STUDIES OF THE CHEMOPREVENTIVE EFFICACY AND PHARMACOKINETICS OF CURCUMIN IN A MURINE MODEL OF COLORECTAL CANCER

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Studies of the chemopreventive efficacy and pharmacokinetics of curcumin in a murine model of colorectal cancer

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Curcumin, the major yellow pigment in turmeric, prevents the development of adenomas in the intestinal tract of the C57Bl/6J Min/+ mouse, a model of human colorectal cancer with an *Apc* gene mutation. In order to aid the rational development of curcumin as a colorectal cancer preventive agent, the link between its chemopreventive potency in the Min/+ mouse and levels of drug and metabolites in target tissue and plasma was explored.

Curcumin was administered in the diet at a variety of dose levels and time periods to assess the effect of this phytochemical on tumour burden in Min/+ mice. The growth, survival and haematocrit of the mice were investigated. The effect of a combination of aspirin and curcumin was also explored as well as the effect of altering the dietary matrix. Long-term administration of curcumin (0.2%) was efficacious at reducing tumour multiplicity in the small intestine by 39%. This dosing regime was utilised to study the effect of curcumin on potential markers of carcinogenesis. These included cyclooxygenase-2 (COX-2) expression, malondialdehyde (MDA), and deoxyguanosine adducts, M_1G and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG). The studies tested the potential application of these markers to carcinogenesis and investigated the antioxidant properties of curcumin. Potential markers of carcinogenesis and mechanisms of action of curcumin were also studied using cDNA microarray. The results highlighted significant gene changes in 420 genes, many of which are germane to chemoprevention, most notably genes involved in antioxidation. Pharmacokinetics of dietary administration of curcumin suggests that this phytochemical is poorly bioavailable with the majority of the agent excreted unchanged in the faeces.

Curcumin reduces tumour burden in Min/+ mouse, at least in part through reduction in oxidative DNA damage and suppression of COX-2 activity. In conclusion the results support the clinical evaluation of curcumin as a colorectal cancer chemopreventive agent.

To my family

" being as I know you"

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Abbreviations

AAPC	Attenuated adenomatous polyposis coli
ABC	Avidin biotin complex
ACF	Aberrant crypt foci
Ala	Alanin
ANOVA	Analysis of variance
AP-1	Activator protein 1
Apc	Adenomatous polyposis coli
APES	3-Aminopropyltriethoxy silane
BSA	Bovine serum albumin
CHRPE	Congential hypertrophy of the retinal pigment epithelium
COX	Cyclooxygenase
CRC	Colorectal cancer
CYP	Cytochrome P450
DAB	Diaminobenzidine
DCC	Deleted in colorectal cancer (gene)
DMSO	Dimethyl sulphoxide
dNTP	2´-Deoxyribonucleoside-5´-triphosphate
DTT	Dithiothreitol
dUTP	2'-Deoxyuridine 5'-triphosphate
ECL	Enhance chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ERK	Extracellular regulated kinase
EST	Expressed sequence tag
FAP	Familial adenomatous polyposis
GSK-3β	Glycogen synthase kinase 3β
GST	Glutathione s-transferase
H & E	Haematoxylin and eosin
HPLC	High performance liquid chromatography
HRP	Horse radish peroxidase
IKK	IκB kinase
iNOS	Inducible nitric oxide synthase

IL	Interleukin
IP	Intraperitoneal
LC-MS	Liquid chromatography/ mass spectrometry
LOH	Loss of heterozygosity
LRP6	Lipoprotein receptor related protein
JNK	c-JUN NH_2 terminal kinase
MDA	Malondialdehyde
Met	Methionine
M_1G	Malondialdehyde deoxyguanosine
Min/+	Multiple intestinal neoplasis
m-THPC	5,10,15,20-Tetra-(m-hydrophenyl)-chlorine
NAT	N-acetyl transferase
NFκB	Nuclear factor κB
NO	Nitric oxide
NOS	Nitric oxide synthase
NSAIDs	Non steroidal anti-inflammatory drug
ODC	Ornithine decarboxylase
8-Oxo-dG	8-oxo-7-8-dihydro-2'-deoxyguanosine
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pd	Per day
PG	Prostaglandin
PhIP	2-amino-1-methyl-6-phenylimidazo [4,5*b*] pyridine
PI (3) K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PPAR	Peroxisome proliferating activated receptor
Pro	Proline
RM3	Rat and mouse breeding diet
ROS	Reactive oxygen species
SD	Standard deviation
SDS	Sodium dodecyl sulphate
Ser	Serine
SSC	Saline sodium citrate

SSPE	Saline sodium phosphate EDTA
TBE	Tris borate EDTA
TBS	Tris buffered saline
TBST	Tris buffered saline tween
TCF	T-cell factor
TEMED	N, N, N', N,'-tretramethylethylenediamine
TNF	Tumour necrosis factor
VEGF	Vascular epithelial growth factor

CHAPTER 1
INTRODUCTION

1.1 Cancer and chemoprevention

With one in three individuals in Britain now developing cancer and with a steady rise in incidence, an increasing focus on cancer research is imperative (Cancer Research Campaign, 1994). At present, four strategies are used to combat the disease: prevention, early diagnosis and intervention, treatment of localised cancer, and management of non-localised cancer (Challa *et al.*, 1997). Even with many effective treatments, 25% of deaths in the Western world may be attributable to malignant disease making the development of robust preventative measures immensely important.

In 1948, Berenblum and Shublik concluded that "the recognition that carcinogenesis is at least a two-stage process should invariably be borne in mind" (Berenblum and Shublik, 1948). Today we appreciate that carcinogenesis proceeds *via* multiple distinguishable steps of molecular and cellular alterations. These events can be separated into three distinct phases: initiation, promotion, and progression (Figure 1.1.1). The primary step, initiation, is rapid, involving direct carcinogen binding to the target DNA and subsequent DNA damage. Unless this damage is repaired or the cell is eliminated by apoptosis, the carcinogen may cause irreversible genetic mutations. The initiated or mutated cells remain latent until subsequent agents acting as mitogens induce clonal expansion, leading to proliferation of the initiated cells. This stage is generally reversible. During the ensuing progression, the initiated clone of cells suffers additional mutations. Throughout tumour progression genotypically and phenotypically altered cells gradually emerge. Both promotion and progression phases are prolonged.

Cancer chemoprevention may be defined as the inhibition, retardation or reversal of the carcinogenic process by chemical means (Lippman *et al.*, 1994). Although the concept of cancer chemoprevention is relatively new, since its emergence thirty years ago hundreds of studies have been reported on the identification of efficacious agents. Examples are the vitamin A analogues, isotretinoin and retinol palmitate, in the prevention of second primary cancers in patients with malignancies of the lung and head/neck (Hong *et al.*, 1990; Pastorino *et al.*, 1993).

