and represent the mean ± SD (n = 3). The table below represents the raw data of PGE_2 levels expressed as pg PGE_2 / mg protein.

PGE_2 levels in HT29 cells were not affected by either resveratrol or DMU212. However the levels in control cells were only just above the limit of detection for the assay.

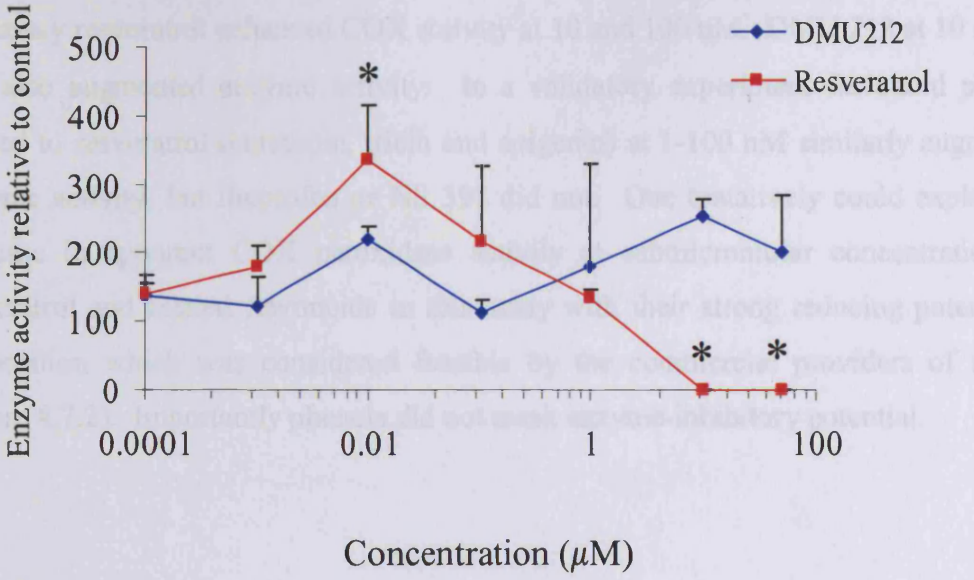
PGE_2 levels in HCA-7 cells were depressed by both resveratrol and DMU212 at all three time points, although the decrease did not reach statistical significance at 96 h because of considerable variability of the control values.

PGE_2 levels in the HCEC cells were depressed by both stilbenes as observed in HCA-7 cells, albeit most noticeable at 50 μM, although the reduction was not significant, because of considerable variability of the values.

4.7 Effect of resveratrol and DMU212 on COX activity in a cell free system

We wished to explore whether the ability of the stilbenes to inhibit PGE_2 generation in HCA-7 and HCEC cells is the outcome of their enzyme-inhibitory potential. Inhibition of COX activity by the stilbenes was investigated using a Chemiluminescent Cyclooxygenase Activity Kit. Enzyme activity was measured following the instructions of the manufacturer (section 2.5.6). In this assay the peroxidative activity of COX enzymes is measured after addition of arachidonic acid and a luminescent substrate. Enzyme preparations were incubated with stilbenes (0.0001–50 μM) for 2 h. Results of this experiment are shown in figure 4.7.1.

COX-1



COX-2

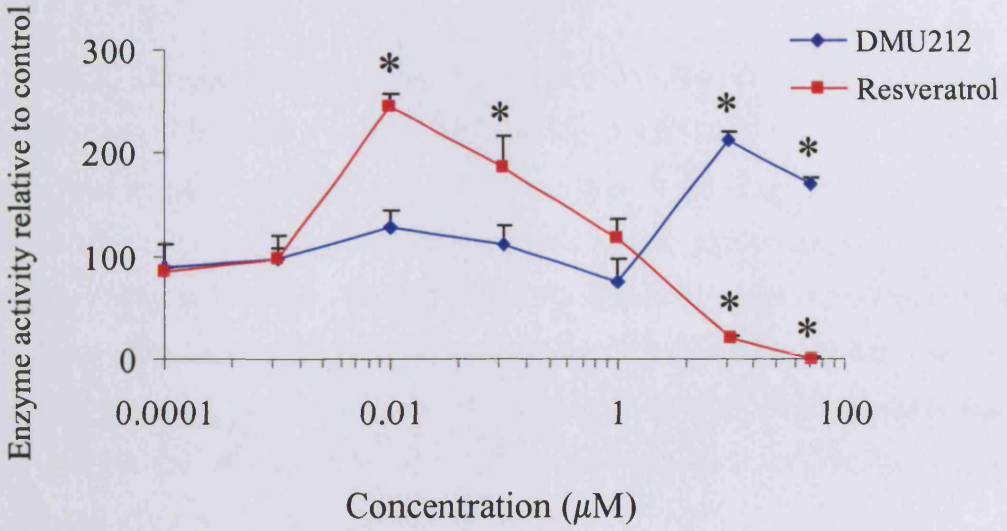


Figure 4.7.1. Effect of resveratrol and DMU212 on activity of COX-1 and COX-2 in purified enzyme preparation. Enzyme activity is expressed relative to control (=100) and are the mean ± SD (n = 3-6). "*" indicates that values differ significantly from controls ($p < 0.05$).

Resveratrol appears to potently inhibit COX-1 and COX-2 activity with an IC_{50} value between 1 and 10 μM. DMU212, on the other hand has no inhibitory effect at all. In this assay resveratrol enhanced COX activity at 10 and 100 nM. DMU 212 at 10 and 50 μM also augmented enzyme activity. In a validatory experiment flavonoid phenols related to resveratrol (curcumin, tricin and apigenin) at 1-100 nM similarly augmented enzyme activity, but ibuprofen or NS 398 did not. One tentatively could explain the increase in apparent COX peroxidase activity at submicromolar concentrations of resveratrol and related flavonoids in this assay with their strong reducing potential, a supposition which was considered feasible by the commercial providers of the kit (figure 4.7.2). Importantly phenols did not mask enzyme-inhibitory potential.

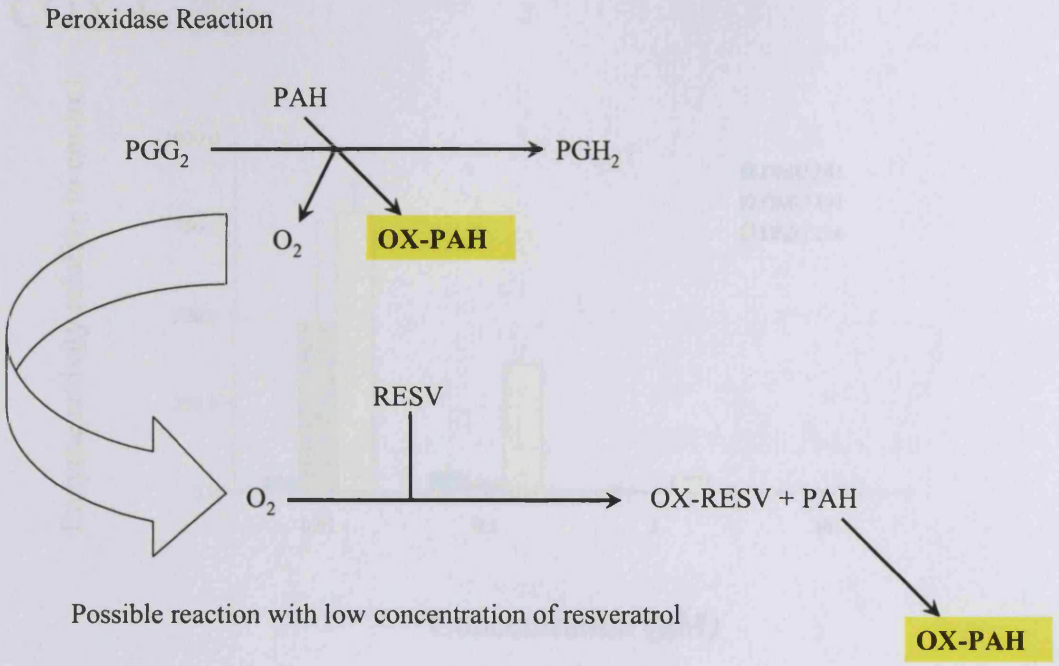
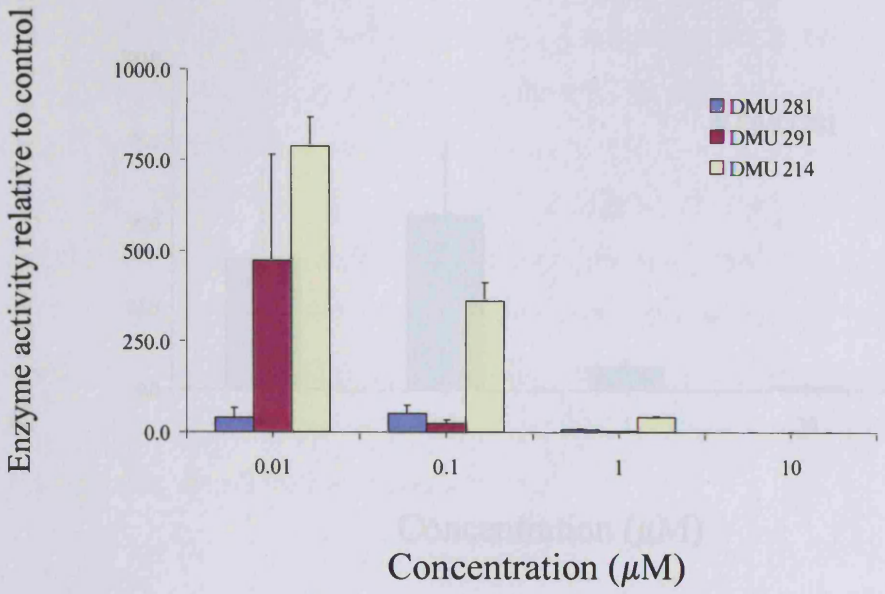


Figure 4.7.2. The peroxidase reaction that converts PGG_2 to PGH_2 is controlled by the COX enzyme. The COX activity kit supplies a substrate (PAH – polyaromatic hydrocarbon) which when oxidised emits light which is measurable. Oxygen is a by-product of this reaction and it is conceivable that at low concentrations of resveratrol, resveratrol becomes oxidised. The PAH could then possibly compete with the oxidised form of resveratrol and itself be oxidised causing further light emission which would explain the enhanced COX activity at low concentrations with all polyphenols tested in this assay. The O_2 and OX-RESV, in the figure, indicate forms of reactive oxygen and unstable oxidised resveratrol, respectively.

DMU212 inhibited PGE_2 production *in vitro,* however in this cell free system DMU212 had no inhibitory effect. When DMU212 was administed orally to mice oxidative metabolites of DMU212 were detected in the gastrointestinal tract (Chapter 3). It was therefore hypothesised that a metabolite of DMU212 causes the inhibitory effect on COX-2 activity seen *in vitro.* To that end, three metabolites of DMU212 were investigated in the same purified enzyme preparation system as that used above. These metabolites were identified as the three most prevalent in the pharmacokinetic study in mice described in Chapter 3.

COX-1



COX-2

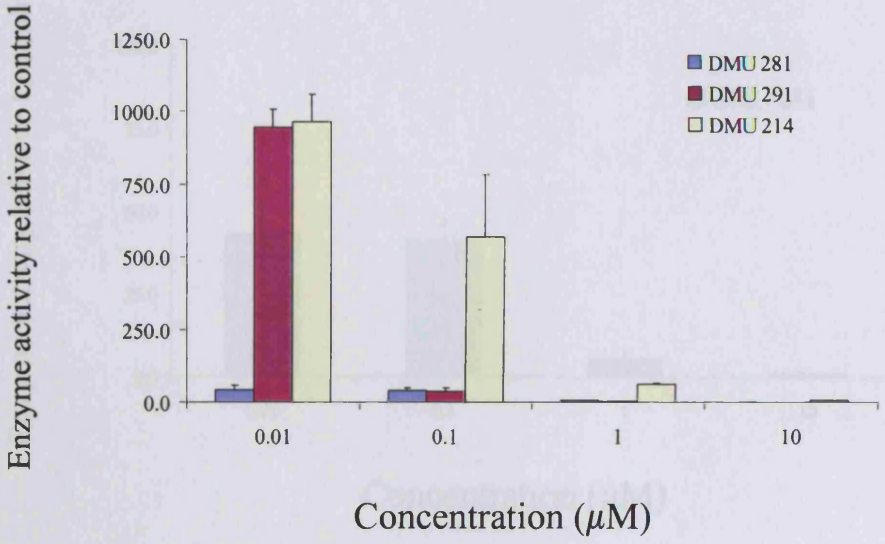
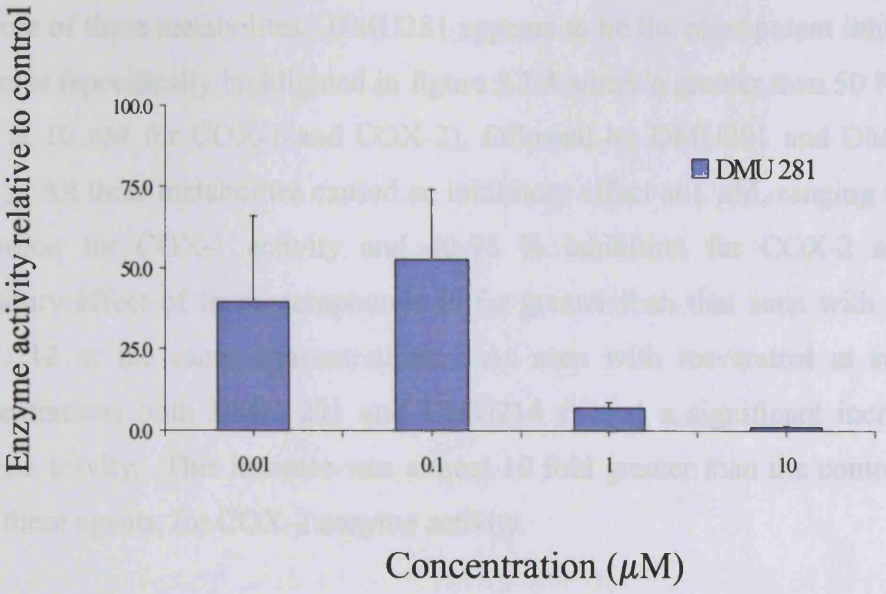


Figure 4.7.3. Effect of DMU281, DMU291 and DMU214, three known metabolites of DMU212, on activity of COX-1 and COX-2. Enzyme activity is expressed relative to control (=100) and are the mean ± SD (n = 3-4). For chemical structures of these metabolites see figure 2.3.1.2.

COX-1



COX-2

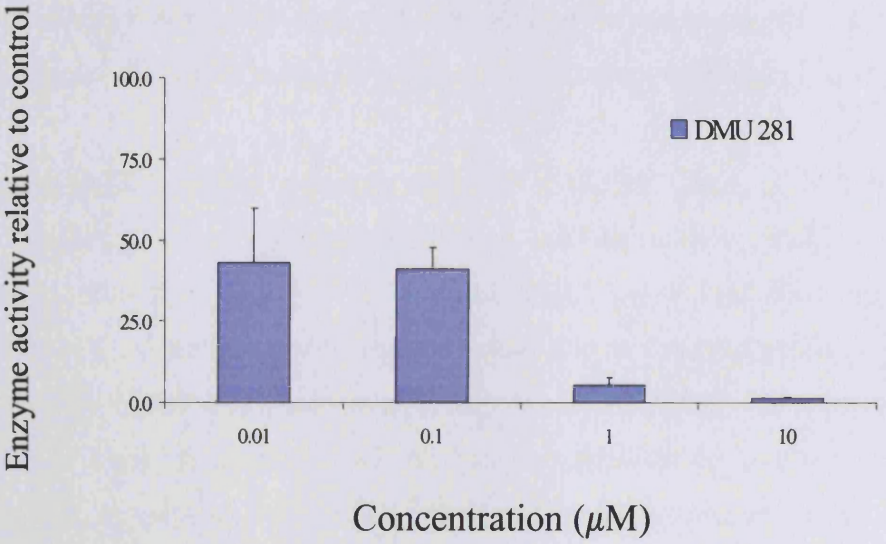


Figure 4.7.4. Effect of DMU281, a metabolite of DMU212, on activity of COX-1 and COX-2. Enzyme activity is expressed relative to control (=100) and are the mean ± SD (n = 3-4). This figure is a magnified copy of DMU281 taken from Figure 4.7.3 to further highlight the potent inhibitory effect that is has on COX activity.

The results, in figures 4.7.3 and 4.7.4, suggest that the inhibitory effect of DMU212 on PGE_2 levels *in vitro* could be a result of the parent compound being metabolised to one or more of these metabolites. DMU281 appears to be the most potent inhibitor of COX enzymes (specifically highlighted in figure 5.7.4 where a greater than 50 % inhibition is seen at 10 nM for COX-1 and COX-2), followed by DMU291 and DMU214 (figure 4.7.3). All three metabolites caused an inhibitory effect at 1 μM, ranging from 59-99 % inhibition for COX-1 activity and 40-98 % inhibition for COX-2 activity. The inhibitory effect of these compounds is far greater than that seen with resveratrol or DMU212 at the same concentrations. As seen with resveratrol at submicromolar concentrations both DMU 291 and DMU214 caused a significant increase in COX enzyme actvity. This increase was almost 10 fold greater than the control value, with both these agents, for COX-2 enzyme activity.

4.8 Discussion

The results described above can be summarized as follows: i) differences exist between resveratrol and DMU212 in terms of inhibition of colon cell growth *in vitro*; ii) resveratrol and DMU212 do not interfere with the apoptosis and/or cell cycle pathways in the HCA-7 cell line at the time points investigated; iii) both resveratrol and DMU212 interfered with PGE_2 generation in colon-derived cells; iv) resveratrol attenuated cellular COX-2 protein expression, whilst DMU-212 did not, v) resveratrol, but not DMU212 is a potent inhibitor of COX activity in a purified enzyme preparation, vi) the COX-inhibitory effects seen *in vitro* with DMU212, could in part, be due to DMU212 being metabolised to a more potent inhibitor of COX activity.

The growth inhibitory potential of DMU212 was superior to that of resveratrol in both colon cancer cells investigated. This finding is consistent with a previous study which reported that DMU212 was capable of preferentially interfering with proliferation and survival of transformed human lung-derived cells, with much lower growth–inhibitory and apoptogenic properties than with their untransformed counterparts (Lu et al., 2001). Resveratrol, in the same study, did not express this same capability (Lu et al., 2001).

Although COX-2 protein was detected in all of the cell lines, COX-2 enzyme activity was variable. As an estimate of COX-2 enzyme activity, PGE_2 production was measured in the HT29, HCA-7 and stimulated HCEC cell line. PGE_2 was chosen as an indicator of COX activity as this has been shown to be the most predominant eicosanoid produced in HCA-7 and other colon cells (Shao et al., 2000). This study also reported that HT29 cells, in contrast, did not produce appreciable levels of prostaglandins, despite the presence of low levels of COX-2 protein (Shao et al., 2000). The findings from our study are consistent with those shown previously albeit we did detect very low levels of PGE_2, however, these were very close to the limit of detection for this assay kit and were probably too low to distinguish any differences between the treated and untreated groups (Figure 4.6.1). Our studies with all three cell lines failed to detect any appreciable levels of COX-1 protein (data not shown). Also, other work in our group showed that PGE_2 production by the cells was totally inhibited by celecoxib (Tunstall, Sale, Steward and Gescher, unpublished data). These two findings suggest that the PGE_2 produced by all of these cells is generated through the activity of COX-2.

Figures 4.5.4-4.5.6 show that in the HCA-7 cells, resveratrol downregulated COX-2 expression after 48 or 96 h. Such a decrease in protein level was not seen after 24 h, suggesting that during this short time span the rate of degradation of pre-existing protein was too slow to allow inhibition of COX-2 expression to be observed. Similarly, in PMA-treated HCEC cells (Figure 4.5.7) resveratrol inhibited COX-2 expression with an IC_{50} of between 5 and 10 μM, consistent with previous results in human mammary and oral epithelial cells (Subbaramaiah et al., 1998). DMU212 failed to affect COX-2 levels in HCA-7 or HCEC cells. PGE_2 levels in HCA-7 cells were depressed by both resveratrol and DMU212 at all three time points, although the decrease did not reach statistical significance at 96 h because of the considerable variability of the values (Figure 4.6.2). PGE_2 levels in HCEC cells were also depressed by both stilbenes, albeit only at 50 μM, and the reduction was not statistically significant, because of the considerable variability of the values.

These results suggest that these two agents may have different mechanisms of action. Resveratrol was capable of both the inhibition of COX-2 expression and the inhibition of COX-2 activity, whereas DMU212 had no effect on the transcription of the COX-2 protein but was able to inhibit COX-2 activity but not in the cell free system.

The results, regarding the effects resveratrol and DMU212 have on COX expression and activity, allow some deductions to be made as to stilbene structure-activity relationships. Alteration of the resveratrol molecule by methylation of the three hydroxy moieties and introduction of another methoxy in position 4, which generates DMU212, abolishes ability to downregulate COX-2 expression but does not abrogate the potential to inhibit COX enzyme activity, even though the precise mechanism of COX inhibition seems to differ between the two molecules.

Whilst resveratrol inhibited COX enzymes with an IC_{50} of between 1 and 10 μM, DMU212 had no inhibitory activity in the cell free system (Figure 4.7.1.). Intriguingly, both stilbenes at concentrations of up to 1 μM enhanced enzyme activity significantly. A similar observation was made when curcumin or the flavone apigenin, both polyphenols with potent oxidising properties, were evaluated in the assay as inhibitors, but not with ibuprofen or the COX-2 inhibitor NS-398 (Tunstall, Sale, Steward and Gescher, unpublished data). Therefore, we tentatively implicate oxidative potential with the augmentation of COX enzyme activity at low polyphenol concentrations

(Figure 4.7.2). Resveratrol has been reported to be a better inhibitor of COX-1 than of COX-2 (Johnson & Maddipati, 1998; Szewczuk et al., 2004). It interferes with COX-1 peroxidase and inactivates COX-1 cyclooxygenase, whilst inhibiting only the peroxidase activity of COX-2 *via* its role as a co-substrate, but not COX-2 cyclooxygenase (Jang et al., 1997; Johnson & Maddipati, 1998). The enzyme assay kit used here gauged overall COX enzyme activity as reflected by its peroxidase function and thus did not permit detection of the differential susceptibility of COX-1 and COX-2 towards inhibition and inactivation by resveratrol.

The ability of resveratrol to interfere with COX activity as described above is broadly consistent with published results in which several different sources of COX enzyme were used. The IC_{50} value for COX inhibition observed here was between 1 and 10 μM, which compares with values reported in the literature of 20 μM for COX from ram seminal vesicles (Maccarrone et al., 1999), 32 μM for human recombinant COX-2 (Subbaramaiah et al., 1998) and between 0.3 and 3 μM for PMA- or lipopolysaccharide-induced COX-2 from murine peritoneal macrophages (Martinez & Moreno, 2000). In contrast, at 439 μM (100 μg/ml) resveratrol apparently failed to inhibit recombinant COX-2 whilst totally blocking COX-1 (Zhang et al., 2004). Potent inhibition of COX-1 by resveratrol has recently been shown to be the result of mechanism-based enzyme inactivation (Szewczuk et al., 2004). The meta-dihydroxy functionality in the B ring of resveratrol has been suggested to be essential for COX inactivation (Johnson & Maddipati, 1998), which suggests that DMU212 does not share COX-inactivatory ability with resveratrol. Consistent with this finding DMU212 failed to interfere with COX activity in the isolated enzyme preparation, even though it inhibited PGE_2 production in cells. There are at least two explanations which rationalize these apparently paradoxical findings. Firstly DMU212 might interfere with the metabolic generation of PGE_2 from PGH_2, the end product of COX-catalyzed metabolism of arachidonic acid in the assay kit used here, or alternatively DMU212 might be metabolically *O*-demethylated to species which exert COX-enzyme-inhibitory properties in intact cells but not in a cell-free system.

The second hypothesis is supported by the inhibition of COX enzyme by three metabolites of DMU212. The inhibition of COX activity with these metabolites was more potent than that seen with resveratrol or DMU212. These findings that hydroxylated metabolites of DMU212 caused potent inhibition of COX activity is

consistent with a recently published study (Murias et al., 2004). Here they report that methoxy derivatives of resveratrol were all weaker inhibitors of COX activity than resveratrol itself, a finding we also report in this study with DMU212. However, more interesting is the fact they reported that the hydroxylated resveratrol derivatives were all more potent inhibitors of COX activity than resveratrol itself (Murias et al., 2004). The study also revealed that some of the hydroxylated derivatives of resveratrol actually showed significantly lower IC_{50} values against COX-2 than celecoxib. They hypothesized that this finding should result in lower doses necessary to achieve the same efficacy in clinical trials.

In summary, resveratrol and to a greater extent DMU212 inhibited the growth of the tumour derived HT29 and HCA-7 colon cancer cell lines. The fact that DMU212 is more potent than resveratrol at inhibiting the growth of these cell lines and also the fact that it is found in much higher concentrations and cleared less rapidly in target tissues *in vivo* (chapter 4), suggest that this compound may be of greater therapeutic value than resveratrol. Neither of these compounds had any significant effect on inducing apoptosis or inhibiting the cell cycle process. Resveratrol was able to inhibit COX-2 protein expression in the HCA-7 cell line and also in the PMA induced HCEC cell line whereas DMU212 had little effect with only a slight inhibition at the high dose of 50 μM in the stimulated HCEC cell line. Both compounds decreased PGE_2 production in the HCA-7 and HCEC cell lines indicating their ability to interfere with the activity of COX-2, however, only resveratrol showed inhibitory effects on COX-1 and COX-2 in the cell free system employed to measure COX activity. These findings tentatively suggest that their chemopreventive effects may be mediated via different mechanisms. Having identified possible biomarkers of intervention *in vitro*, it seems reasonable to test these compounds side by side in an animal model of colorectal cancer to see if they are able to interfere with tumour development and/or interfere with COX expression and/or activity.

CHAPTER 5

EFFECT OF RESVERATROL AND DMU212 ON ADENOMA FORMATION AND PHARMACODYNAMIC BIOMARKERS OF EFFICACY IN THE MIN/+ MOUSE

5.1 Introduction

The chemopreventive efficacy of resveratrol in the Min/+ mouse is a topic of debate. As outlined in section 1.7.2, when administered to animals via the drinking water (0.01% w:v), it decreased adenoma number by 70% in the small intestine and completely inhibited adenoma development in the colon (Schneider et al., 2001); however resveratrol administered in the diet (up to 90 mg/kg) was ineffective (Ziegler et al., 2004). Pharmacodynamic changes associated with colorectal cancer development have yet to be proposed. Schneider *et al* (Schneider et al., 2001) explored gene changes by microarray which may accompany resveratrol action in the target tissue. The work conducted as part of the project described here in cells *in vitro* suggests that COX-2 may be a target of both agents, resveratrol and DMU212 (Chapter 5).

In order to assess the potential of a chemopreventive agent for use in the clinic, specific pharmacodynamic biomarkers of efficacy need to be identified. Such biomarkers should allow monitoring of the effect of the agent on neoplasia, and can provide surrogate endpoints to assess the efficacy of treatment. Potential biomarkers should be key components intrinsically linked to carcinogenic mechanisms, and therefore also a target for chemopreventive intervention. Alterations and changes in the presence or expression of such biomarkers in the normal or preneoplastic colorectal mucosa should predict any treatment response (Keller & Giardiello, 2003). The biomarkers investigated in these studies were COX-2 and PGE_2, both known to play a pivotal role in the development of colorectal cancer.

In the light of these considerations the aims of the work described in this chapter are as follows:

1. to elucidate whether administration of resveratrol or DMU212 in the drinking water may reduce adenoma formation and be a viable option for administration in the Min/+ mouse and,

2. to compare the efficacy of dietary intervention with resveratrol and DMU212 in the Min/+ mouse by measuring adenoma formation and,

3. to quantify the expression and activity of COX-2 as a target of these agents in Min/+ mouse adenomas and,

4. to explore the ability of these agents to interfere with intestinal PGE_2 production in intestinal mucosa of mice.

5.2. Effect of resveratrol and DMU212 dissolved in drinking water.

A previous study has shown that a 0.01 % (w:v) dose of resveratrol dissolved in 0.4 % ethanol in drinking water was sufficient to significantly inhibit adenoma development (Schneider et al., 2001). As resveratrol was being evaluated side by side with DMU212, in this study, the dose of 0.01 % dissolved in drinking water seemed to be a sensible starting point to assess the compound's stability. It was important to determine the stability of these compounds since animal drinking water is changed on a weekly basis and compound degradation could affect results.

5.2.1. Feasibility of administration of resveratrol and DMU212 in drinking water.

Solutions of resveratrol and DMU212 were dissolved in tap water and analysed for stability over a one week period using reversed phase HPLC. Peak areas were measured daily and plotted as a percentage of the day 1 value against time for each compound (Figure 5.3.1).

After only 24 h the percentage of DMU212 measured had decreased by over 75 % (figure 5.2.1). Upon examination, solutions of DMU212 were opaque suggesting DMU212 did not stay in solution in tap water. For this reason, DMU212 was considered unsuitable for administration in drinking water. The levels of resveratrol in solution remained consistent over the week with only a small reduction (<20 %) on the final day of analysis which was not significantly different from the control. It was therefore feasible to assess the efficacy of resveratrol in the Min/+ mouse using a 0.01 % (w:v) dose in drinking water.

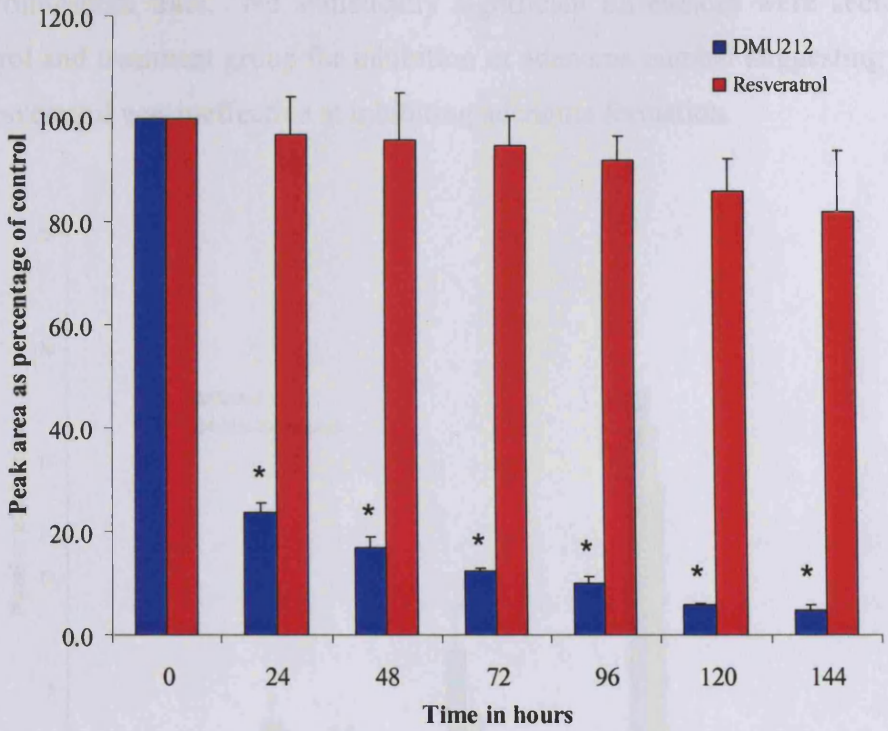


Figure 5.2.1. Stability of solutions of resveratrol (red bars) and DMU212 (blue bars) in drinking water at 0.01 % with 0.4 % ethanol over a one week period. Levels of each compound are plotted as a percentage of the 0 h peak area, determined immediately after solution preparation. Values represent mean ± SD (n = 3), and "*" indicate values which differ significantly from 0 h values ($p < 0.05$).