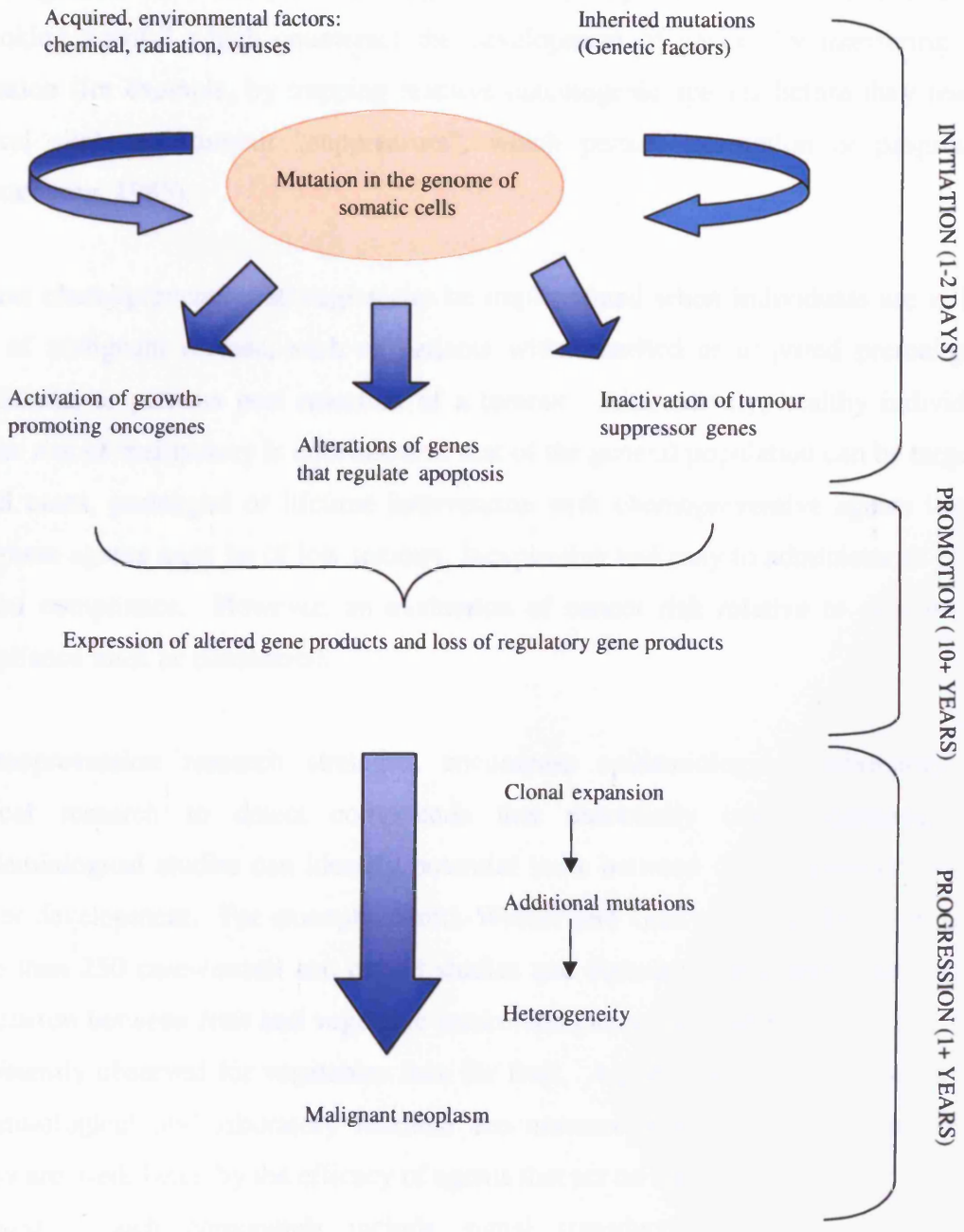

Figure 1.1.1 A simplified scheme of multistep carcinogenesis (Modified from Cotran *et al.*, 1994).

The multiple stages of carcinogenesis offer potential targets for chemopreventive agents to halt, hinder or possibly reverse the process. Depending on which phase of carcinogenesis the chemopreventive agents effect, they can be classified into tumour "blocking agents" which counteract the development of cancer by interfering with initiation (for example, by trapping reactive carcinogenic species before they reach a critical site), and tumour "suppressors", which perturb promotion or progression (Wattenberg, 1985).

Cancer chemoprevention strategies can be implemented when individuals are at high risk of malignant disease, such as patients with inherited or acquired premalignant conditions, or patients post resection of a tumour. Alternatively, healthy individuals whose risk of malignancy is equivalent to that of the general population can be targeted. In all cases, prolonged or lifetime intervention with chemopreventive agents implies that these agents must be of low toxicity, inexpensive and easy to administer to ensure patient compliance. However, an evaluation of cancer risk relative to expense and compliance must be considered.

Chemoprevention research strategies encompass epidemiological, laboratory and clinical research to detect compounds that potentially inhibit carcinogenesis. Epidemiological studies can identify potential links between dietary components and cancer development. For example, Smith-Warner and Gianvannucci (1999) reviewed more than 250 case-control and cohort studies and demonstrated a significant inverse association between fruit and vegetable intake and cancer risk, with associations more consistently observed for vegetables than for fruit. Agents initially identified through epidemiological and laboratory research are assessed systematically. Mechanistic assays are used, whereby the efficacy of agents that act on specific molecular targets are assessed. Such compounds include signal transduction modulators, hormone modulators, anti-inflammatories that inhibit the promotion and progression of neoplasia, anti-mutagens that inhibit initiation and anti-oxidants that inhibit initiation and promotion (Kelloff, 2000). Furthermore, agents initially used as cancer treatments may be investigated as potential chemopreventive agents. Examples include finasteride, a 5-α-reductase inhibitor, used to treat benign prostatic hyperplasia for

prostate cancer, and tamoxifen, a selective oestrogen receptor modulator, for breast cancer (Greenwald, 2002).

Once identified as potential chemopreventive agents, preclinical studies assess chemopreventive efficacy using *in vitro* assays (Steele, 1994a) and *in vivo* studies which use animal models that exhibit precancerous lesions and are representative of human neoplasia (Steele, 1994b). Table 1.1.1 provides examples of carcinogen induced *in vivo* models. Characterisation in the animal models involves determination of dose response curves, dosing regimes, and potential combination strategies (Kelloff, 2000). Agents considered for clinical trial must demonstrate high efficacy and low toxicity in these models.

Clinical chemoprevention trials are divided into three phases and the salient features are described in Table 1.1.2. Most clinical trials focus on the prevention of carcinogenesis at a premalignant stage or an early phase of malignancy. This is termed "primary" or "secondary" chemoprevention, depending on whether it involves normal or high-risk individuals. The treatment of patients who have undergone successful therapy of a primary malignancy but are at increased risk of a second malignancy with chemopreventive agents is referred to "tertiary" chemoprevention (De Flora *et al.*, 1996). Listed in Table 1.1.2 are selected agents undergoing colon cancer prevention trials sponsored by the US National Cancer Institute.

Table 1.1.1 Animal models for preclinical cancer chemoprevention studies (taken from Greenwald, 2000; adapted from Kelloff, 2000)

ORGAN MODEL	SPECIES	CARCINOGEN	END POINT
Buccal Pouch	Hamster	DMBA	Squamous cell carcinoma, papilloma
Colon	Mouse	AOM, DMH, MAM	Adenomcarcinoma, adenoma, aberrant crypt foci
	Rat	AOM, DMH, MAM, MNU	Adenocarcinoma, adenoma
Oesophagus	Rat	Nitrosamines	Squamous cell carcinoma, papilloma
Forestomach	Mouse	B[*a*}P	Squamous cell carcinoma, papilloma
Intestines	Rat	AOM, DMH	Adenocarcinoma, adenoma
Liver	Mouse/ rat	AAF, DEN, DMN, Me-DAB	Hepatocellular carcinoma, adenoma
Lung	Mouse	B[*a*]P, DMBA, NNK, urethane	Adenoma
	Hamster	DEN, MNU (trachea)	Squamous cell carcinoma, adenosquamous carcinoma
Mammary Glands	Mouse/ rat	DMBA, MNU	Adenocarcinoma, adenoma
Pancreas	Hamster	BOP	Ductal adenocarcinoma, adenoma
	Rat	*l*-Azaserine	Acinar cell carcinoma
Skin	Mouse	UV radiation, B[*a*]P, B[*a*]P/TPA, DMBA, DMBA/TPA, MCA	Carcinoma, papilloma
Stomach	Rat	MNNG	Adenocarcinoma
Urinary bladder	Mouse/ rat	OH-BBN, MNU	Transitional cell carcinoma

Abbreviations: AAF, acetylaminofluorene; AOM, azoxymethane; B[*a*]P, benzo[*a*] pyrene; BOP, *N*-nitrobis(2-oxopropyl)amine; DEN, *N, N*-diethylnitrosamine; DMBA, 7.,12-dimethylbenz[*a*]anthracene; DMH, dimethylhydrazine; DMN, *N, N*, dimethylnitrosamine; MAM, methlazoymethanol; MCA, methylcholanthrene; MeDAB, methl-*N, N*-dimethyl-4-aminobenzene; MNNG, *N*-methyl-*N*'-nitro-*N*-nitroguanidine; MNU, *N*-methyl-*N*-nitrosourea; NNK, *N*-nitrosonornicotine; OH-BBN, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine; TPA, 12-O-tetradecanolphorbol-13-acetate

Table 1.1.2 Basic generalised design for chemoprevention trials (Modified from Sharma *et al.*, 2001a; Greenwald, 2002).

PHASE	NUMBER OF SUBJECTS	DESIGN	PRIMARY ENDPOINT	EXAMPLES OF AGENTS IN COLORECTAL CANCER TRIALS
I	15-30	Small, short-term pilot study in cancer patients with rapid dose escalation if no toxicity	1. Pharmacokinetics 2. Toxicity 3. Preliminary data on preneoplastic biomarkers	Curcumin Ursodiol Phenethylisothiocyanate
IIa	30-60	Small, short-term dose de-escalation study	Sensitivity and response of preneoplastic biomarkers	Celecoxib Folic acid Vitamin D and calcium Celecoxib and 2-difluoromethylornithine 2- difluoromethylornithine and sulindac
IIb	500-1500	Larger, medium term randomised study in high risk individuals	Response in definitive preneoplstaic markers	
III	Several thousand	Large, long-term randomised study with low and high risk levels	1. Response in definitve preneoplsatic markers 2. Cancer incidence and mortality	Celecoxib

1.2 Biomarkers of chemoprevention

One of the inadequacies of many chemoprevention studies is the lack of suitable biomarkers of efficacy. Biomarkers can be defined as biological events, which take place between exposure to external or endogenous carcinogens and the subsequent development of cancer (Kelloff *et al.,* 1994). It is important to differentiate these 'preneoplastic' biomarkers from tumour markers that are used to diagnose or monitor malignancy (Sharma, 2000). A biomarker may be a discrete event, such as the formation of a colonic adenoma, or a quantitative change, such as an increase in cell proliferation (Kelloff *et al.,* 1994). Such biomarkers should be based on mechanistic hypotheses, then developed and validated in preclinical models and small pilot clinical studies. The study of biomarkers facilitates both the development of further mechanistic hypotheses regarding the actions of chemopreventive agents and the progression of future clinical trials of such agents. Criteria used to identify and judge the potential efficacy of biomarkers of colon carcinogenesis, as defined by Einspahr *et al.,* (1997), are outlined below:

1. Variability of expression between phases of the carcinogenic process (i.e. normal, premalignant and malignant), preferably increasing with carcinogenic progression;
2. Ability to be detected early in the carcinogenic pathway;
3. Association with risk of developing cancer or recurrence of tumour;
4. Presence in tissues that are easily accessible for multiple biopsies;
5. Potential for modification by a chemopreventive agent;
6. Capability of developing adequate quality control procedures.

The interrelationship between epidemiology and molecular biology underlying colorectal cancer is now becoming clearer (Potter, 1999), enabling the potential intervention at varying stages in the carcinogenic process. The investigation of biochemical biomarkers related to the genetic pathways implicated in colorectal carcinogenesis may provide a broader understanding of the mechanisms of action and pharmacodynamics of the agent under scrutiny. For example, the development of aspirin as a chemopreventive agent has included measurement of colon mucosal levels of prostaglandins and cyclooxygenase (COX)-2 in conjunction with histological measures of cell proliferation (Krishnan *et al.,* 1997). Increasing numbers of pilot

screening programmes for sporadic cancer in normal populations are likely to identify individuals with early stages of this disease, which may enhance the importance of chemoprevention (Kronberg *et al.*, 1996). Studies of human premalignant lesions also offer opportunities to elucidate the relationship between putative preneoplastic biomarkers and the subsequent risk of cancer.

1.3 Pathology of colorectal adenomas

The intestine begins at the pylorus and terminates at the anal-rectal junction and is separated into the small and large portions by the ileocaecal valve. The small intestine is composed of the duodenum, jejunum, and ileum. The large intestine is composed of the caecum, ascending, descending, and sigmoid colon and the rectum. The primary function of the intestine is the digestion and absorption of essential components from ingested food, eliminating the waste at defaecation. The intestinal wall has four layers: 1) mucosa, which is lined by glandular epithelium and characterised by the presence of crypts and villi, which increase the surface area for absorption in the small intestine, 2) submucosa, which contains blood and lymphatic vessels and the submucosal nerve plexus, 3) muscularis externa, and 4) serosa (Figure 1.3.1).

Within the mucosal layer, the crypts (invaginations of the epithelium) 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 four cellular types: columnar absorptive cells, goblet (mucus-secreting) cells, neuroepithelial cells and Paneth cells. These differentiated cell types are located in the upper two thirds of the crypt, continually migrating upwards until they are eventually exfoliated into the lumen by an apoptotic mechanism. This process of epithelial renewal takes 3-6 days, and the rate of mitosis equals the rate of epithelial loss. It is only when this ratio increases, favouring renewal that intestinal tumours develop. The earliest manifestations of neoplasia are the aberrant crypt foci (ACF), which are only visible by methylene blue staining or microscopy. ACF usually encompass few crypts and can be composed of either normal (nondysplastic) or dysplastic cells. Dysplasia refers to the disordered growth and organisation of the cell. These alterations include size, shape, hypochromatic nuclei, and architectural orientation. Dysplastic cells are more likely to progress to become a polyp, a benign tumour mass that protrudes into the lumen from

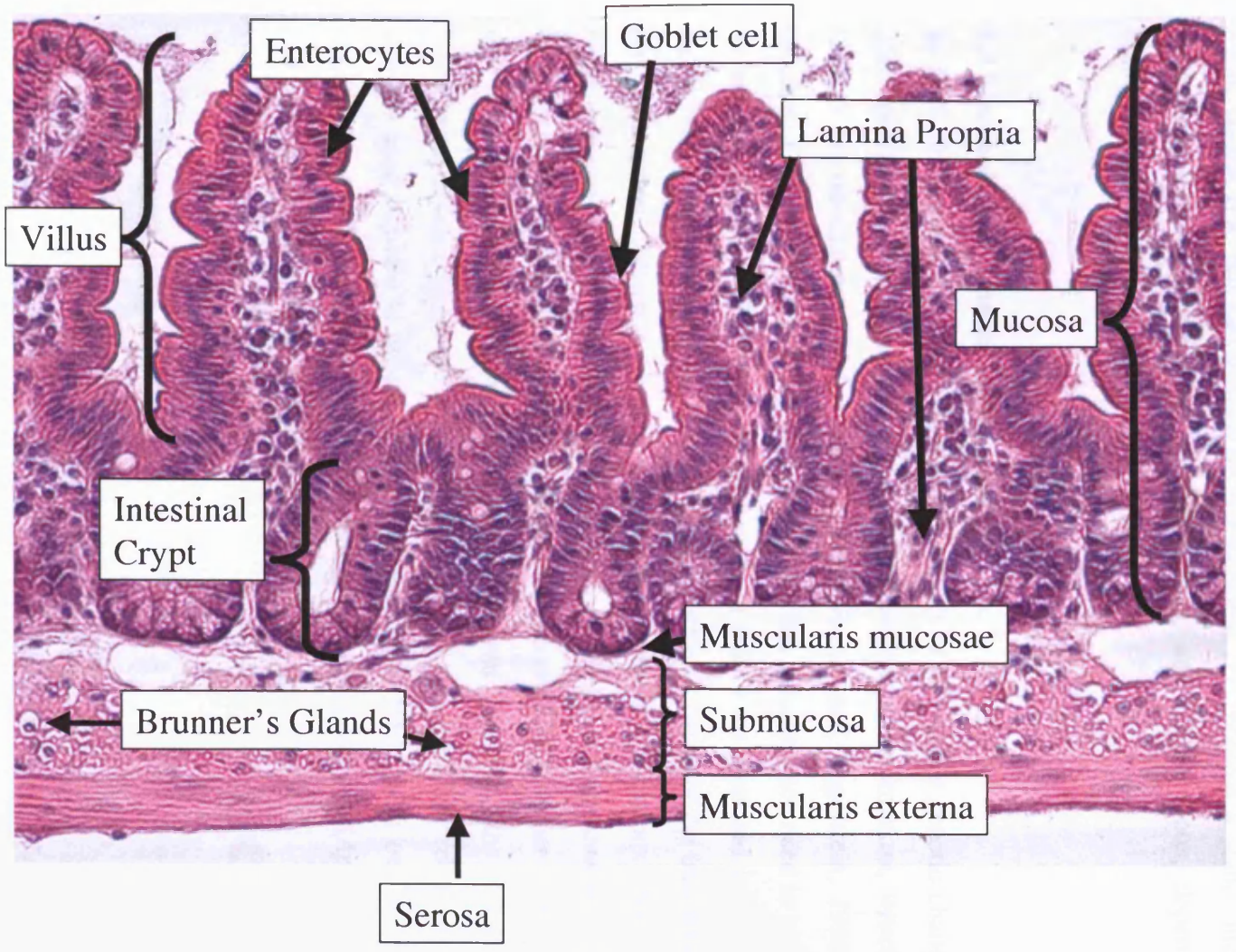

Figure 1.3.1 Structure of the small intestine (Methodology of hematoxylin and eosin immunohistochemistry, section 2.2.8)

the intestinal epithelium. Polyps may also be classified into hyperplastic (nondysplastic) and adenomatous (dysplastic). Hyperplastic polyps preserve normal morphology, whereas adenomatous polyps are characterised by both inter- and intracellular abnormalities. The epithelium is organised into multiple layers, nuclei are enlarged, and their alignment at the basal membrane is lost. The adenomatous polyps may be either sessile (i.e. flat elevations), or pedunculated (i.e. possess a stalk) and can be segregated into three subtypes: tubular, villous, and tubulovillous, depending on epithelial architecture.

1.4 Mechanism of colon carcinogenesis.

In 1990 Fearon and Vogelstein proposed a model of successive genetic changes each accompanied by a well-characterised histopathological transformation, which would lead to the development of colorectal cancer (Fearon and Vogelstein, 1990). This concept of a "multistep model of carcinogenesis" has been characterised in some detail for colorectal cancer (Figure 1.4.1). Although, in reality the proposed scheme may be more flexible in its progression, the model has provided a basis for the understanding of the interaction between genetic alterations and modifications by potential chemopreventive agents. The original proposal stressed that mutations in these genes were essential for the development of colorectal cancer (CRC), rather than specifying the exact sequence of changes. With subsequent research, the functions and interactions of these genes are now better understood. At least four sequential genetic changes need to occur to ensure CRC evolution (Figure 1.4.1), with one oncogene, *K-ras*, and three tumour suppressor genes, adenomatous polyposis coli (*Apc*), *SMAD4* and *p53* the main targets of these genetic alterations.