5.2.2. Effect of resveratrol administered in drinking water on the Min/+ mouse.

Adenoma number

Male and female Min/+ mice were randomly divided, at 5 weeks of age, into two groups (n=10 in each group). Group 1, the control group, were maintained on AIN93G, a synthetic powdered diet and received drinking water containing 0.4% ethanol for a 7 week period. Group 2, the treatment group, were maintained on AIN93G diet and received drinking water containing 0.01% resveratrol dissolved in 0.4% ethanol for 7 weeks. Diet and drinking water were changed every 4 days. At 12 weeks of age mice were culled by cardiac exsanguination, the intestinal tract removed and washed with PBS and adenoma number, size and distribution scored as described previously (section 2.6.2.5).

Figure 5.2.2.1 shows the number, size and distribution of adenomas in the gastrointestinal tract. No statistically significant differences were seen between the control and treatment group for inhibition of adenoma number suggesting that this dose of resveratrol was ineffective at inhibiting adenoma formation.

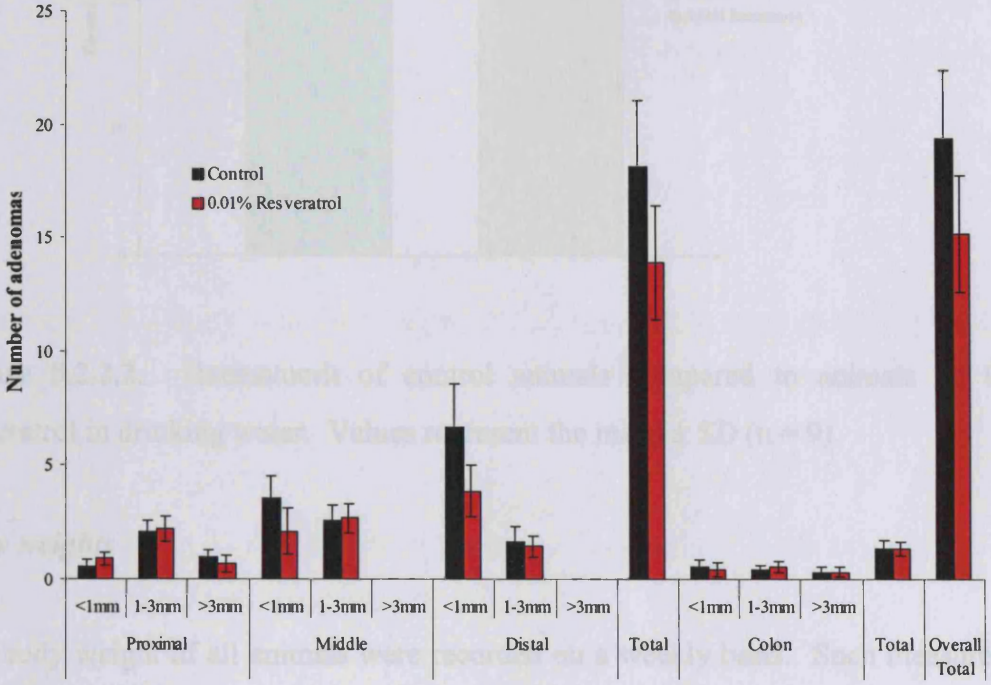


Figure 5.2.2.1. Effects of 0.01% resveratrol in the drinking water on adenoma formation in the Min/+ mouse. The data shows the number, size and location of adenomas within the proximal, middle and distal regions of the small intestine and also the colon. Total adenoma number in the small intestine, and colon, and the overall adenoma count are also shown. Values represent the mean ± SD (n = 9).

Haematocrit

Min/+ mice are prone to anaemia due to haemorrhaging from adenomas within the intestinal tract. In these circumstances the degree of anaemia can reasonably accurately be determined by measuring the haematocrit, provided there is not too much of an increase or decrease in erythrocyte cell size. Figure 5.2.2.2 shows that no significant differences were detected in haematocrit between control and treatment animals.

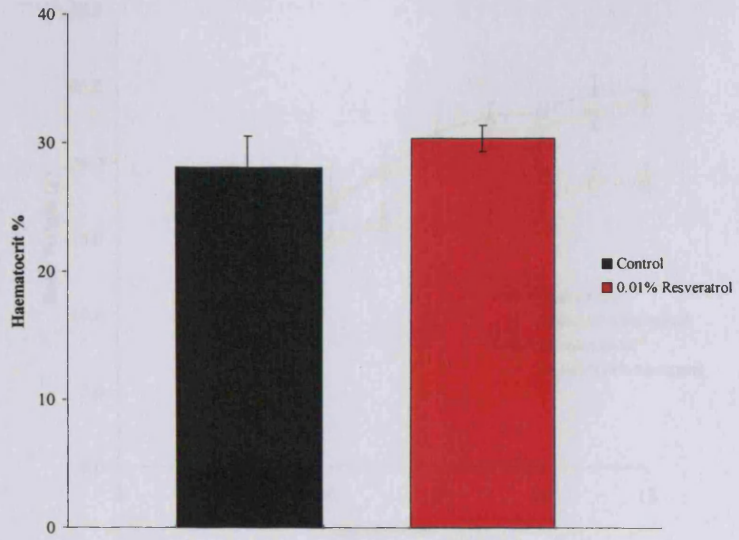


Figure 5.2.2.2. Haematocrit of control animals compared to animals on 0.01% resveratrol in drinking water. Values represent the mean ± SD (n = 9).

Body weights

The body weight of all animals were recorded on a weekly basis. Such measurements can give an indirect indication as to whether the animals consume water or diet in similar quantities to controls. In addition water and diet consumption was measured weekly. There were no differences in water or diet consumption between control and treatment animals (results not shown).

Resveratrol was administered in drinking water for seven weeks and during this time animals did not show any adverse effects.

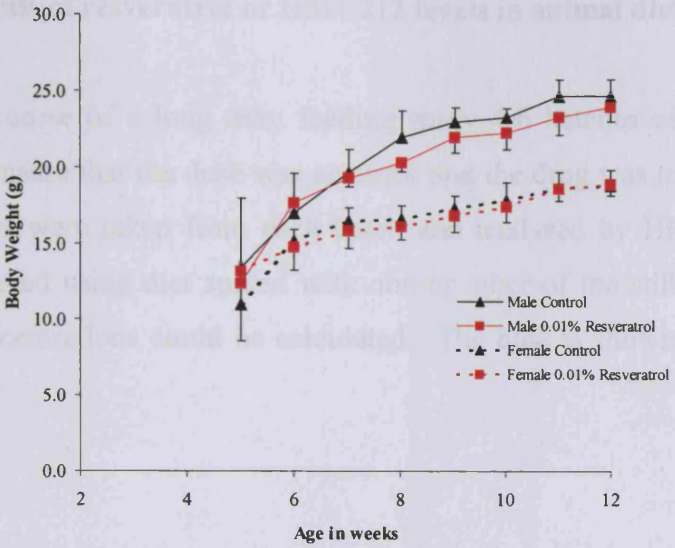


Figure 5.2.2.3. Body weights of male and female Min/+ mice that received control drinking water or water containing 0.01% resveratrol from 5-12 weeks of age. Values represent the mean ± SD (n = 3-7).

Resveratrol had no significant effect on the body weight of male or female mice as compared to control animals (figure 5.2.2.3.).

5.3. Effect of long term administration of resveratrol and DMU212 on the Min/+ mouse.

Animals were bred as described previously (section 2.6.2) and offspring of both genders from batch matings were randomly assigned to study groups. At 4 weeks of age animals were given the study diets. Study diets were changed weekly, and excess study diet was stored in the dark at 4°C. At the end of the experimental period Min/+ mice were culled by cardiac exsanguination. The entire gastrointestinal tract was removed and flushed to remove intestinal content. The gut was opened longitudinally and washed. The stomach and caecum were omitted from the analysis. The small intestine and colon were fixed, and examined for adenomas. The adenomas in the colon were counted with relation to size: <1 mm, 1-3 mm and >3 mm. The small intestine was divided into proximal, middle and distal regions and the adenomas were again discriminated on size. Total adenomas in the small intestine, colon and as an overall total were counted and analysed

5.3.1. Analysis of resveratrol or DMU212 levels in animal diet

During the course of a long term feeding study 4-6 batches of each study diet were mixed. To ensure that the dose was accurate and the drug was mixed thoroughly in the feed, samples were taken from each batch and analysed by HPLC. Standard curves were established using diet spiked with one or other of the stilbene derivative so that accurate concentrations could be calculated. The data is shown in figures 5.3.1.1 and 5.3.1.2.

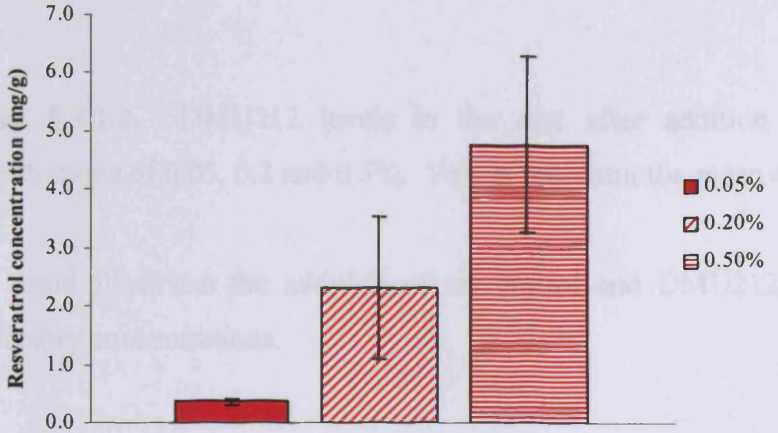


Figure 5.3.1.1. Resveratrol levels in the diet after addition to the feed to give concentrations of 0.05, 0.2 and 0.5%. Values represent the mean ± SD (n = 4-6).

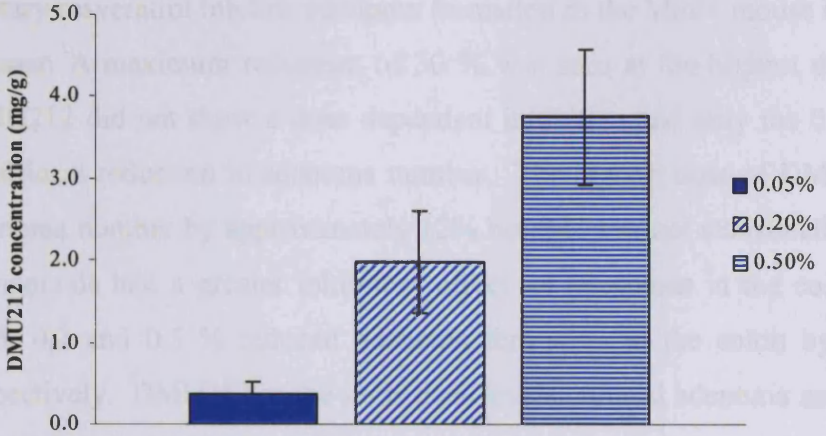


Figure 5.3.1.2. DMU212 levels in the diet after addition to the feed to give concentrations of 0.05, 0.2 and 0.5%. Values represent the mean ± SD (n = 4-6).

This result illustrates the addition of resveratrol and DMU212 to the diet achieved satisfactory concentrations.

5.3.2 Effects of resveratrol and DMU212 admixed in the diet in the Min/+ mouse.

5.3.2.1 Effect of resveratrol and DMU212 on adenoma formation in the Min/+ mouse

These experiments were conducted with two different cohorts of mice and were performed 8 months apart. Control animals from the experiment exploring the effects of 0.2 % dietary resveratrol and DMU212 on adenoma number averaged 105.8 ± 8.5 adenomas in the small intestine and 8.7 ± 1.5 in the colon (n = 10), whereas the control animals from the experiment exploring adenoma number following the dietary administration of 0.05 and 0.5 % resveratrol and DMU212 averaged 55.0 ± 9.2 adenomas in the small intestine and 4.4 ± 0.9 in the colon (n = 12). For this reason, findings are presented as percentage of control rather than crude adenoma count. Also due to the initial cohort of mice developing so many more adenomas, the duration of treatment with resveratrol or DMU212 for these animals was four weeks less

(Administered study diet from 4-14 weeks of age rather than 4-18 weeks of age for the second cohort of mice) as the animals feel ill sooner due to tumour load and burden.

Dietary resveratrol inhibits adenoma formation in the Min/+ mouse in a dose-dependent manner. A maximum reduction of 30 % was seen at the highest dose level of 0.5 %. DMU212 did not show a dose dependent inhibition and only the 0.2 % dose caused a significant reduction in adenoma number. The highest dose of DMU212 reduced total adenoma number by approximately 12% but this was not statistically significant. Both compounds had a greater inhibitory effect on adenomas in the colon. Resveratrol at 0.05, 0.2 and 0.5 % reduced adenoma formation in the colon by 26, 53 and 64 % respectively. DMU212 at the same dose levels reduced adenoma number by 21, 48 and 37 % respectively (Figure 5.3.2.1.1).