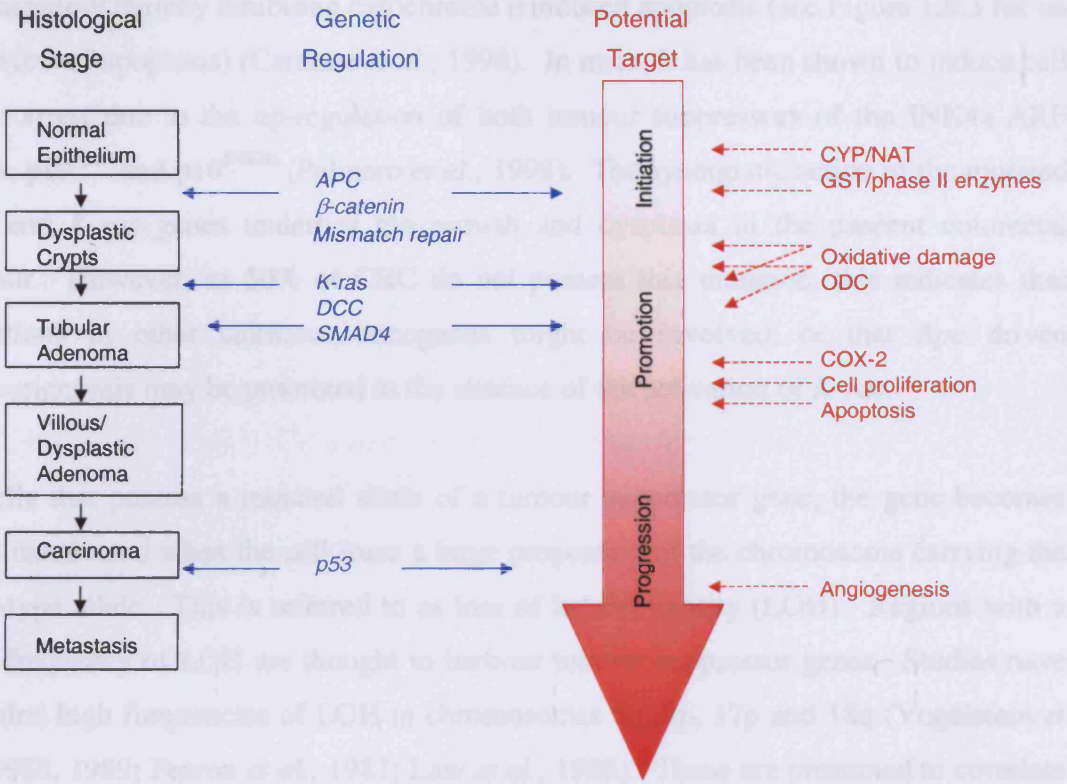

Figure 1.4. 1. Multistep model of carcinogenesis with targets for chemopreventive agents (modified from Fearon and Vogelstein, 1990, Sharma *et al.*, 2001a).

The earliest genetic alterations, required to initiate clonal evolution, are mutations in the *Apc* gene, often referred to as the "gatekeeper" gene in the development of CRC (Kinzler and Vogelstein, 1996). These mutations are found in the very earliest stage of the adenoma-carcinoma sequence, the aberrant crypt foci (ACF). The *Apc* mutations are closely associated with the degree of dysplasia of these small lesions, which correlates with the propensity for the ACF to develop into polyps (Fodde *et al.*, 2001). These mutations are discussed in more detail in section 1.6.

Acquired, activating *K-ras* mutations are found in approximately 50% of sporadic colorectal adenomas and carcinomas (Forrester *et al.*, 1987; Bos *et al.*, 1987; Vogelstein *et al.*, 1988). *K-ras* mutations are also found in a large proportion of non-dysplastic lesions that have a more limited potential to develop into larger tumours including non-dysplastic ACF or hyperplastic polyps. As Ras is involved in the EGFR-Ras-Raf-ERK-JUN/FOS pathway, which generally conducts stimulatory signals, hyperactive mutant Ras induces cellular proliferation. Furthermore, mutant Ras can phosphorylate

procaspase-9 thereby inhibiting cytochrome *c* induced apoptosis (see Figure 1.9.3 for an overview of apoptosis) (Cardone *et al.*, 1998). In mice, it has been shown to induce cell cycle arrest due to the up-regulation of both tumour suppressors of the INK4a-ARF locus, $p19^{ARF}$ and $p16^{INK4a}$ (Palmero *et al.*, 1998). The synergistic action of the mutated *Apc* and *K-ras* genes underlies the growth and dysplasia in the nascent colorectal tumour. However, as 50% of CRC do not possess this mutation, this indicates that mutations in other unknown oncogenes might be involved, or that *Apc* driven tumourigenesis may be promoted in the absence of the activation of *K-ras*.

In cells that possess a mutated allele of a tumour suppressor gene, the gene becomes fully inactivated when the cell loses a large proportion of the chromosome carrying the wild-type allele. This is referred to as loss of heterozygosity (LOH). Regions with a high frequency of LOH are thought to harbour tumour suppressor genes. Studies have revealed high frequencies of LOH in chromosomes 5q, 8p, 17p and 18q (Vogelstein *et al.*, 1988, 1989; Fearon *et al.*, 1987; Law *et al.*, 1988). These are presumed to correlate with *Apc* in 5q, *p53* in 17p and *SMAD4* in 18q. It is thought that these additional mutations and allelic losses in *p53*, *SMAD4*, and other 18q tumour suppressor genes drive malignant transformation. For example, loss of chromosome 17p is found in approximately 75% of colorectal carcinomas, but infrequently in benign lesions, suggesting its role in progression rather than initiation (Vogelstein *et al.*, 1988; Rodrigues *et al.*, 1990). *SMAD2* and *SMAD4*, which can both be mapped to chromosome 18q, are now regarded as tumour suppressor genes involved in malignant transformation of colorectal tumours and are important components of the TGF-β signal transduction pathway (Attisano and Wrana, 2002). Tumours acquire TGF-β resistance at a relatively late stage, and interestingly $Apc^{+/\Delta 716}/SMAD4^{+/-}$ compound heterozygous mice develop more malignant intestinal polyps than those of $Apc^{+/\Delta 716}$ counterparts. Although Smith *et al.*, (2002), have recently suggested that alternative pathways to CRC exist after analysis of mutation spectra in 106 CRC patients, the Vogelstein model and its subsequent modifications depict the general adenoma-carcinoma sequence for CRC. The model suggests that several genetic hits are required, and this is reflected by the time span (20-40years) required for the first ACF to develop into an invasive carcinoma.

1.5 Familial Adenomatous Polyposis (FAP) and Adenomatous Polyposis Coli (APC)

Familial Adenomatous Polyposis (FAP) was first described as a disease with clear dominant inheritance by Lockhart-Mummery in 1925 (Lockhart-Mummery, 1925). The incidence of FAP in the population is approximately 1 in 8000 (Bisgaard *et al.*, 1994), and it is nearly 100% penetrant (Bisgaard *et al.*, 1994), meaning that almost every individual who inherits the genetic defect will develop the disease. Although the majority of FAP cases are inherited, 22-46% result from *de novo* mutations without an associated family history of the disease (Bisgaard *et al.*, 1994).

FAP is the archetypal example of an adenomatous polyposis syndrome in that FAP patients typically develop 500-2500 colonic adenomas, which carpet the mucosal surface. In addition, adenomas may also occur in the upper gastrointestinal tract: in the duodenum in over 50% of cases and sometimes in the jejunum, ileum and stomach (Offerhaus *et al.*, 1999). Histologically, the vast majority of polyps, which measure <1 cm, are tubular adenomas; occasionally polyps may have a villous feature. Polyps begin to appear at 10 - 20 years of age. The risk of polyp formation increases with age with about 15% of individuals developing polyps by age 10, and 90% by age 30. Although a minority of patients do not develop polyps until they are over 70 years of age, the average age of polyp detection is around 15 years (Lynch *et al.*, 1996). Rectal bleeding is the commonest presenting feature. Colon carcinoma (malignant epithelial neoplasm) supervenes in 100% of cases (Offerhaus *et al.*, 1999). The mean age at development of carcinoma is 35-40 years, more than 30 years earlier than the average age of diagnosis of colorectal cancer in the general population. The risk of colorectal cancer is proportional to the size and number of polyps. Total colectomy to prevent cancer is essential (Offerhaus *et al.*, 1999).

The identification of an interstitial deletion of the chromosomal band 5q21 in a patient with colorectal polyposis in association with congenital abnormalities led to the initial identification of the FAP locus in 1986 (Herrera *et al.*, 1986). Positional cloning subsequently identified the adenomatous polyposis coli (*Apc*) gene (Kinzler *et al.*, 1991; Nishisho et *al.*, 1991; Groden *et al.*, 1991; Joslyn *et al.*, 1991). The *Apc* gene consists of 8535 base pairs spanning 21 exons (Thliveris *et al.*, 1996) and encodes a 2843 amino acid protein with a molecular weight of 310 kD in its commonest isoform

(Horii *et al.*, 1993). *Apc* is classified as a tumour suppressor gene and in accordance with Knudson's two-hit hypothesis, the inactivation of both alleles results in the loss of control of cell growth and proliferation (Knudson, 1971). Patients with FAP have a germ-line mutation in one of the two alleles of the *Apc* gene. The majority of these (95%) are nonsense or frame shift mutations that result in a truncated protein product with abnormal function (Groden *et al.*, 1991; Joslyn *et al.*, 1991). Colorectal tumours from FAP patients also carry additional somatic *Apc* mutations or loss of heterozygosity at this locus (Miyoshi *et al.*, 1992; Soloman *et al.*, 1987; Ichii *et al.*, 1993; Levy *et al.*, 1994). More than 300 different *Apc* mutations have been described (Beroud *et al.*, 1996). Exon 15 comprises > 75% of the coding sequence of *Apc* and is the most common target for both germline and somatic mutations (Beroud *et al.*, 1996).

The FAP phenotype (age of onset, type and number of intestinal polyps and extra-colonic manifestations) often correlates with the position and type of inherited *Apc* mutation and is summarised in Figure 1.5.1. FAP is also associated with a number of benign and malignant extra-colonic lesions. Benign extra-colonic manifestations include epidermoid cysts, which characteristically develop in young FAP patients on the face, scalp and extremities, congenital hypertrophy of the retinal pigment epithelium (CHRPE), and osteomas. Extra-colonic tumours include desmoid tumours, upper intestinal neoplasms, hepatobiliary neoplasms and thyroid carcinomas (Lal and Gallinger, 2000). Of note, attenuated adenomatous polyposis coli (AAPC), associated with mutations located at the extreme 5' end of the gene (exons 3 and 4) and distal to codon 1578 at the 3' end of the gene, is characterised by a reduced number of colorectal polyps (≤ 100 polyps) and an age of onset that is 10-15 years later than in classical FAP patients (Spirio *et al.*, 1992). The lifetime risk of colon cancer is still very high and gastric and duodenal polyps are also frequent (Leppert *et al.*, 1990; Soravis *et al.*, 1998).

Mis-sense germline variants of *Apc* have also been described. For example, a weakly penetrant *Apc* variant, I1307K, has been found in Ashkenazi Jews. The variant consists of a T→A substitution, and carries an elevated risk of colorectal polyp and cancer development (Laken *et al.*, 1997; Frayling *et al.*, 1998; Woodage *et al.*, 1998; Gryfe *et al.*, 1999; Prior *et al.*, 1999; Rozen *et al.*, 1999). Somatic mutations in *Apc* are also

prevalent in around 75% of colorectal adenomas and carcinomas (Miyaki *et al.*, 1994), including adenomas < 5 mm in size (Powell *et al.*, 1992), therefore emphasising the likelihood that this gene plays an important role in the aetiology of early CRC.

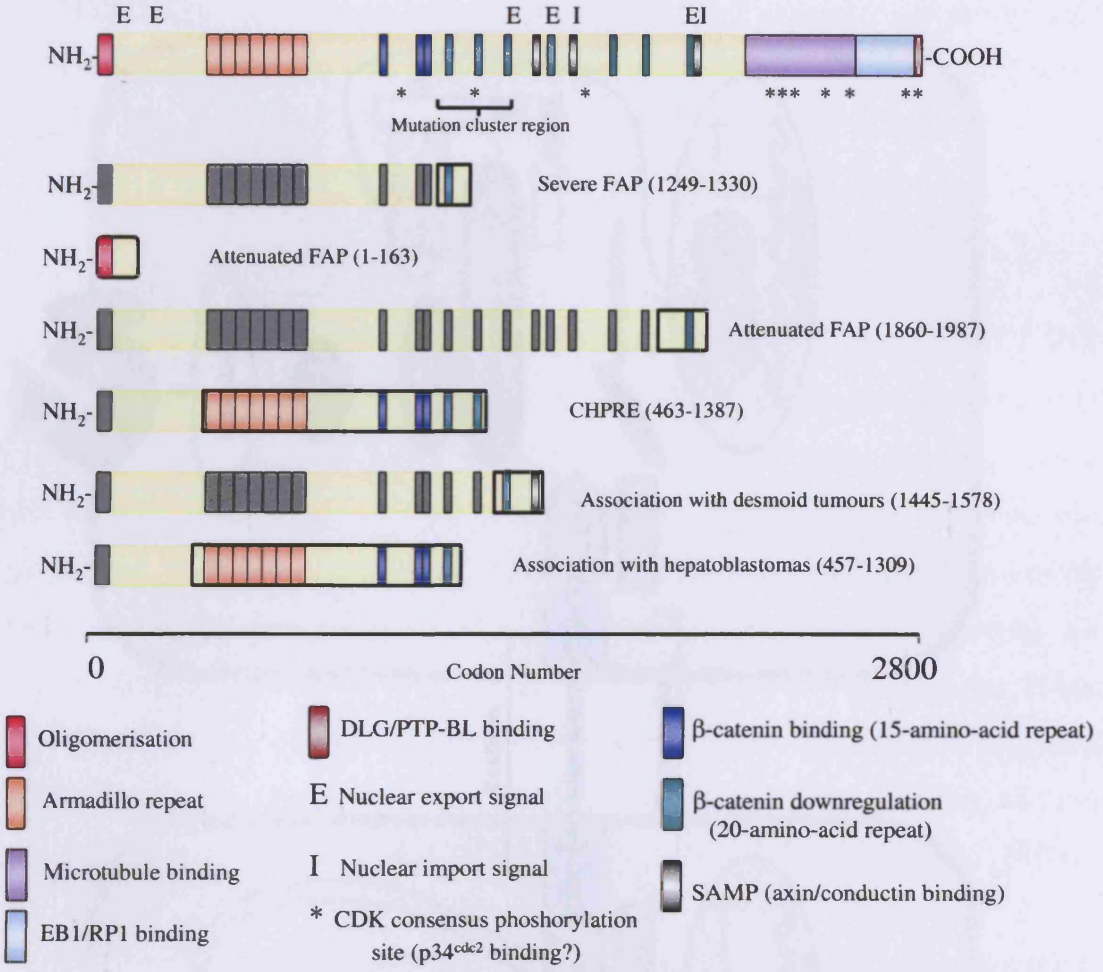

Figure 1.5.1. Diagram showing the association between FAP phenotype and the position of *Apc* mutation. The grey area indicates translated region of protein. The coloured area indicates region of mutation in associated FAP phenotype. Truncated APC products would include both grey and coloured areas (Adapted from Fearnhead *et al.*, 2001)

1.6 APC protein: functions and the Wnt signalling pathway.

The APC protein consists of multiple domains, which allow it to interact with numerous protein partners, conferring the large number of roles of APC in the cell. The APC protein is an integral part of the Wnt signalling pathway, but also plays a role in cell-cell adhesion, stability of the microtubular cytoskeleton, cell cycle regulation and possibly apoptosis (Fearnhead *et al.*, 2001).

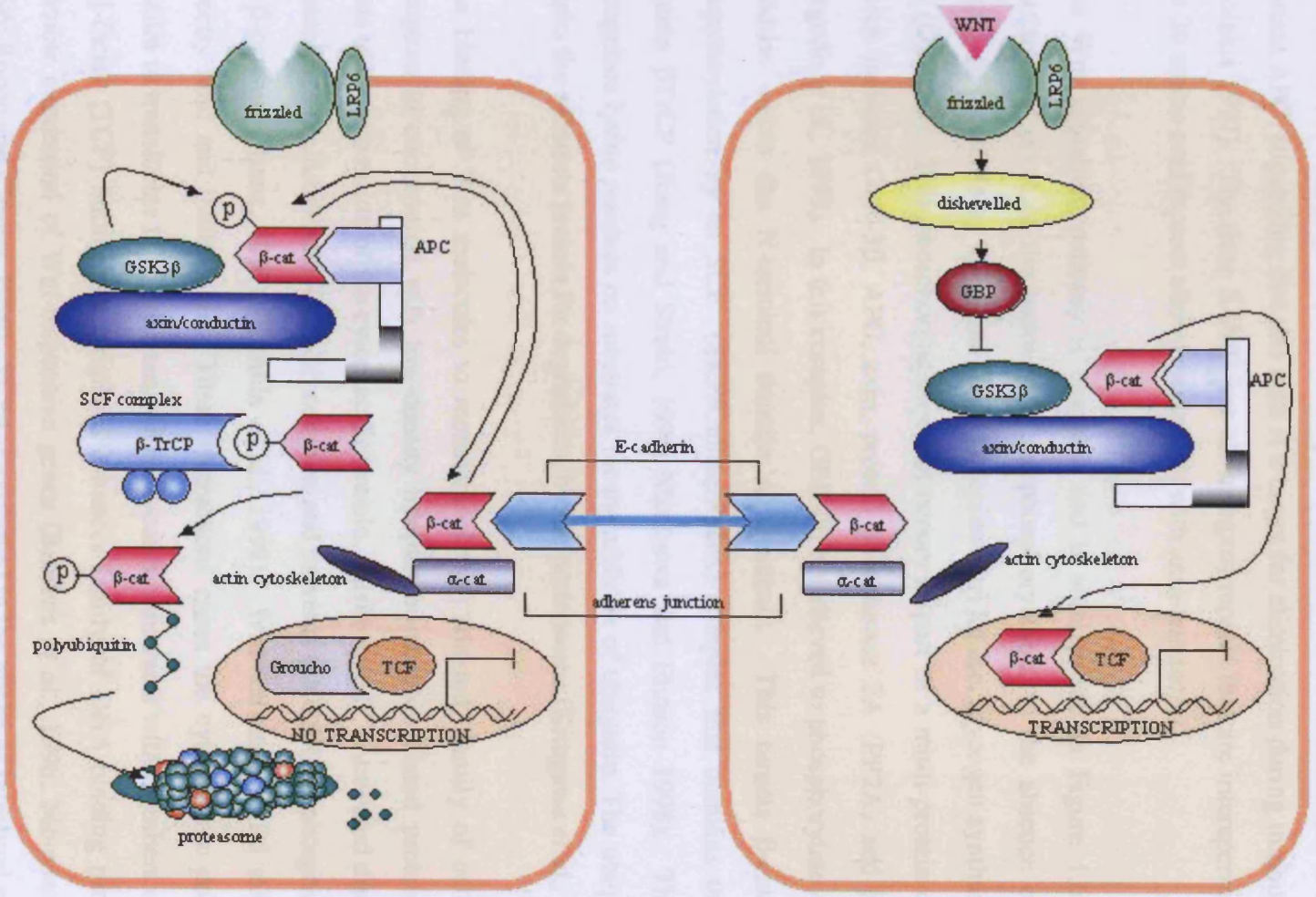

Figure 1.6.1 Wnt signalling pathway (Adapted from Fodde *et al.*, 2001; abbreviations are explained in the text)