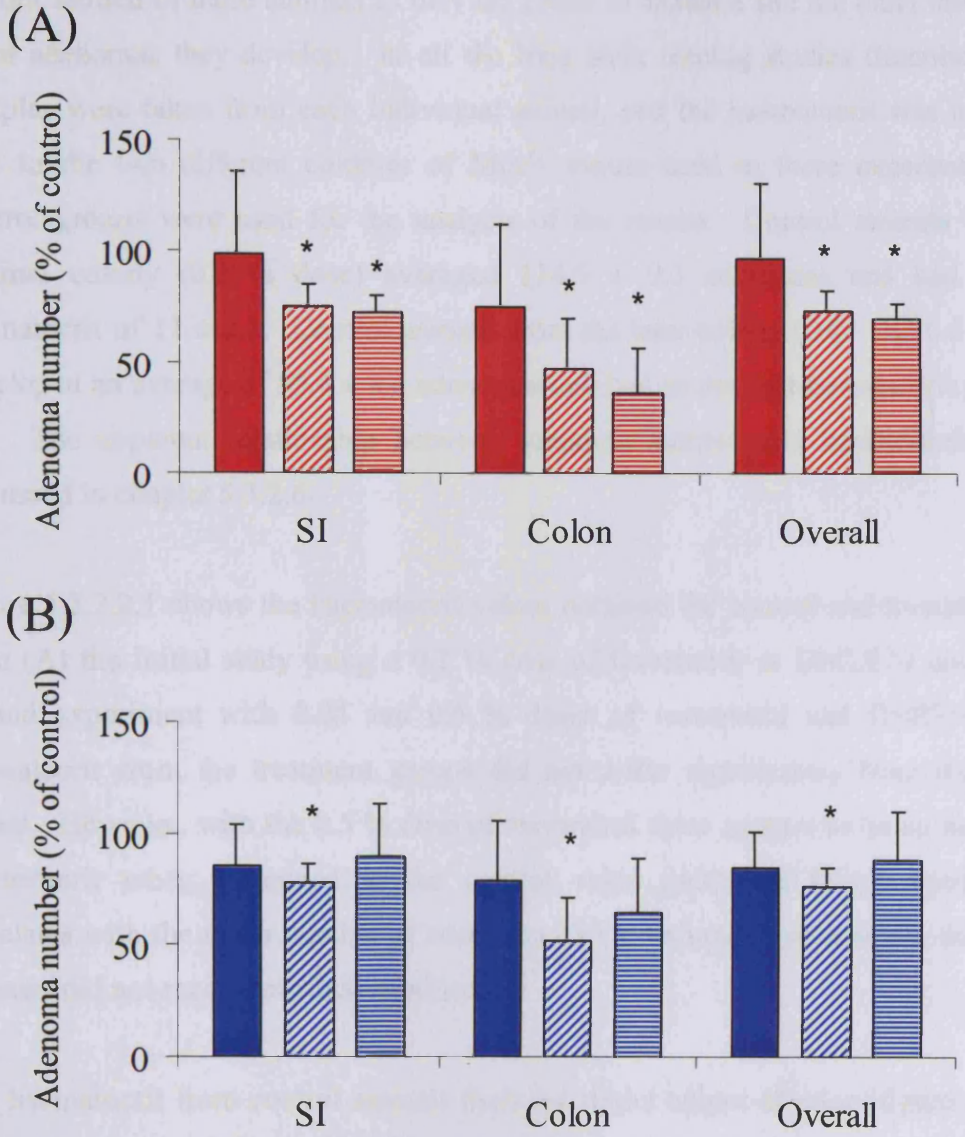


Figure 5.3.2.1.1. Adenoma number following long term administration of (A) resveratrol or (B) DMU212 in the diet at 0.05 (closed bars), 0.2 (diagonally hatched bars) or 0.5% (horizontally hatched bars) in the small intestine (SI) and colon of Min/+ mice. Values are the mean ± SD (n = 10-14). "*" indicates values differ significantly from control untreated animals (one way ANOVA followed by Bonferroni *post hoc* test $p < 0.05$).

5.3.2.2 Effect of resveratrol and DMU212 on haematocrit in the Min/+ mouse.

As already described in section 5.3.2, haematocrit values gives an indication as to the tumour burden of these animals as they are prone to anaemia and the older they get the more adenomas they develop. In all the long term feeding studies described, blood samples were taken from each individual animal, and the haematocrit was measured. Due to the two different colonies of Min/+ mouse used in these experiments, two control groups were used for the analysis of the results. Control animals from the original colony (0.2 % dose) averaged 114.5 ± 9.3 adenomas and had a mean haematocrit of 11 ± 2.1. Control animals from the later colony (0.05 and 0.5 % dose) developed an average of 59.4 ± 8.6 adenomas and had an average haematocrit of 14.5 ± 3.6. The apparent relationship between adenoma number and haematocrit will be discussed in chapter 5.3.2.6.

Figure 5.3.2.2.1 shows the haematocrit values obtained for control and treated animals from (A) the initial study using a 0.2 % dose of resveratrol or DMU212 and (B) the second experiment with 0.05 and 0.5 % doses of resveratrol and DMU212. The haematocrit from the treatment groups did not differ significantly from the control values. However, with the 0.5 % dose of resveratrol there appears to be an increase in haematocrit when compared to the control value (untreated Min/+ mice) which correlates with the fewer number of adenomas (30 % reduction) seen at this dose. This increase did not reach statistical significance.

The haematocrit from control animals from the initial cohort of mice (figure 5.3.2.2.1 (A)) is lower than that seen with the second cohort of mice. An explanation for this difference is due to the greater number of adenomas seen in the intestinal tract of these animals.

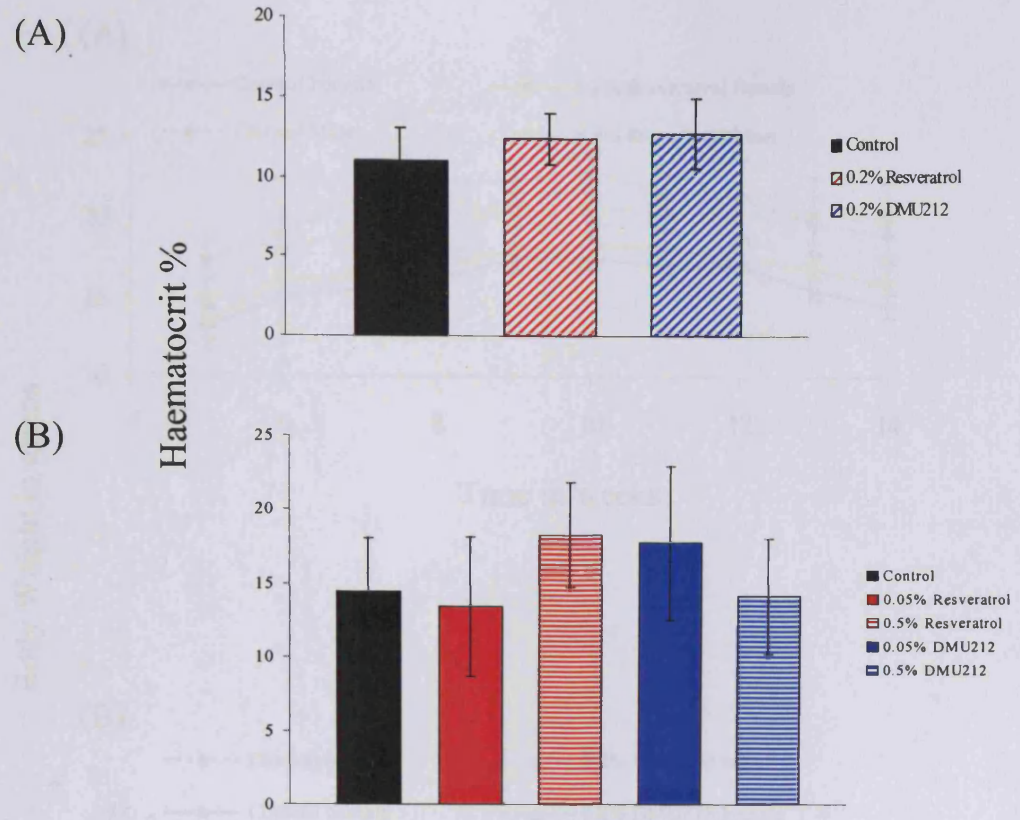


Figure 5.3.2.2.1. Min/+ mouse haematocrit following long-term administration with resveratrol or DMU212. (A) shows the effect of 0.2% resveratrol or DMU212 on haematocrit in animals from the initial Min/+ breeding colony, (B) are haematocrit values for animals from the second breeding colony receiving 0.05 and 0.5% resveratrol or DMU212 (section 2.6.2. regarding Min/+ breeding colonies). Values represent the mean ± SD (n = 10-14)

5.3.2.3 Effect of resveratrol and DMU212 on body weight.

All three doses of resveratrol were well tolerated by the animals, and no difference in body weight was observed between control and treatment groups for male or female mice (figures 5.3.2.3.1A and 5.3.2.3.2A). Figures 5.3.2.3.1 B and 5.3.2.3.2 B show the effect of 0.05, 0.2 and 0.5 % DMU212 on body weight of male and female mice. The 0.5 % dose of DMU212 significantly reduced the body weight of male mice when compared to the control ($p < 0.05$). This difference may not be clear from this figure and it has therefore been highlighted in figure 5.3.2.3.3.

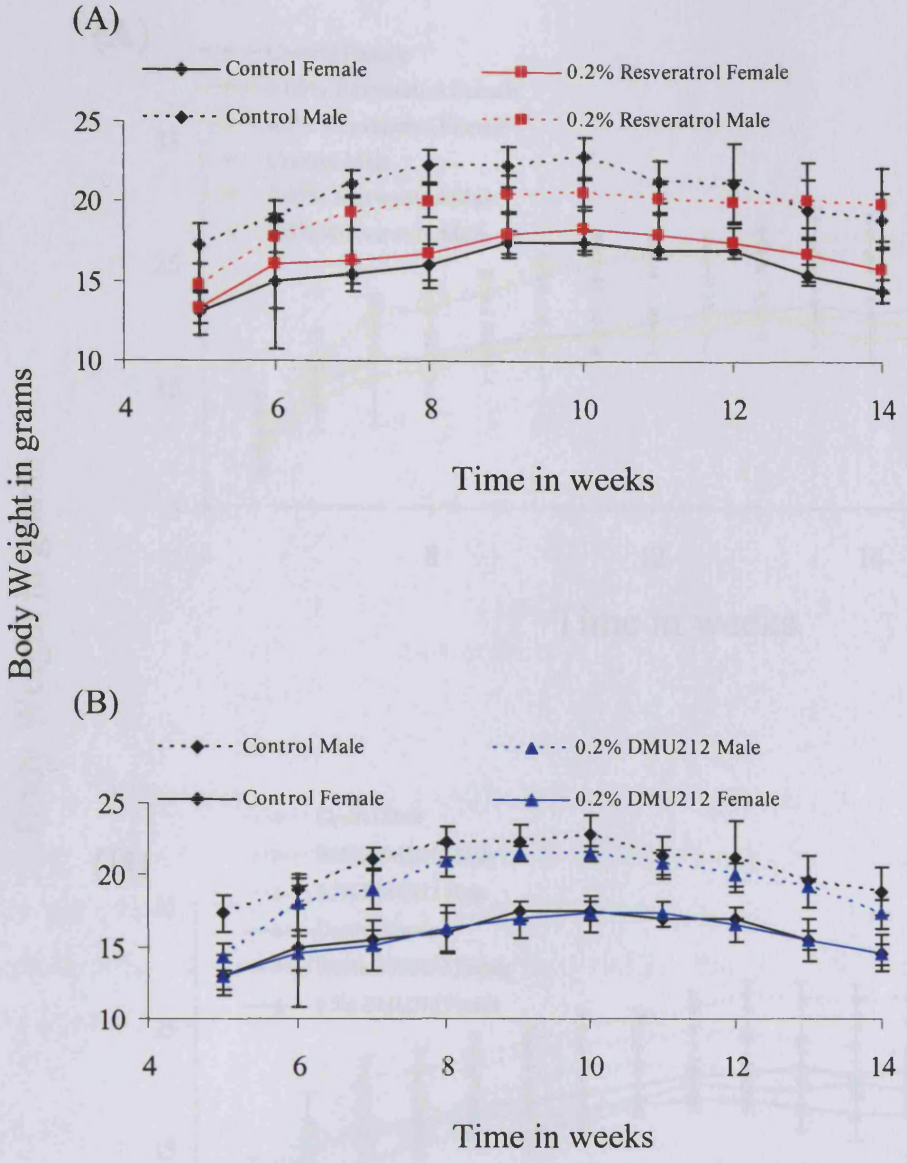


Figure 5.3.2.3.1. Effect of 0.2 % (A) resveratrol or (B) DMU212 on the body weight of male (dashed lines) and female (solid lines) mice during a long term feeding study. Values are the mean ± SD (n = 4-7).

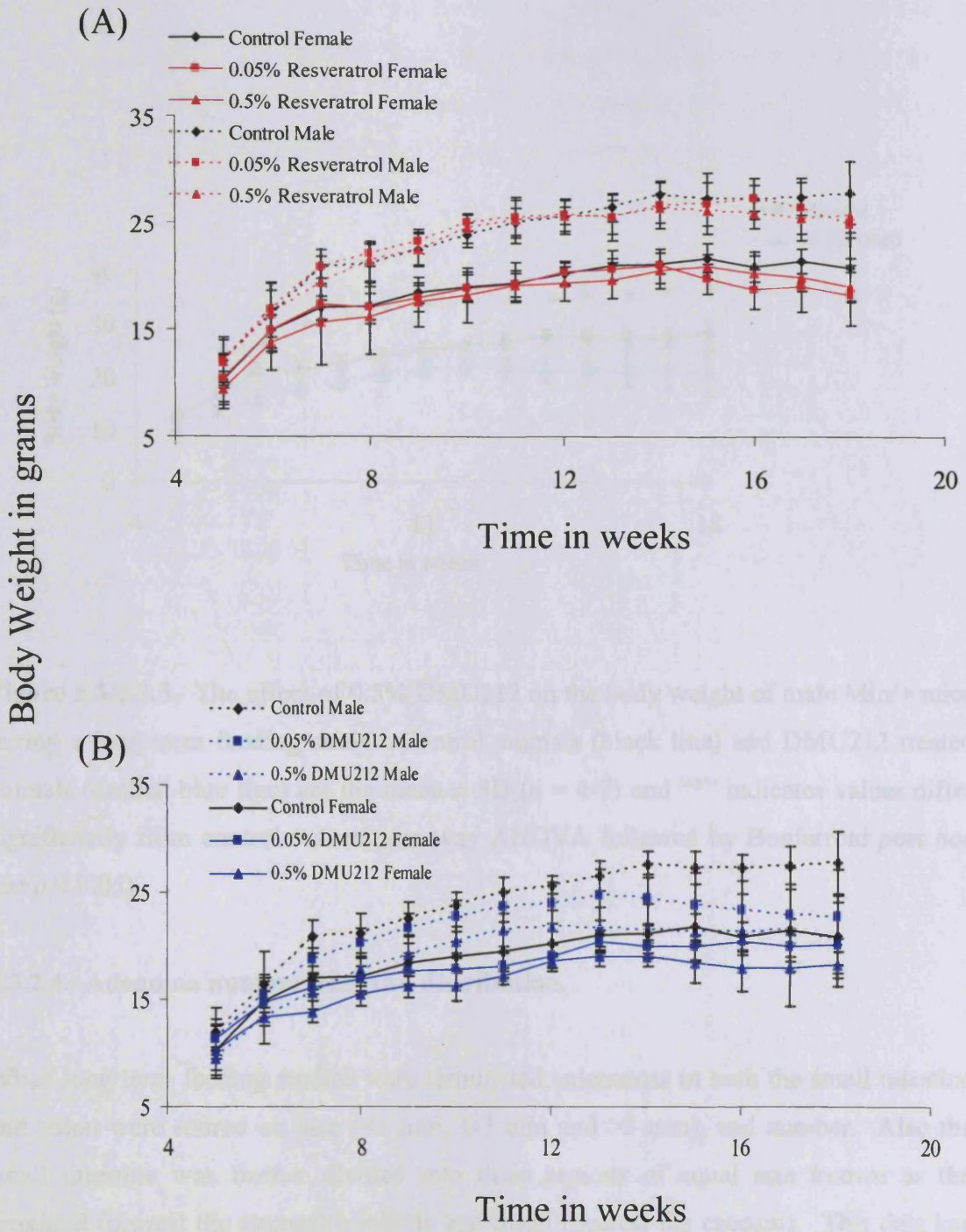


Figure 5.3.2.3.2. Effect of 0.05 and 0.5 % (A) resveratrol or (B) DMU212 on the body weight of male (dashed lines) and female (solid lines) Min/+ mice during a long term feeding study. Values are the mean ± SD (n = 4-7).