Two important regions of the APC protein are the 15 and 20 amino acid repeats, which mediate the binding and down-regulation of β-catenin respectively. The 15 amino acid repeats are retained in the majority of mutant APC proteins (Rubinfeld *et al.*, 1993; Su *et al.*, 1993) unlike the 20 amino acid repeats, most or all of which are lost in truncated mutant APC, suggesting that this area is a target for elimination during tumourigenesis (Polakis 1997). The three SAMP (ser-ala-met-pro) repeats that are interspersed among the 20 amino acid repeats allow interactions with axin-conductin.

The Wnt signalling pathway is complex and is summarised in Figure 1.6.1. The fundamental pivot of the pathway is the phosphorylation, in the absence of a Wnt signal, of β-catenin by the serine (ser)/ threonine (thr) kinase, glycogen synthase kinase 3β (GSK-3β). This phosphorylation event occurs as part of a multi-protein complex, which includes GSK-3β, APC, axin, protein phosphatase 2A (PP2A) and β-catenin (Fagotto *et al.*, 1999). In this complex, GSK-3β is believed to phosphorylate 4 ser/ thr residues within the N-terminal domain of β-catenin. This targets β-catenin for ubiquitinylation by an SCF (SKP/Culline/F-Box) complex that contains the F-box protein βTrCP (Jiang and Struhl, 1998; Marikawa and Elinson 1998). The F-box recognises lysine residues on substrates for the addition of ubiquitin. The ubiquitin tail marks the substrate protein for degradation by the proteosome (Kitagawa *et al.*, 1999).

The binding of Wnt molecules to members of the "frizzled" family of cell-surface receptors in cooperation with low-density lipoprotein-receptor-related protein LRP6, leads to the activation of the cytoplasmic protein, "dishevelled". Activated dishevelled destabilises the Axin-APC-GSK-3β complex and prevents the GSK-3β phosphorylation of β-catenin (Bienz, 1999) (Kishida *et al.*, 1999). Wnt-activated LRP6 might also directly bind and inhibit axin. These interactions cause the cytoplasmic pool of β-catenin to translocate to the nucleus. Here, β-catenin interacts with members of the T-cell-factor (TCF) family and lymphoid enhancer family of DNA-binding proteins to promote expression of Wnt-responsive genes (Behrens *et al.*, 1996; Molenaar *et al.*, 1996; Roose and Clevers, 1999). TCF4 is expressed in the nuclei of intestinal epithelial cells and its activity is tightly controlled in complexes with potent corepressors, such as Groucho (Roose *et al.*, 1998; Cavallo *et al.*, 1998). Groucho proteins repress transcriptional activation by β-catenin/Tcf complexes, probably by interacting directly

with Tcf transcription factors. Constitutive Wnt signalling occurs when mutations in β-catenin that cannot be phosphorylated by GSK-3β or mutations in APC that result in elevated and modified levels of β-catenin are present (Miller *et al.*, 1999).

In addition to controlling the Wnt pathway, APC has numerous other functions. β-Catenin is an essential component of adherens junctions, where it provides the link between E-cadherin and α-catenin, which in turn bind actin and actin-associated proteins. Therefore, APC controls intercellular adhesion by regulating the stability and subcellular localisation of β-catenin (Ben-Ze'ev and Geiger, 1998). Modulation of cell adhesion appears to be a common mechanism of neoplastic change, and altered E-cadherin activity has been found in a number of cancers of epithelial origin including colorectal cancer (Birchmeier and Behrens 1994).

1.7 Rodent models of CRC And FAP

Carcinogen induced preclinical models have elucidated the efficacy of chemopreventive agents. ACF assays have been used widely in the short-term screening of putative chemopreventive agents, based on the assumption that dysplastic areas may become adenomas (Bird, 1987). Chemically induced colon cancer models use large doses of certain carcinogens to induce tumours, and are therefore different from the multifactorial aetiology considered important to humans (Potter, 1999). However, they are useful models of the post-initiation period of carcinogenesis and of established neoplasia and the subsequent effect of intervention (Wargovich *et al.*, 2000). For example, the F344 rat treated with azoxymethane develops ten or fewer large intestinal polyps, which develop into adenocarcinomas at 36 weeks following treatment (Sharma *et al.*, 1994). The main disadvantage of these models is that the genetic basis of the disease does not reflect the observed human condition. Recently, new rodent models have emerged that produce preneoplastic and neoplastic lesions in the gastrointestinal tract without chemical carcinogens. The use of animals harbouring the gene defect or defects pivotal to the human disease is an attractive device as it provided insights into the chemopreventive activity germane to the human situation.

In 1989, Moser and colleagues reported that they had developed a mutation in the mouse genome that led to multiple intestinal tumours in all carriers (Moser *et al.*, 1989). This lineage was established when an ethyl-nitrosourea-treated C57bl/6j (B6) male mouse was mated with AKR/J females. Progressive adult-onset anaemia was noted in some of the progeny and this anaemia appeared to be an autosomal dominant trait. The anaemic animals frequently passed bloody faeces and had numerous visible tumours of the large and small intestine and were the first multiple intestinal neoplasia (Min) mice. Further linkage analysis elucidated that the murine homolog of the *Apc* gene (*mApc*) was tightly linked to the Min locus (Su *et al.*, 1992). Sequence comparison of the *mApc* between normal and Min-affected mice identified a nonsense mutation in codon 850 of the *Apc* gene, which leads to a truncated APC polypeptide of approximately 95 kDa. Only heterozygous Min animals (Min/+) are viable as the homozygous Min mice die during embryogenesis (Moser *et al.*, 1995). The Min/+ animals develop around 100 adenomas per animal, depending on the genetic background, mainly located in the small intestine. However, the animals become moribund before carcinomas develop. Min/+ mice seldom live longer than 140 days. In this short life span, the Min/+ mouse shows a low penetrance of mammary adenosquamous carcinomas and desmoids (Moser *et al.*, 1993; Shoemaker *et al.*, 1997; Smits *et al.*, 1998; Halberg *et al.*, 2000).

At present, there are two other commonly used animal models for FAP, each bearing germline mutations in *Apc*.

1) *Apc* $^{\Delta 716}$ mouse. Generated by introducing neomycin cassette in *Apc* codon 716, these mice develop small intestine adenomas similar to the Min/+ mice and numerous extra-intestinal tumours. The resulting APC protein is truncated to approximately 80 kDa.

2) *Apc* $^{\Delta 1638N}$ mouse. Generated by introducing neomycin cassette in *Apc* codon 1638, in the transcriptional orientation opposite to that of *Apc*. These mice develop 5-6 intestinal tumours and a range of extra intestinal manifestations. These include multifocal desmoids (soft tissue tumours) and cutaneous cysts. For unknown reasons, homozygous *Apc* $^{1638N/+}$ cell lines contain very low amounts (1-2%) of the predicted 182 kDa truncated protein.

A further APC mouse model, *Apc* ^{1638T}, has been generated by inserting the cassette in codon 1638 in the same transcriptional orientation as *Apc*. The resulting expression of the truncated 182 kDa protein is in a 1:1 ratio with the wild-type 312 kDa protein. The truncated protein lacks the binding region for tubulin, EB1-like proteins and DLG (*Drosophila* discs large). *Apc* $^{1638T/+}$ mice do not develop tumours. Unlike the other APC mouse models, homozygous *Apc* ^{1638T} mice survive into adulthood and these mice do not show any increased tumour susceptibility.

The formation of mainly small intestinal tumours rather than colon tumours in these mice differs from the disease state in FAP patients. This discrepancy may arise from differences in the microenvironment of the murine intestine, which has a different distribution of bacterial content and a faster rate of cell turnover than that of humans (Bertagnolli, 1999). In spite of this difference, these animal models exhibit important phenotypic differences produced by *Apc* mutations; these distinct phenotypes provide additional insight into the mechanism of *Apc* –associated carcinogenesis.

1.8 Effect of diet on colon cancer development

With 20-30 deaths per 100,000 people, colorectal cancer is the second most common malignancy in developed countries (Ries *et al.*, 2000). The lifetime colorectal cancer risk in the general population is 5%, but this figure rises dramatically with age with approximately half of the Western population having developed an adenoma by the age of 70 (Pisani *et al.*, 1999). In general, the incidence of colorectal cancer is high in the developed countries such as Australia, North America and Northern and Western Europe, and low in developing countries of Africa and Asia (Parkin *et al.*, 1999). There is also a rapid increase in the incidence of colorectal cancer in the late twentieth century in many countries such as Italy, Japan and urban China (Muir *et al.*, 1987) that have occurred within one generation. These observations suggest an important role for environmental influence in the aetiology of this disease. Dietary factors appear to be a major influence on a person's predisposition to developing colorectal cancer (Cummings and Bingham, 1998). Diets low in vegetables and folate and high in fat, red meat and alcohol appear to increase the relative risk although this relative risk ratio is small in comparison to the risk of lung cancer in smokers. Large prospective studies of dietary risk factors are continuing, such as the European prospective Investigation of

Cancer, in which data from over 4 million individuals are being evaluated (Riboli, 1992). It is because of such studies, coupled with an understanding of the molecular biology underpinning neoplasias that the case for chemopreventive agents will become stronger.

1.9 Curcumin

The phytochemical, curcumin, is an example of a dietary derived agent under evaluation as a chemopreventive agent. Curcumin is the major yellow pigment extracted from turmeric, the dried ground rhizome of the herb *Curcuma longa.* Turmeric is extensively used to impart colour and flavour to food, although it has been used in Asian medicine for four thousand years as a treatment for a diverse range of conditions, including wound healing, haemorrhage, inflammation and cancer (Brouk, 1975). In India, normal dietary intake of turmeric has been found to range from 0.1 g to 3.8 g/adult/day, of which 2 - 8% is curcumin. First characterised in 1910, curcumin (diferuloylmethane) is a low molecular weight polyphenol (MW 368) generally regarded as the most active constituent of most turmeric preparations. Furthermore, preparations of curcumin contain small amounts of curcuminoids. Levels range from 3-15% desmethoxycurcumin and 1-5% bisdesmethoxycurcumin. Studies have shown that bisdemethoxycurcumin is less potent than curcumin with respect to chemoprevention (Huang *et al.,* 1995) and contributes little to the pharmacological activity of over the counter curcumin.

Curcumin, 1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3, 5-dione, is a bis-α,β-unsaturated β-diketone, which exists in equilibrium with its enol tautomer (Figure 1.9.1). At pH>8, the enolate form of the heptadienone chain between the two methoxyphenol rings predominates. Under these conditions, curcumin acts mainly as an electron donor, a characteristic of phenolic antioxidants leading to their scavenging activity (Jovanovic *et al.,* 1999). Curcumin appears to be unstable at basic pH, and is degraded within 30 minutes in buffer systems to trans-6-(4'-hydroxy-3'-methoxyphenyl) 2-2,4-dioxo-5-hexanal, ferulic acid, feruloylmethane and vanillin (Lin *et al.,* 2000). The presence of foetal calf serum or human blood, or addition of antioxidants such as ascorbic acid, N-acetylcysteine or glutathione, completely blocks

this degradation in culture media or phosphate buffer above pH7. At pH 3 to 7, curcumin acts as a potent H-atom donor and it is in these acidic and neutral conditions (and in the cell membrane) that the bis-keto form predominates. In the keto form of curcumin, the heptadieone linkage contains a highly activated carbon atom. The adjoining C-H carbon bonds on this carbon are very weak due to delocalisation of the unpaired electron on the adjacent oxygens. Under acidic conditions, the degradation of curcumin is slow, with less than 20% of total curcumin decomposed at 1 hour (h).

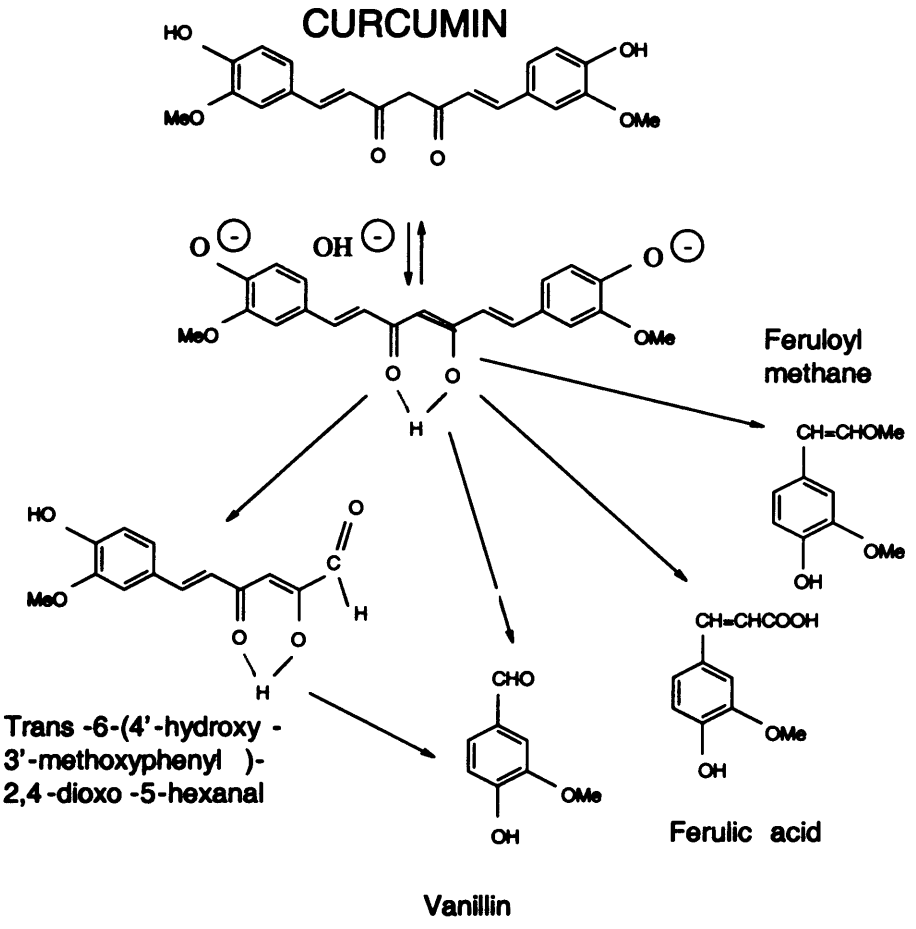

Figure 1.9.1. Tautomerism of curcumin and subsequent degradation products in alkaline medium

1.9.1 Toxicity Of Curcumin

As chemopreventive agents are likely to be administered to individuals for prolonged periods of time it is imperative that curcumin should possess negligible adverse effects. Both in human and animal studies, curcumin has demonstrated a lack of significant toxicity. Although one early study reports dietary curcumin inducing ulcerogenic activity in the stomach of an albino rat (Gupta *et al.*, 1980), later studies in which up to 3.5 g/ kg body weight curcumin was given to rats, dogs or monkeys for 3 months were without effect (NCI Report, 1996). Similarly, Wahlstrom and Blenow (1978) also administered doses of up to 5 g/ kg curcumin to rats without any adverse effects. In human studies, results from phase 1 clinical trial indicate that oral doses of 8 g daily was without side effects (Cheng *et al.*, 1998). No toxicity was observed after administration of 1.2 g to 2.1 g of curcumin for 2-6 weeks in patients with rheumatoid arthritis (Deodhar *et al.*, 1980).

1.9.2 Mechanisms of the chemopreventive efficacy of curcumin

The chemopreventive efficacy of curcumin in preclinical rodent models is well documented. Table 1.9.2 shows that curcumin can affect all three stages of carcinogenesis: initiation, promotion and progression. This efficacy may be explained by the broad spectrum of actions of curcumin. Important pharmacological activities of curcumin with respect to anticarcinogenesis are described below.

The inappropriate regulation of signalling pathways, such as those that involve the nuclear factor-kappa B (NF-κB) is associated with acute inflammation and cancer (Manson *et al.*, 2000). This NF-κB pathway is depicted in Figure 1.9.2. NF-κB is a cytosolic transcription factor which plays an important role in the regulation of immune response, embryo and cell lineage development, apoptosis, inflammation, cell-cycle progression, oncogenesis, viral replication and various autoimmune diseases (Sha *et al.*, 1998; Baeuerle and Henkel, 1994; Baeuerle and Baltimore 1996; O'Neill and Kaltschmidt, 1997). The pathway can be induced by a variety of stimuli. These inducers include tumour necrosis factor (TNF), which mediates inflammation, cellular immune responses, and protects several cell types against apoptosis; Interleukin-1 (IL1)

Table 1.9.2. Examples of preclinical studies of curcumin using rodent models of carcinogenesis