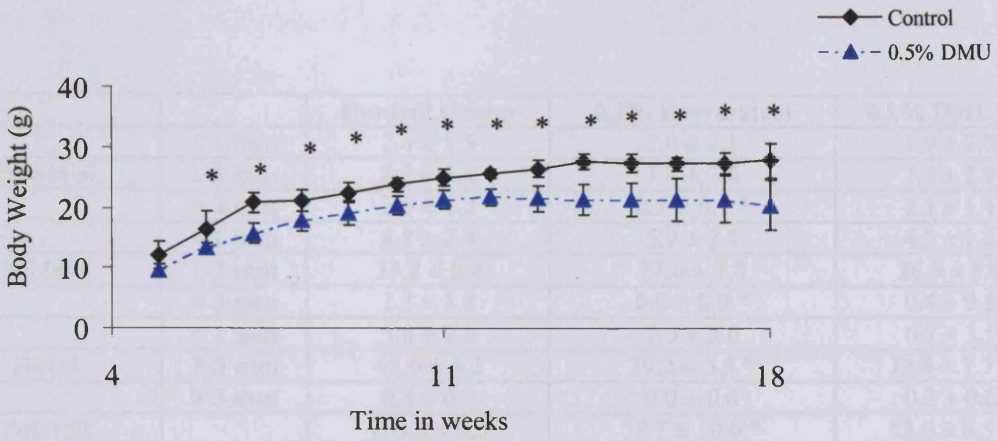


Figure 5.3.2.3.3. The effect of 0.5% DMU212 on the body weight of male Min/+ mice during a long term feeding study. Control animals (black line) and DMU212 treated animals (dashed blue line) are the mean ± SD (n = 4-7) and "*" indicates values differ significantly from control values (one way ANOVA followed by Bonferroni *post hoc* test $p < 0.05$).

5.3.2.4. Adenoma number, size and distribution.

When long term feeding studies were terminated, adenomas in both the small intestine and colon were scored on size (<1 mm, 1-3 mm and >3 mm), and number. Also the small intestine was further divided into three regions of equal size known as the proximal (nearest the stomach), middle and distal (nearest the caecum). This data has been tabulated (Table 5.3.2.4.1).

Table 5.3.2.4.1. Total adenoma numbers per treatment group when expressed based on size and location. (A) Results from initial cohort of mice where 0.2 % dietary resveratrol or DMU212 was administered and (B) the second cohort of mice where 0.05 and 0.5% dietary resveratrol or DMU212 was administered.

(A)

		Control Group	0.2% Resveratrol	0.2% DMU212
	< 1 mm	2.4 ± 1.5	2.0 ± 2.1	1.9 ± 2.0
Proximal	1-3 mm	8.2 ± 3.1	7.8 ± 2.6	7.5 ± 2.9
	> 3 mm	2.6 ± 1.2	0.3 ± 0.7 *	2.1 ± 1.4
	< 1 mm	4.2 ± 2.4	5.9 ± 2.5	4.3 ± 1.2
Middle	1-3 mm	33.2 ± 8.4	27.6 ± 7.0	26.9 ± 8.0
	> 3 mm	1.3 ± 1.8	0.0 ± 0.0 *	0.4 ± 0.5
	< 1 mm	5.6 ± 2.0	7.0 ± 3.6	6.7 ± 3.5
Distal	1-3 mm	48.0 ± 8.3	29.2 ± 5.5 *	32.6 ± 7.7 *
	> 3 mm	0.3 ± 0.5	0.0 ± 0.0	0.3 ± 0.6
Total SI		105.8 ± 8.5	79.7 ± 10.0 *	82.6 ± 8.5 *
	< 1 mm	2.6 ± 1.6	1.3 ± 1.1	1.3 ± 1.4
Colon	1-3 mm	3.4 ± 1.0	0.9 ± 1.4 *	1.7 ± 0.7 *
	> 3 mm	2.7 ± 1.4	1.9 ± 1.1	1.5 ± 1.4
Total Colon		8.7 ± 1.5	4.1 ± 2.0 *	4.5 ± 1.7 *
Total Overall		114.5 ± 9.3	83.8 ± 11.0 *	87.1 ± 9.4 *

(B)

		Control Group	0.05% Resv	0.5% Resv	0.05% DMU212	0.5% DMU212
	< 1 mm	3.6 ± 2.2	1.9 ± 1.8	0.4 ± 0.7 *	2.4 ± 1.9	1.2 ± 1.6
Proximal	1-3 mm	4.9 ± 2.1	6.3 ± 4.5	4.8 ± 2.3	4.5 ± 2.5	2.8 ± 2.0
	> 3 mm	1.7 ± 1.2	1.8 ± 2.0	1.5 ± 1.4	1.8 ± 1.0	1.6 ± 0.9
	< 1 mm	6.8 ± 4.8	5.1 ± 5.5	3.5 ± 2.5	2.9 ± 1.6	3.2 ± 1.5
Middle	1-3 mm	12.7 ± 5.0	11.6± 6.8	9.3 ± 2.1	11.8 ± 6.0	10.5 ± 4.7
	> 3 mm	0.4 ± 0.5	0.1 ± 0.3	0.0 ± 0.0 *	0.2 ± 0.6	0.5 ± 0.9
	< 1 mm	5.2 ± 2.7	4.3 ± 4.1	5.5 ± 5.4	4.3 ± 2.0	3.8 ± 2.0
Distal	1-3 mm	18.8 ± 8.5	23.2 ± 11.4	14.8 ± 5.4	18.8 ± 6.6	24.6 ± 8.3
	> 3 mm	0.8 ± 1.2	0.0 ± 0.0 *	0.0 ± 0.0 *	0.4 ± 0.7	1.2 ± 2.2
Total SI		55.0 ± 9.2	54.1 ± 18.5	39.8 ± 7.5 *	47.0 ± 10.2	49.5 ± 13.0
	< 1 mm	2.8 ± 1.3	1.9 ± 1.8	1.1 ± 1.1	2.1 ± 1.6	1.8 ± 1.2
Colon	1-3 mm	0.7 ± 1.0	0.6 ± 0.7	0.4 ± 0.7	0.8 ± 0.7	0.5 ± 0.8
	> 3 mm	0.8 ± 0.8	0.8 ± 0.9	0.1 ± 0.3	0.6 ± 1.0	0.6 ± 0.7
Total Colon		4.4 ± 0.9	3.3 ± 1.9	1.6 ± 1.0 *	3.5 ± 1.2	2.8 ± 1.1
Total Overall		59.4 ± 8.6	57.4 ± 18.5	41.4 ± 7.5 *	50.5 ± 9.5	52.3 ± 13.0

Values are the mean ± SD (n = 10-13) and "*" indicates values differ significantly from controls. (One way ANOVA followed by Bonferroni *post hoc* test $p < 0.05$)

Figure 5.3.2.4.1 shows the effect of dietary resveratrol on adenoma number, size and distribution in a graph using the data that has been shown in Table 5.3.2.4.1. There appears to be a trend in which resveratrol reduces the number of larger sized adenomas (> 3 mm) at both the 0.2 and 0.5 % doses. At the 0.05 % dose there was also a significant reduction in the number of large adenomas but only in the distal region of the small intestine. Resveratrol at 0.2% significantly reduced the number of 1–3 mm adenomas in the distal region of the small intestine and also in the colon. There was also a significant reduction in small adenomas (<1 mm) in the proximal region of the small intestine in mice given 0.5 % resveratrol. However, this reduction in small adenomas was not seen in any other location or with any other dose of resveratrol.

Figure 5.3.2.4.2 shows the effect of dietary DMU212 on adenoma number, size and distribution. DMU212 at a dose of 0.2 % (figure 5.3.2.4.2 A) significantly reduced the number of 1-3 mm adenomas in the distal region of the small intestine and also in the colon. A significant reduction was also seen in the total number of adenomas in the small intestine, the colon and the overall total. DMU212 did not alter the number of adenomas significantly from the controls at either the 0.05 or 0.5 % doses (figure 5.3.2.4.2 B). Small reductions were seen in total adenomas in the small intestine, colon and overall total, but the reduction did not reach statistical significance for any of these results.

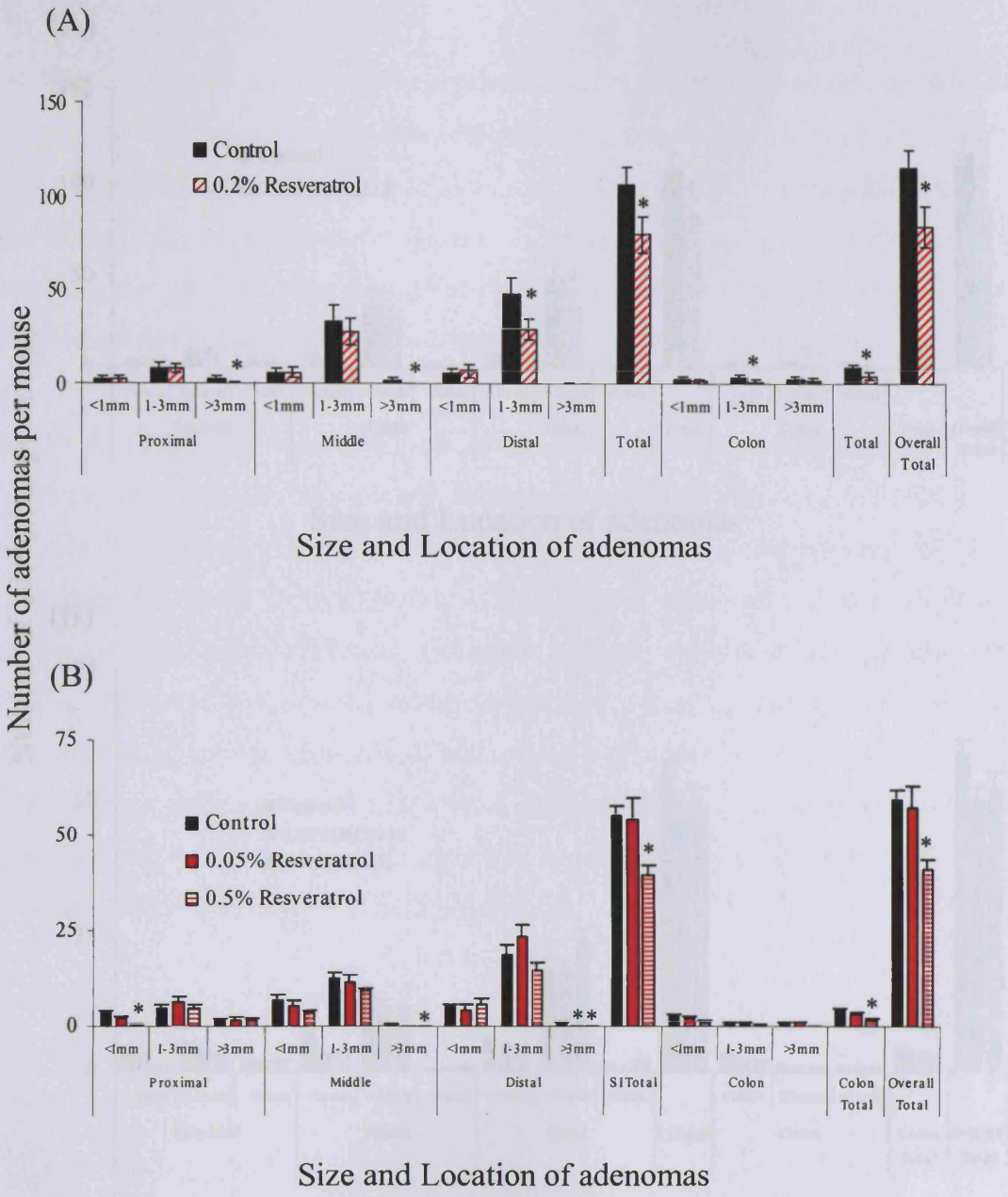


Figure 5.3.2.4.1. Adenoma number, size and distribution in the Min/+ mouse after long-term administration of (A) 0.2 % or (B) 0.05 and 0.5 % resveratrol admixed in the diet. The small intestine was divided equally into three regions: proximal, middle and distal. Adenomas were scored on size as <1 mm, 1-3 mm and >3 mm. Values are the mean ± SEM (n = 10-13). "*" Indicates that the values differ significantly from control (one way ANOVA followed by Bonferroni *post hoc* test $p< 0.05$). The overall total represents the sum of the total adenoma number in the small intestine and the colon.

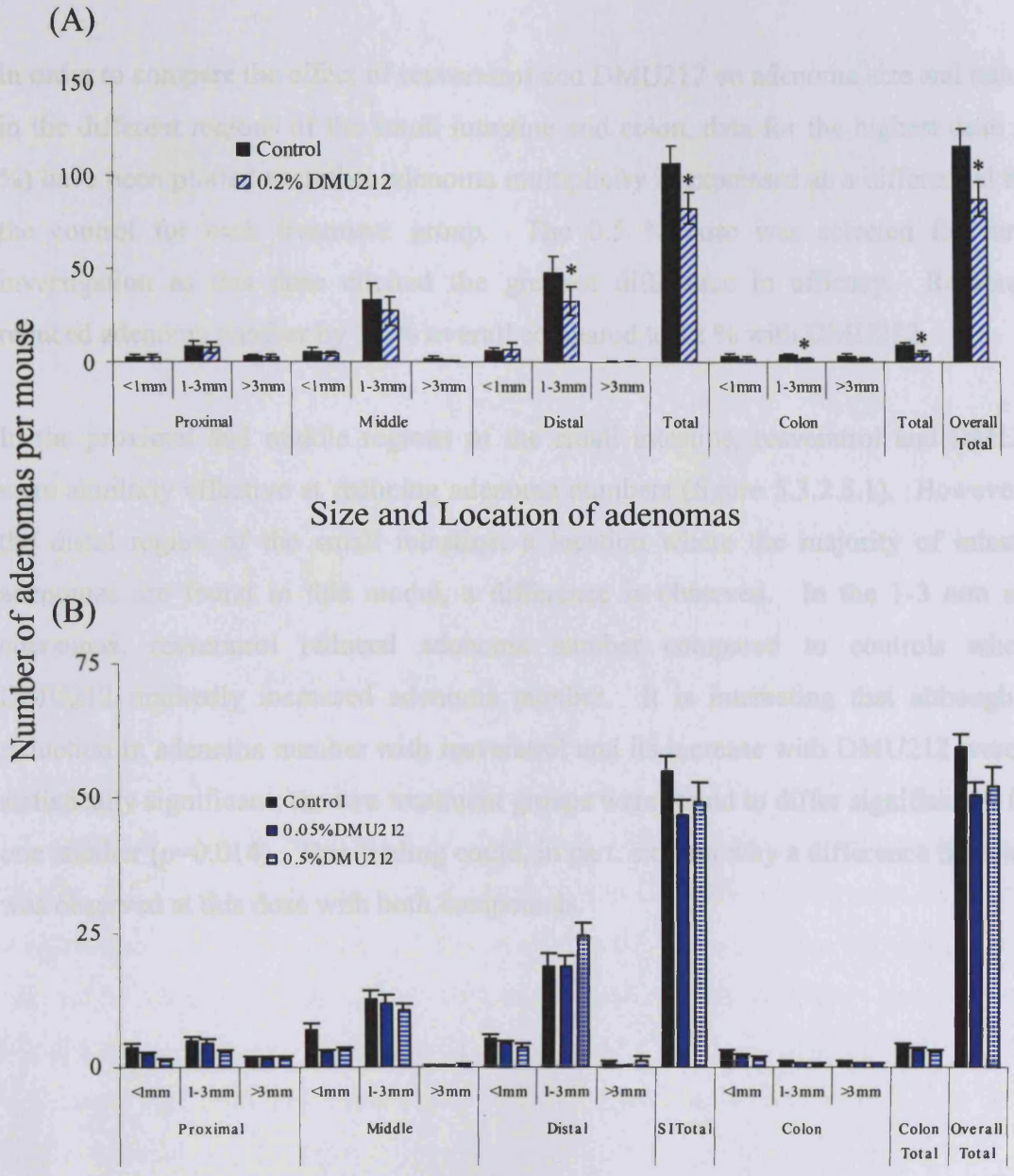


Figure 5.3.2.4.2. Adenoma number, size and distribution in the Min/+ mouse after long-term administration of (A) 0.2 % or (B) 0.05 and 0.5 % DMU212 admixed in the diet. The small intestine was divided equally into three regions: proximal, middle and distal. Adenomas were scored on size as <1 mm, 1-3 mm and >3 mm. Values are the mean ± SEM (n = 10-13). "*" Indicates that the values differ significantly from control (one way ANOVA followed by Bonferroni *post hoc* test $p< 0.05$). The overall total represents the sum of the total adenoma number in the small intestine and the colon.

5.3.2.5. Effect of dietary resveratrol and DMU212 on adenoma multiplicity

In order to compare the effect of resveratrol and DMU212 on adenoma size and number in the different regions of the small intestine and colon, data for the highest dose (0.5 %) have been plotted such that adenoma multiplicity is expressed as a differential from the control for each treatment group. The 0.5 % dose was selected for further investigation as this dose elicited the greatest difference in efficacy. Resveratrol reduced adenoma number by 30 % overall compared to 12 % with DMU212.

In the proximal and middle regions of the small intestine, resveratrol and DMU212 were similarly effective at reducing adenoma numbers (figure 5.3.2.5.1). However, in the distal region of the small intestine, a location where the majority of intestinal adenomas are found in this model, a difference is observed. In the 1-3 mm sized adenomas, resveratrol reduced adenoma number compared to controls whereas DMU212 markedly increased adenoma number. It is interesting that although the reduction in adenoma number with resveratrol and its increase with DMU212 were not statistically significant; the two treatment groups were found to differ significantly from one another (p=0.014). This finding could, in part, explain why a difference in efficacy was observed at this dose with both compounds.

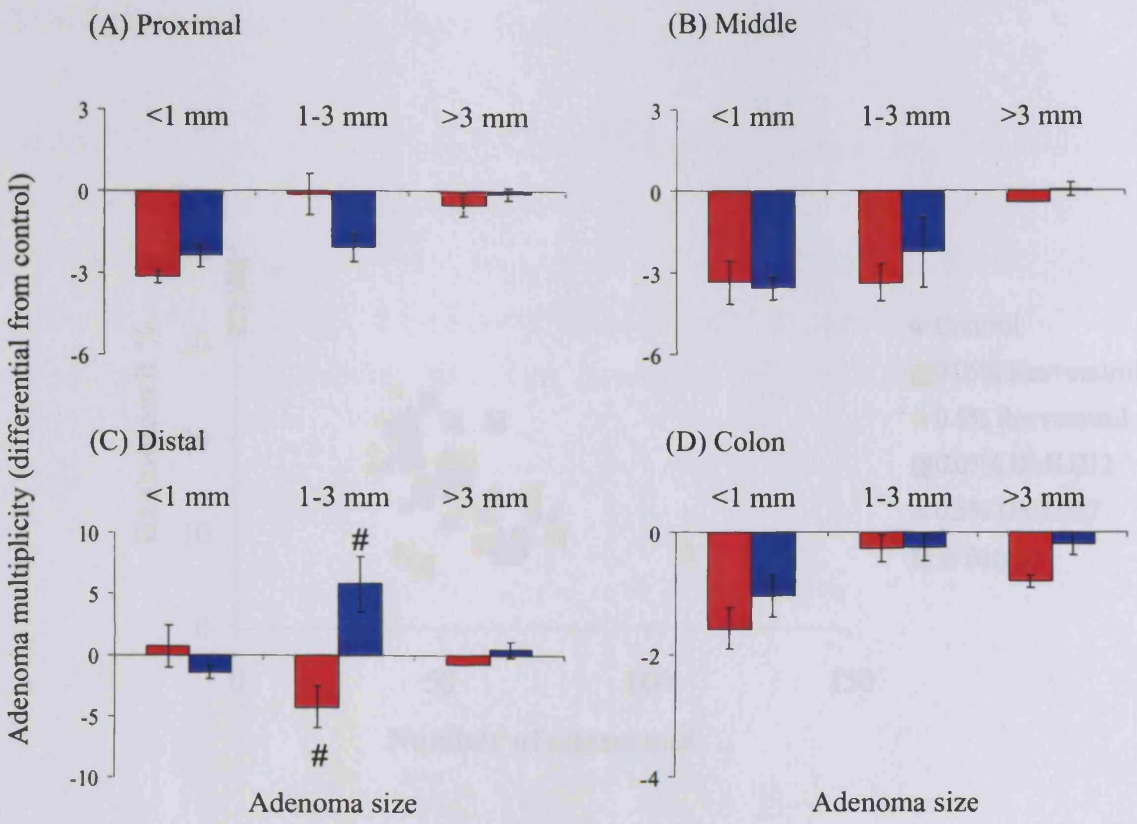


Figure 5.3.2.5.1. Effect of 0.5 % dietary resveratrol (red bars) or DMU212 (blue bars) on adenoma multiplicity (shown as differential from control) in (A) proximal, (B) middle, (C) distal region of the small intestine and (D) colon. Adenomas were graded with respect to size as <1 mm, 1-3 mm and >3 mm. The results are expressed as mean number of adenoma above or below mean numbers in untreated Min/+ mice. Number of mice per group were between 10 and 14. The "#" indicates that values for the resveratrol and DMU212 treated groups differ significantly from one another but not from controls.

5.3.2.6. Relationship between adenoma number and haematocrit.

As the haematocrit is an indirect indicator of adenoma burden, the relationship between adenoma number and haematocrit was investigated. The haematocrit value and adenoma number were compared for control and treated animals including wild type C57BL/6J mice without adenomas (Figure 5.3.2.6.1).

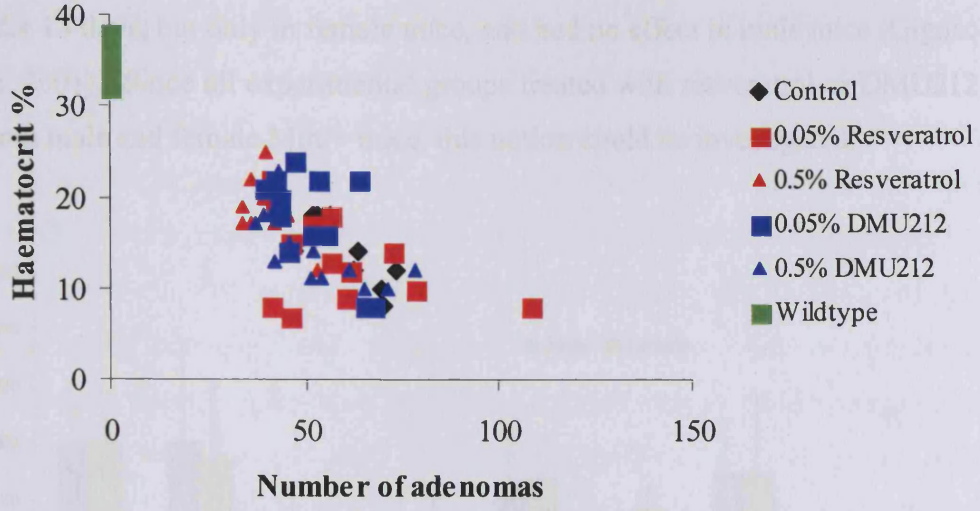


Figure 5.3.2.6.1. Relationship between total adenoma number and haematocrit following dietary administration of resveratrol or DMU212. Control animals and wild type C57BL/6J mice with no adenomas are also represented. Each data point represents a single animal.

Although there was no significant difference between the haematocrit of control and treated animals (section 5.3.2.2.) there is a relationship between haematocrit and adenoma number. It is known that Min/+ mice can become anaemic as adenomas develop (Moser et al., 1992). These data suggest that the more adenomas these animals develop the lower the haematocrit. Using a two tailed Pearson's correlation co-efficient the relationship was found to have a correlation co-efficient of -0.85, a result that was statistically significant ($p < 0.01$).

5.3.2.7. Gender differences in efficacy of resveratrol and DMU212

It has previously been reported that resveratrol may be more efficacious in female mice. It was shown that resveratrol reduced adenoma numbers by 50 % with a dietary dose of 500 mg/kg for 14 days, but only in female mice, and had no effect in male mice (Gignac & Bourquin, 2001). Since all experimental groups treated with resveratrol or DMU212 contained both male and female Min/+ mice, this notion could be investigated.

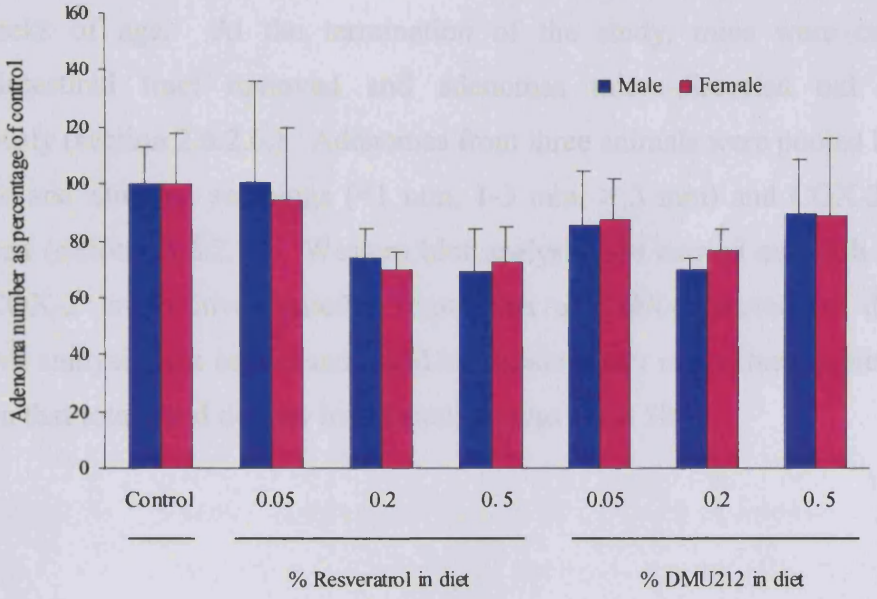


Figure 5.3.2.7.1. Relationship between adenoma number and gender in Min/+ mice administered resveratrol or DMU212. Adenoma number is expressed as percentage of control (where control = 100). Values are the mean ± SD (n = 4-8).

There was no sex-linked effect of resveratrol or DMU212 when administered to Min/+ mice. Figure 5.3.2.7.1 shows that the adenoma numbers at all three doses (0.05, 0.2 and 0.5 % of resveratrol or DMU212) for both male and female mice did not differ significantly from one another.

5.4. Biomarkers of efficacy *in vivo*.

5.4.1. Cyclooxygenase-2: its role in adenoma development.

Exploration of the ability of either resveratrol or DMU212 to downregulate COX-2 protein expression in Min/+ mice adenomas *in vivo* was confounded by an enormous variability in COX-2 protein expression between adenomas. This variability was investigated at two experimental time periods. In the first, mice received control diet (AIN93G) from 4 to 12 weeks of age and in the second, diet was administered from 4 to 18 weeks of age. At the termination of the study, mice were culled and the gastrointestinal tract removed and adenomas micro-dissected out as described previously (section 2.6.2.6.). Adenomas from three animals were pooled but segregated by size and mucosal scrapings (<1 mm, 1-3 mm, > 3 mm) and COX-2 lysates were prepared (chapter 3.6.2.7.). Western blot analysis was carried out with these samples and COX-2 levels investigated. Expression of COX-2 protein as determined by Western analysis was semi-quantitated by densitometry and values quoted as arbituary units in that total band density in the analysis was set at 100.

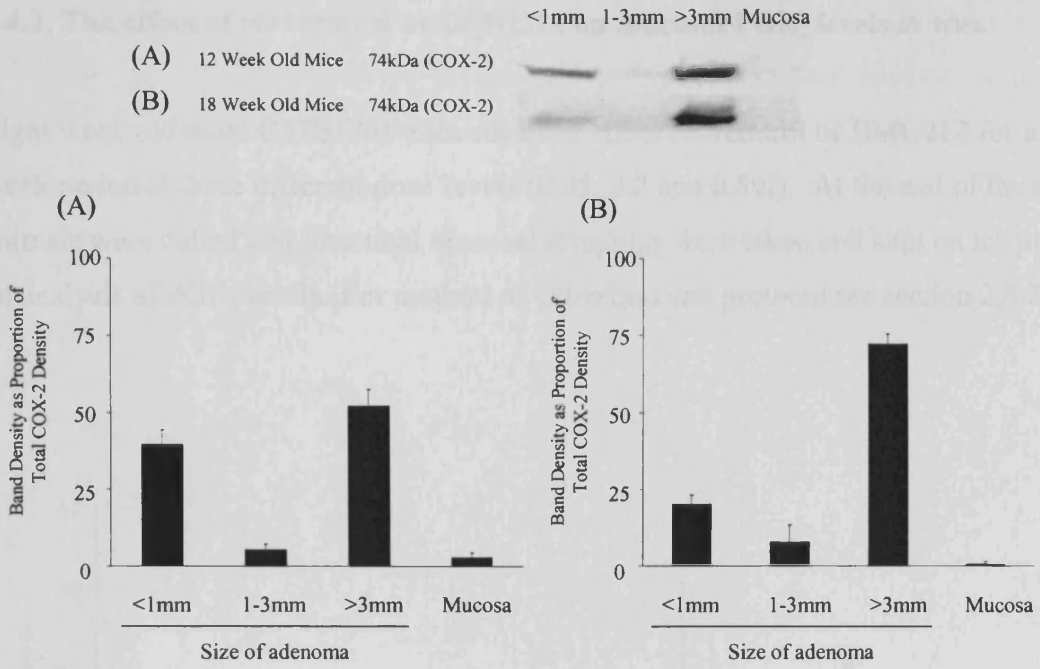


Figure 5.4.1.1. Expression of COX-2 protein in normal intestinal mucosa and differentially sized adenomas (<1mm, 1-3mm and >3mm) of mice aged (A) 12 weeks and (B) 18 weeks, respectively. Values are shown as a proportion of the total COX-2 density and all data have been corrected for equal protein loading. Values are the mean ± SD (n = 3) of adenomas and intestinal mucosa from three pooled animals.

There was a difference between COX-2 protein expression in the different sized adenomas (figure 6.4.1.1). At 12 weeks of age the proportion of COX-2 expression in the <1 mm adenomas was 40 ± 5, however this dropped to 20 ± 3 by the age of 18 weeks. There was a slight increase in the 1-3 mm group in which the proportion of COX-2 expression rose from 5 ± 2 to 8 ± 6. The most noticeable change was seen in the >3 mm group where the relative proportion of COX-2 expression rose from 52 ± 5 at 12 weeks of age to 72 ± 4 at 18 weeks of age. These findings suggest COX-2 levels were highly dependent on tumour size (figure 5.4.1.1), with large adenomas displaying high, small tumours intermediate and medium-sized tumours low COX-2 expression. This result is in principle consistent with the reported size-dependency of COX-2 expression in adenomas of $Apc^{\Delta 716}$ mice (Seno et al., 2002; Takeda et al., 2003), even

though the gradual increase of COX-2 expression with size in $Apc^{\Delta 716}$ adenomas was not faithfully imitated in adenomas of the Min/+ mice used in this study.

5.4.2. The effect of resveratrol or DMU212 on mucosal PGE_2 levels *in vivo*.

Eight week old male C57BL/6J mice received either resveratrol or DMU212 for a three week period at three different dose levels (0.05, 0.2 and 0.5%). At the end of the study, animals were culled and intestinal mucosal scrapings were taken and kept on ice prior to an analysis of PGE_2 levels (For method of extraction and protocol see section 2.6.2.8.)

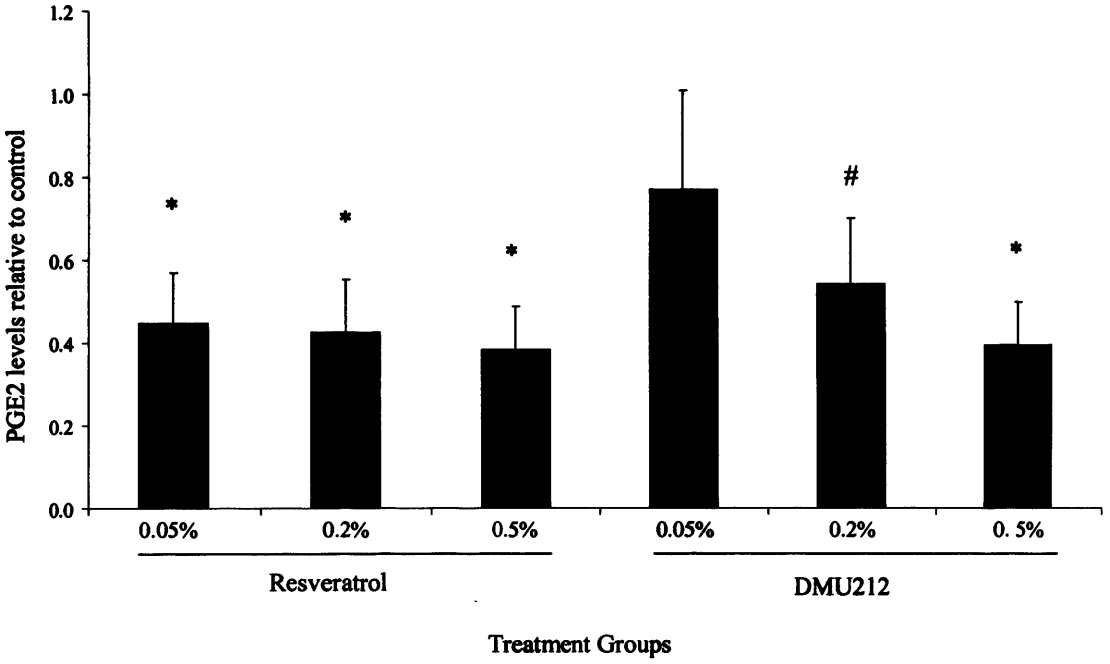


Figure 5.4.2.1. The effect of dietary resveratrol or DMU212 on mucosal PGE_2 levels in C57BL/6J mice. Mucosal PGE_2 levels for each treatment group are expressed relative to the control value (=1) and represent the mean ± SD, (n=5). "#" and "*"indicate values differ significantly from original values ($p<0.05$ and $p<0.01$ respectively). One way ANOVA followed by a Bonferroni *post hoc* test.

As shown in figure 5.4.2.1 resveratrol and DMU212 caused a reduction in mucosal PGE_2 levels at all three doses of resveratrol and the two highest doses (0.2 and 0.5 %) of DMU212. In DMU212-treated animals the data supports a dose dependent decrease in PGE_2 levels with the maximum reduction of approximately 60 % seen at the highest dose (0.5 %). All three doses of resveratrol resulted in a similar decrease in mucosal PGE_2 levels (51-56 % reduction) which did not differ statistically from each treatment group ($p = 1$).

5.5. Discussion

In the work described in this chapter the effects of resveratrol and DMU212 on the growth and development of intestinal adenomas *in vivo* were investigated. An attempt was made to elucidate the possible mechanisms of action by which these agents exert their chemopreventive effect. Three different experimental paradigms were investigated including a drinking water study using 0.01 % (w:v) resveratrol, long term feeding studies comparing the efficacy of resveratrol with that of DMU212 and a third study designed to measure intestinal mucosal PGE_2 levels following intervention with either resveratrol or DMU212.

DMU212 was relatively insoluble in water and after only 24 h the majority had "fallen out" of solution. For this reason it was not possible to conduct a comparative study of resveratrol and DMU212 administered in the drinking water. Therefore, long term feeding studies were conducted with both compounds admixed in the diet. In addition, resveratrol was administered to animals in the drinking water at a dose level shown previously to cause 70 % inhibition of adenoma formation in the small intestine and complete inhibition in the colon (Schneider et al., 2001).

Contrary to the previous report, 0.01 % (w:v) resveratrol in the drinking water did not significantly alter adenoma number compared to the controls. The resveratrol treated animals also developed colonic adenomas in similar numbers to the controls. It is difficult to explain why we were unable to reproduce the resveratrol efficacy previously reported by Schneider et al. Our finding is consistent, however in terms of dose response, with the dietary administration of 0.05 % resveratrol, which also did not result in any effect on adenoma number (section 6.3.2). One explanation for the lack of efficacy could be that the concentration of resveratrol is too low to exert chemopreventive activity in the target tissue.

The 0.01 % (w:v) dose of resveratrol in the drinking water relates to an oral dose of approximately 12 mg/kg, and was not found to be toxic or induce any adverse effects in the animals. This finding is similar to a previous study (Juan et al., 2002b), in which

orally administered resveratrol (20 mg/kg a day) was well tolerated and caused no alterations in any of the haematological and biochemical variables tested.

Dietary intervention with resveratrol or DMU212 did not induce any gross adverse effects. These findings are consistent with those of a previous study (Gignac & Bourquin, 2001), where doses of resveratrol up to 500 mg/kg had no ill effects. The exception to these findings was that DMU212 administration at the highest dose level (0.5 %) caused a significant reduction in the body weight of male mice when compared to the controls, an observation that requires further investigation. The reason for this reduction remains unclear. With regard to this finding in a clinical setting, it should be interpreted with caution, as it intimates that high oral dosing of DMU212 to human males may produce unwanted weight loss.

Dietary resveratrol caused a dose-dependent decrease in adenoma number producing a maximum 30 % reduction at the highest dose level of 0.5 % (w:w). This is consistent with results reported from a previous study (Ziegler et al., 2004), the dose of 0.05 % resveratrol, equivalent to 60 mg/kg, had no effect on adenoma number.

The resveratrol and DMU212 treatment groups that had chemopreventive efficacy, notably the 0.2 % dose for both and the 0.5 % dose for resveratrol, appeared to target the population of small and medium sized adenomas, particularly in the distal region of the small intestine, where the majority of adenomas occurred. This finding is consistent with previous studies in the Min/+ mouse model using resveratrol (Gignac & Bourquin, 2001), curcumin (Perkins et al., 2002), piroxicam (Jacoby et al., 1996) or the selective COX-2 inhibitor celecoxib (Jacoby et al., 2000b). The efficacy of piroxicam varied greatly depending on the region of the intestine: proximal-middle-distal. Piroxicam was much more effective in the distal region of the small intestine (ileum). The cause of this difference in the regional response is unknown, but it has been suggested that it could be due either to luminal substances such as biliary secretions, or growth factors, or to intrinsic differences in gene expression in each intestinal region (Jacoby et al., 2000a). These findings are of particular importance as they tentatively suggest that resveratrol and DMU212 may exert their chemopreventive efficacy through mechanisms similar to that of the compounds mentioned above. This, therefore, supports the rationale for

investigating COX-2 and PGE_2 levels *in vivo* as potential targets for intervention. As mentioned in the introduction, COX-2 has been implicated in the development and progression of colorectal cancer (section 1.4.1). For this reason inhibition of either of these molecular targets would suggest a potential for chemopreventive activity.

As reported in chapter 4 both resveratrol and DMU212 reduced PGE_2 levels in *vitro*. However, only resveratrol was able to modulate COX-2 protein expression significantly. A reduction in PGE_2 levels was also observed in the mucosa of mice following dietary administration of resveratrol and DMU212. The fact that no inhibition of COX-1 or COX-2 activity was observed in purified enzyme preparations with DMU212 but that inhibition was seen in cells *in vitro* and *in vivo* suggests that it is not the parent compound that is exerting the inhibitory effect. DMU212 in the cells and *in vivo* may be metabolised into more potent inhibitors of COX-2 activity and it is these metabolites which inhibit COX activity. In the previous chapter, the effect of DMU281, a metabolite of DMU212, on COX activity was shown to be more potent than either resveratrol or DMU212. If similar metabolites are produced, *in vivo,* this result might explain the effect of DMU212 seen in the Min/+ mouse. In chapter 3 liver microsome preparations were used to identify oxidative metabolites of DMU212, one of these metabolites was identified as DMU281. DMU281 was also identified as a major metabolite in the small intestine following oral dosing of mice with DMU212 (section 3.3.2). These results would support the hypothesis that both in cells *in vitro* and in mice *in vivo* DMU212 undergoes oxidative metabolism to generate metabolites whose COX-2 inhibiting activity may be more potent than that of DMU212 itself. Confirmation of this hypothesis would require further investigation.

A recent abstract reported that the effects of resveratrol may be sex linked as only female mice had reduced adenoma number following dietary intervention with resveratrol (Gignac & Bourquin, 2001). The findings detailed here contradict this. At all three dose levels of both resveratrol and DMU212 there were no significant differences in adenoma number between male and female animals. To date this is the first study investigating resveratrol analogues in the Min/+ mouse model. The superior bioavailability of DMU212 over resveratrol, described in chapter 3, is not reflected in improved efficacy *in vivo* and the only significant effect of dietary DMU212 was seen at the 0.2 % dose. The reason why the 0.5 % dose may not have been as effective may be

related to the significantly lower body weights of male mice in this group as compared to controls. When food consumption was measured by monitoring weekly diet weight changes for the first four weeks of the study, no difference was apparent. However, the possible "bad" taste of DMU212 at the highest dose may have caused the mice to sort and dig through the feed causing excessive waste and spillage thereby giving the impression that food was being consumed, although since only the weights of male mice were affected and not those of females this explanation seems unlikely. An obvious reason for this finding therefore remains unclear.

It is important to find out why a response was seen only at the 0.2 % dose of DMU212. One explanation could be that two different cohorts of mice were used in these experiments. As the mice in the second cohort only developed approximately 60 adenomas, the subtle reduction in adenoma numbers observed with DMU212 did not reach significance.

From these studies it appears that measurement of the haematocrit could be used as a biomarker of adenoma burden as there is a significant correlation between the haematocrit reading and adenoma number. Similarly, another polyphenolic compound, curcumin, when given at 0.2 and 0.5 % in the diet reduced adenoma number by 39 and 40 % respectively and also caused a significant increase in haematocrit when compared to controls (Perkins et al., 2002). A NSAID, *R*-flurbiprofen, has also been shown to reduce adenoma number compared to control animals and at the same time cause a significant increase in haematocrit (Wechter et al., 2000). An explanation for these findings is that as adenomas develop the Min/+ mice become anaemic through intestinal bleeding. With reduced adenoma number and size as a result of chemopreventive intervention, intestinal bleeding is reduced and the haematocrit is at least partially restored. This work, together with the previous studies highlighted above, has shown that adenoma number is negatively correlated to haematocrit. Haematocrit may therefore act as a useful biomarker of efficacy for chemopreventive agents that are investigated in this animal model.

The expression of COX-2 protein in the adenomas and surrounding mucosa of the Min/+ mouse was investigated to probe its potential use as a biomarker of the efficacy

of resveratrol and DMU212. COX-2 has been shown to be expressed at different levels in differently sized adenomas (Tunstall et al., 2005). At 12 weeks of age the majority of COX-2 expression was seen in adenomas of <1 mm and >3 mm. By 18 weeks the majority of COX-2 expression was seen in adenomas of >3 mm. These findings indicate that COX-2 protein is expressed more prominently in the smallest adenomas at the early time point. COX-2 levels in the large adenomas appears to increase from 12 to 18 weeks of age. It is conceivable that COX-2 is needed to allow these adenomas to develop a blood supply and undergo angiogenesis as other studies have shown a possible link between COX-2 overexpression and tumour angiogenesis (Koga et al., 2004; Shtivelband et al., 2003). These findings confound the notion that COX-2 is a poor biomarker for efficacy in this murine model.

Due to COX-2 protein being differentially expressed in the differently sized adenomas in our Min/+ mice it was not assessed as a biomarker in animals treated with resveratrol or DMU212. The majority of control animal intestines contained adenomas in the 1-3 mm size range, a size shown to express low levels of COX-2. In dietary intervention studies with 0.2 % resveratrol or DMU212 it was shown that both compounds significantly reduced the number of adenomas of 1-3 mm, especially in the distal region of the gastrointestinal tract, where the majority of adenomas are found in this model. As there are fewer 1-3 mm adenomas in the treated groups compared to the controls there may be a bias towards higher COX-2 protein expression in the treated groups. Since, when analysed by Western blot analysis, a larger proportion of the adenomas dissected from the small intestine of the treated mice are of <1 mm size, the size of adenomas which have been shown to express higher levels of COX-2 protein (figure 5.4.1.1). Preliminary findings in our laboratory investigating mRNA levels from the differently sized adenomas showed no difference between the groups (Tunstall, Sale, Steward and Gescher, unpublished observation). This finding would suggest that the regulation of COX-2 protein expression in the adenomas of Min/+ mice occurs at the post-transcriptional level and does not involve a transcriptional mechanism.

The differences between resveratrol and DMU212 with regards to colon cancer cell growth inhibition and their abilities to downregulate COX-2 are not reflected in their ability to inhibit PGE_2 production *in vitro*. A similar observation was made *in vivo*. COX-1 is expressed throughout the gastrointestinal tract whereas COX-2 is not

(Kargman et al., 1996). It has also been shown that a COX-1 inhibitor, SC-560, inhibited 80 % of the production of basal gastric mucosal PGE_2, whereas celecoxib, a COX-2 inhibitor, did not suppress PGE_2 production. These data suggest that the production of basal gastric mucosal prostaglandin is controlled by COX-1 (Smith et al., 1998). In the HCA-7 colon cell line COX-2 is expressed to a much greater extent than COX-1 (Goldman et al., 1998; Sharma et al., 2001a). *In vivo*, PGE_2 levels were measured in basal gastric mucosa, where PGE_2 generation is predominantly COX-1-induced. Inhibition of PGE_2 production was observed with both compounds but to a lesser extent with DMU212. From these findings we can tentatively conclude that the anti-proliferative effect and COX-2 downregulation induced by resveratrol and DMU212 *in vitro* are not major mechanistic mediators of the intestinal adenoma-retarding potency *in vivo*, but their ability to inhibit adenoma formation may in part, involve the inhibition of PGE_2 production. If this hypothesis is proved correct, resveratrol might be considered a better chemopreventive agent, as in a purified COX enzyme preparation, resveratrol inhibited the activity of the COX enzyme with an IC_{50} between 1 and 10 μM, whereas DMU212 was completely ineffective and at high concentrations even significantly enhanced COX activity (figure 5.7.1). Interestingly, DMU281, the oxidative metabolite of DMU212, may prove to be a highly effective chemopreventive agent if investigated in the Min/+ mouse model as its ability to inhibit COX activity was the most potent of all agents investigated.

When intestinal steady state levels of resveratrol and DMU212 were analysed by HPLC following the administration of the three dietary doses to animals, the levels reached were between 10–300 nmol/g (Chapter 3), equating to 10–300 μM in terms of concentration. When comparing these values to the *in vitro* data in chapter 4, we can see that such concentrations should be sufficient to inhibit PGE_2 production, which coincides with the observations found in the gut mucosa of mice.

From the work described in this chapter resveratrol and DMU212 both provide evidence to suggest that they may be effective as chemopreventive agents in humans as both agents *in vivo* are able to inhibit prostaglandin levels. However, from results with resveratrol and DMU212 investigating the inhibition of COX-2 protein and activity *in vitro* and the efficacious effect on adenoma number in the Min/+ mouse *in vivo* one might tentatively suggest that resveratrol is the better chemopreventive agent. What has

emerged from these studies is the idea that metabolites of DMU212, particularly DMU281, may have a superior chemopreventive activity over DMU212 and it highlights the need for further investigations into resveratrol analogues for the prevention of colorectal cancer.

CHAPTER 6

FINAL DISCUSSION

The information retrieved from preclinical chemoprevention research is utilised to optimise the design of clinical trials. This type of research aims to provide pharmacokinetic, pharmacodynamic, mechanistic and safety data on the agents under investigation. The aim of this project was to provide this type of preclinical data for resveratrol and one of its analogues DMU212, two potential colorectal chemopreventive agents. Information was obtained on the pharmacokinetics and metabolism of resveratrol and DMU212, their anti-proliferative effects *in vitro*, their ability to interfere with COX-2 protein expression and activity, and their pharmacodynamic and chemopreventive efficacy in a murine model of colorectal carcinogenesis.

To my knowledge there has been no attempt to compare the bioavailability of resveratrol to one of its analogues *in vivo*. In the work described here, HPLC methods were employed to investigate the pharmacokinetics and disposition of resveratrol and DMU212 after oral administration to mice (Chapter 3). Administration of resveratrol and, in particular, DMU212 resulted in a number of metabolites (Figure 6.1). Any or all of the metabolites of DMU212 have the potential to mediate, at least in part, the chemopreventive activity of the parent molecule (Chapter 5). In Chapter 4, the potent inhibitory effect of one such metabolite (DMU281) is outlined, whose ability to inhibit COX activity was in the low nanomolar concentration range, and was thus a better inhibitor of COX activity than either resveratrol or DMU212.

Neither resveratrol nor DMU212 exhibited pharmacokinetic properties which would render them suitable pharmaceuticals. Both were rapidly absorbed and eliminated in mice. Therefore to maintain levels of parent compound in the systemic circulation a regular dosing regime would have to be used. The detection of resveratrol and DMU212 as well as their conjugates and/or oxidative metabolites in plasma and target tissues makes the interpretation of the biological and toxic consequences of oral administration of resveratrol and DMU212 particularly difficult. As it would be complicated to distinguish which species brought about which biological or toxic response. The finding that both resveratrol and DMU212 lack systemic availability may prove to be a desirable property if these agents are to be considered as colorectal cancer chemopreventives. This feature would allow the compounds to accumulate in the gastrointestinal tract to concentrations found *in vitro* that have shown pharmacological activity (Chapter 4).

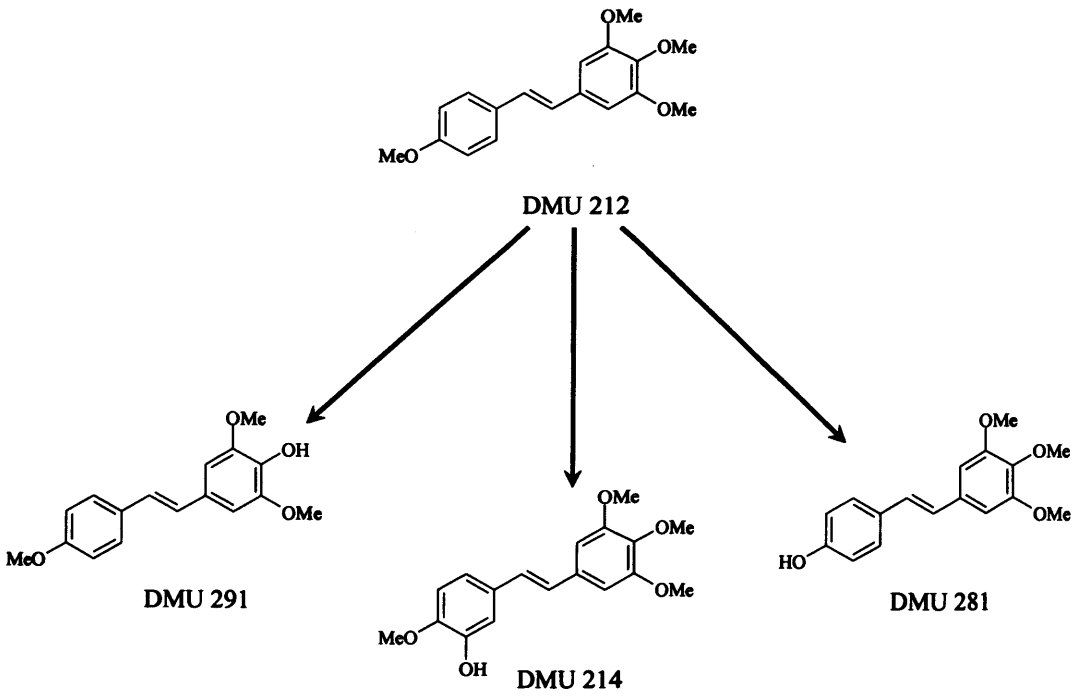


Figure 6.1. Structure of DMU212 and its major metabolites found in plasma and target tissues of mice that received DMU212 by the oral route and also found to be potent inhibitors of COX activity.

Chemoprevention aims to investigate the mechanism of action of new agents primarily through *in vitro* studies in cells. The most promising of these agents are then tested in *in vivo* models of the human condition to assess their efficacy. If the agent tested *in vivo* prove efficacious, it is then put into clinical trials, in this case, FAP patients. To my knowledge it is still unclear as to whether efficacy seen in preneoplastic FAP patients is converted into efficacy in neoplastic colorectal cancer patients. To date NSAIDs and selective COX-2 inhibitors have been the most studied and most promising agents for the chemoprevention of colorectal cancer. However, one problem with these agents, is the fact that they exert undesired side effects. For this reason polyphenols are being considered as a sensible alternative to NSAIDs. A number of polyphenols are currently under preclinical evaluation as alternatives to NSAIDs. It is still unknown whether these polyphenols will also exert undesired side effects and only time will tell.

Studies of COX-2 expression and activity with NSAIDs and COX-2 selective inhibitors show that they potently inhibit PGE_2 production (Chen et al., 2001; Sumitani et al., 2001; Yamazaki et al., 2002) and to a lesser extent COX-2 protein expression depending on the agent (Chen et al., 2001; Goldman et al., 1998). Resveratrol is capable of both the inhibition of COX-2 protein expression and COX-2 activity suggesting the mechanism of action of resveratrol is through pathways similar to that of NSAIDs and COX-2 inhibitors. DMU212 was unable to alter COX-2 protein levels in cells and in the purified COX enzyme preparation had no effect on COX activity, suggesting a mechanism separate from both NSAIDs and COX-2 inhibitors.

It is also important to compare the chemopreventive efficacy seen with resveratrol and DMU212 to that of NSAIDs in the Min/+ mouse model. Numerous studies have been undertaken in the Min/+ mouse with NSAIDs and some of these agents have undergone clinical evaluation. Aspirin is the most widely used NSAID, and many epidemiological studies report a 40-50 % reduction in the incidence of colorectal cancer with regular aspirin use (Rosenberg et al., 1991; Thun et al., 1991). Analyses of the effect of aspirin upon tumorigenesis in the Min/+ mouse model has yielded contrasting results. It has been shown that aspirin at doses ranging from 200-500 ppm caused a maximum reduction in adenoma number of 52 % (Barnes & Lee, 1998; Hansen-Petrik et al., 2002) although, in another study, using 400 ppm aspirin, there was no effect on adenoma number, but interestingly intestinal prostaglandin biosynthesis was inhibited by >70 % (Chiu et al., 2000). It has also been shown that adult dietary exposure to aspirin does not suppress intestinal tumorigenesis, but that continual exposure from the point of conception does (Sansom et al., 2001). Other NSAIDs like sulindac and piroxicam have been shown to reduce intestinal tumour load by 90-95 % and the number of pre-existing adenomas by 80-90 % in the Min/+ mouse (Chiu et al., 2000; Jacoby et al., 1996; Ritland & Gendler, 1999). In the studies described here neither resveratrol nor DMU212 was able to modulate the growth of adenomas in the Min/+ mouse to a similar or greater extent than NSAIDs. It may be that NSAIDs exert their chemopreventive activity through mechanisms different to those engaged by resveratrol and DMU212, and also independent of COX inhibition. The mechanism of action of NSAIDs are still not fully understood. If the hypothesis is true, that resveratrol and DMU212 have mechanisms of action different to that of NSAIDs, it could explain why both resveratrol and DMU212 had inhibitory effects on prostaglandin levels and minimal effects on

adenoma inhibition in the Min/+ mouse. Therefore the chemopreventive activity of resveratrol and DMU212 *in vivo* should be further investigated in other models of colorectal cancer to elucidate their mechanism of action and chemopreventive efficacy. These findings also suggest a rationale for the use of combination therapy with NSAIDs and resveratrol or DMU212 as they might allow the dose of the NSAIDs to be reduced to subtoxic levels but with the same efficacy. Combination therapy may also become a more desirable treatment method following the withdrawal of Vioxx from the clinic due to its unwanted side effects. The 3-year colon polyp prevention study showed that patients on the Vioxx treatment suffered heart attacks and strokes at a much higher rate than the placebo group (Couzin., 2004). It remains unclear as to the cause of these side effects but the use of combinational therapy may allow the dose of COX-2 inhibitor to be reduced and thus eliminate any adverse effects.

In humans, one of the most promising biomarkers for chemoprevention by NSAIDs is downregulation of prostaglandin levels in the colorectal mucosa (Keller & Giardiello, 2003). The degree of downregulation can predict regression of adenomas and suppression of the development of adenomas by sulindac in FAP patients with adenomas, or in presymptomatic individuals (Giardiello et al., 2002; Nugent et al., 1996; Yang et al., 2001). Sulindac has also been shown to induce downregulation of intestinal prostaglandin levels, independent of clinical response, in another study with FAP patients and in Min/+ mice (Chiu et al., 2000; Giardiello et al., 1998). Green tea has been shown, following the administration of a single dose, to rapidly decrease prostaglandin E2 levels in rectal mucosa in humans (August et al., 1999), however it only inhibited adenoma formation by 22 % in Min/+ mice (Suganuma et al., 2001). Resveratrol and DMU212, possibly through a metabolite, inhibited COX-1 and COX-2 induced prostaglandin synthesis *in vitro* and *in vivo* to varying degrees but their effect on adenoma development were similarly minimal, reaching a maximum of 30 % inhibition. As no clinical trials have taken place with resveratrol or DMU212 it is impossible to hypothesize the effect these agents would have on PGE_2 levels in colorectal mucosa, however, if they mediate their effects through pathways similar to green tea these agents may have the potential to inhibit prostaglandin levels in humans.

There are a number of implications derived from the project described here for the potential clinical evaluation of resveratrol and DMU212. The work corroborates the

notion that resveratrol possesses chemopreventive efficacy in the Min/+ mouse model (Schneider et al., 2001) but only at doses of 0.2 % in the diet, or higher. The 0.05 % dose did not cause any significant reduction in adenoma number, consistent with a previous study (Ziegler et al., 2004). This is the first time that an analogue of resveratrol has been tested in the Min/+ mouse model and the results would suggest that DMU212 possesses chemopreventive efficacy, but to a lesser extent than resveratrol. These studies of chemopreventive efficacy encourage the evaluation of both compounds for adenoma-retarding efficacy in FAP patients. The applicability to humans with regard to the relationship between dose level, chemopreventive efficacy and pharmacokinetics defined in this thesis needs further investigation. However, on the assumption that there is a direct comparability, the dose of resveratrol and DMU212 required for efficacy in humans is equivalent to the 0.2 % dietary concentration which was shown to be active in mice. When calculated on the basis of equivalent body surface area (900 mg/m^2 in the mouse), would be 1.6 g per person *pd*, assuming a body surface area of 1.8 m^2 accompanying a body weight of 70 kg (Freireich et al., 1966). A pharmacokinetic Phase I clinical trial is currently underway in our laboratory in Leicester where a single dose of resveratrol is being administered to humans at doses of 0.5 to 7.5 g. To date there have been no reports to suggest any adverse effects with the low doses (Boocock *et al* unpublished data). However, this study might help rationalise future Phase II and III trials, which should provide the answers to whether resveratrol is efficacious in FAP patients when administered at 1.6 g per person *pd.* DMU212 has yet to undergo clinical evaluation, but if tolerated as well as resveratrol this agent and resveratrol could be considered safe and potentially efficacious. If this theory is proved correct then it would warrant their further testing for the treatment and prevention of FAP.

The potential differences between *in vitro* and *in vivo* efficacy seen with resveratrol and DMU212 in this study suggest that additional screening methods needs to be performed in a number of models, both *in vitro* and *in vivo*, before any conclusions can be drawn as to their potential chemopreventive efficacy. Recently, a new murine model for colorectal carcinogenesis has been investigated which supposedly mimics the human condition of colorectal cancer more accurately as the mice develop more adenomas in the colon rather than the small intestine (Hensley et al., 2004). It is unlikely, however, that any animal model can accurately predict the efficacy of these agents in human subjects, this can only be determined by way of clinical trials. Animal models are,

however, useful in that they can highlight indications of efficacy, and demonstrate some of the ways in which the agents behave within the mammalian organism.

The search for novel polyphenolic compounds and in particularly analogues of resveratrol has been going on for many years, but few have proved effective and to date no resveratrol analogue has made it into clinical trials. The synthesis and investigation of novel analogues is, however, beneficial to the furtherment of chemoprevention. Indeed, as has been demonstrated in this study, subtle variations of the stilbene framework can produce great alterations in biological properties. The search for a good chemopreventive agent is still ongoing but from this project the properties that appear essential for a chemopreventive agent are 1) a good bioavailability to the target tissue; 2) they must exert a high level of efficacy in *in vivo* models of colorectal cancer and finally 3) they should be potent inhibitors of molecular targets that are involved in cancer progression. If an agent is able to meet all these criteria then hopefully the prevention or delay of the onset of colorectal cancer is a realistic possibility.

CHAPTER 8

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APPENDIX

Experimental Diet and Ingredients for Laboratory Animals

AIN-93G Purified Rodent Diet

Ingredient	**grams/kilogram**
Casein	200.00
Cornstarch	397.486
Dyetrose	132.00
Sucrose	100.00
Cellulose	50.00
Soybean Oil	70.00
t-Butylhydroquinone	0.014
Salt mix	35.00
Vitamin mix	10.00
L-Cystine	3.00
Choline Bitartrate	2.50

PUBLICATIONS

Papers

Sale S, Tunstall RG, Garcea, G. (2005) Plant-derived polyphenols as colorectal cancer chemopreventive agents: a sensible alternative to NSAIDs? *International Journal of Cancer Research.* 1 (1): 60-70.

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