Organ (Endpoint)	**Model (*species*)**	**Dietary level of curcumin (time of administration)**	**Reference**
Small intestine (Adenomas)	Min/+ mouse (+2.1% PhIP in AIN76A diet)	0.2%	Collett *et al.*, 2001
	Min/+ mouse	0.1%	Mahmoud *et al.*, 2000
	Azoxymethane (*mouse*)	2% (initiation, post-initiation or both)	Huang *et al.*, 1994
Colon – (Aberrant Crypt Foci)	Azoxymethane (*rat*)	0.2%	Rao *et al.*, 1999, 1993
	Azoxymethane (*rat*)	0.2% (initiation and post initiation); 0.2% / 0.6% (promotion progression)	Kawamori *et al.*, 1999
Colon – (Adenocarcinomas)	Azoxymethane (*rat*)	0.8% / 1.6%	Periera *et al.*, 1996
	Azoxymethane (*rat*)	0.2%	Rao *et al.*, 1995,
	Azoxymethane (*mouse*)	2% (initiation, post-initiation or both)	Huang *et al.*, 1994
Stomach	N- methl-N'-nitro-N-nitroguanosine (MNNG) and sodium chloride (*rat*)	0.2%	Ikezaki *et al.*, 2001
	Azoxymethane (*mouse*)	2% (initiation, post-initiation or both)	Huang *et al.*, 1994
Oesophagus	N-nitrosomethylbenzylamine (NMBA) (*rat*)	0.05%	Ushida *et al.*, 2000
Tongue	4-nitroquinoline 1-oxide (4-NQO) (*rat*)	0.05% (initiation or post-initiation)	Tanaka *et al.*, 1994
Mammary	Diethylstilbestrol (DES) induced after radiation initiation (*rat*)	1% (initiation only)	Inano *et al.*, 2000
	Diethylstilbestrol (DES) induced after radiation initiation (*rat*)	1% (promotion only)	Inano *et al.*, 1999
	7,12-dimethylbenz[a]anthracene (DMBA) (*rat*)	0.8% / 1.6%	Periera *et al.*, 1996
Skin	12-O-tetradecanoylphorbol-13-acetate (TPA) promotion after DMBA initiation (*mouse*)	1%	Limtrakul *et al.*, 1997
	12-O-tetradecanoylphorbol-13-acetate (TPA) promotion after DMBA initiation (*mouse*)	1%	Huang *et al.*,1995
Blood (leukaemias) / Lymphastic system (Lymphomas)	7,12-dimethylbenz[a]anthracene (DMBA) (*mouse*)	2%	Huang *et al.*, 1998

which mediates similar effects such as immunoregulation, proinflammatory tumour promoter and haematopoietic activities; and bacterial lipopolysaccharide (LPS), which induce the transcription of various cytokines (e.g. IL-1 and TNF) as an immune response against bacteria. In its inactive form, NF-κB is sequestered in the cytoplasm, bound by IκB inhibitor proteins. Stimulation causes phosphorylation and subsequent ubiquitinylation and degradation of IκB. The now activated transcription factor, NF-κB, translocates to the nucleus where it binds to the consensus sequence (5'GGGACTTTCC-3') of genes, thereby activating their transcription. Curcumin has been shown to inhibit the activation of the NF-κB pathway in a variety of cancer cell lines including prostate cells (Hour *et al.*, 2002), myeloma cells (Bush *et al.*, 2001) and colon cells (Plummer *et al.*, 1999) Furthermore, curcumin prevents phosphorylation of IκB by inhibition of IκB kinases (IKKs) which phosphorylate IκB, directing IκB for degradation (Plummer *et al.*, 1999).

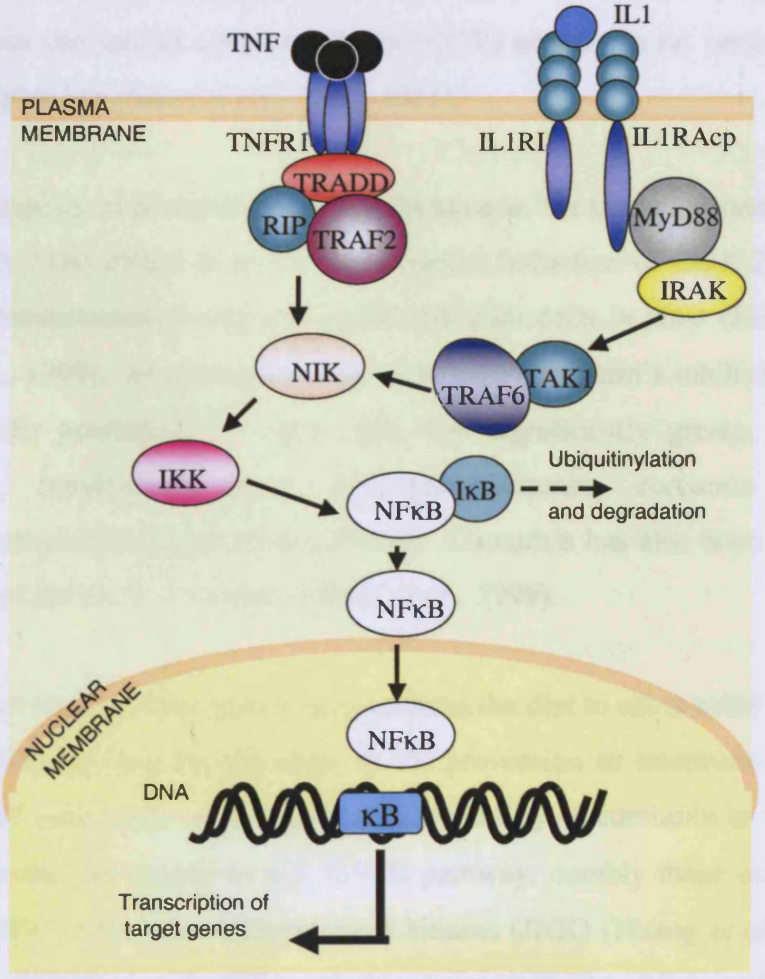

Figure 1.9.2. Summary of the NF-κB signalling pathway.

NF-κB regulates a plethora of genes associated with carcinogenesis, including cyclooxygenase (COX)-2. COX is a key enzyme responsible for the conversion of arachidonic acid to prostaglandins and thromboxanes. It consists of two different isoforms, designated COX-1 and COX-2. COX-1 is a constitutive isoform present in most tissues and is generally regarded as a 'housekeeping' enzyme and its inhibition results in serious side effects such as peptic ulceration or impairment of renal blood flow. In contrast, COX-2 is constitutively expressed only in brain and spinal cord tissue, but can be induced in a wide variety of normal tissues by the hormones of ovulation and pregnancy, cytokines, growth factors, oncogenes, and tumour promoters. COX-2 overexpression has been implicated in the carcinogenesis of tumours of colorectum, breast, head & neck, lung, pancreas, stomach and prostate (Taketo, 1998). Mechanisms of COX-2 associated tumourigenesis are thought to include promotion of tumour-specific angiogenesis, inhibition of apoptosis and induction of proangiogenic factors such as VEGF, iNOS, IL-6, IL-8 and TIE-2. It has been known for a decade that curcumin can inhibit cyclooxygenase (COX) activity in rat peritoneal neutrophils and human platelets (Ammon and Wahl, 1991).

Curcumin appears to decrease COX-2 expression at the transcriptional level (Plummer *et al.*, 1999). The ability of curcumin to inhibit induction of COX-2 gene expression has been demonstrated in oral and colon epithelial cells *in vitro* (Khafif *et al.*, 1998; Zhang et al., 1999). At a concentration of 20 μM, curcumin's inhibition of chemically induced PGE_2 production in colon cells was significantly greater than that of its metabolites, tetrahydrocurcumin, hexahydrocurcumin, curcumin sulphate, and hexahydrocurcuminol (Ireson *et al.*, 2001a). Curcumin has also been shown to inhibit the activity of the COX-2 enzyme (Zhang *et al.*, 1999).

The ability of many natural agents derived from the diet to act at more than one level in a cellular pathway may be important in the prevention or treatment of diseases with multifactorial aetiologies such as cancer. The ability of curcumin to inhibit activation of pathways that are related to the NF-κB pathway, notably those involving activator protein-1 (AP-1) and c-Jun NH_2-terminal kinases (JNK) (Huang *et al.*, 1991; Chen et *al.*, 1998), provides an example of this concept. The AP-1 and JNK pathways constitute an important group of terminal kinases involved in cellular responses to

environmental stress, pro-inflammatory cytokines, mitogen stimulation and apoptotic stimuli.

Reactive oxygen species (ROS), such as singlet oxygen, peroxy radicals, superoxide anions and hydroxyl radicals, play an important role in carcinogenesis (Kensler *et al.*, 1992) as they can cause damage to DNA, protein and lipids. The mode of action may involve direct interaction of specific receptors and/ or redox-activation of members of signalling pathway such as protein kinases, protein phosphatases and transcription factors. For example, ROS has been demonstrated to activate the extracellular regulated kinase 1/ 2 (ERK 1/ 2) pathway, as well as the c-Jun N-terminal kinase (JNK) and p38 MAPK pathways, all of which are known pathways implicated in carcinogenesis (Katsanakis *et al.*, 2002). Furthermore, ROS can attack bases or deoxyribose residues to produce strand breaks or damaged bases or alternatively oxidise lipids or protein molecules to generate intermediates that react with DNA to form adducts (Marnett, 2000). Consequently mopping up activated oxygen species is an important potential mechanism in the prevention of cancer. Impairment of reactive oxygen species generation in rat peritoneal macrophages by curcumin has been shown both *in vitro* and *in vivo* (Joe and Lokesh, 1994), and similar effects have been observed in red blood cells (Tonnesen and Greenhill 1992). More specifically, curcumin has been shown to scavenge superoxide anion radicals (Kunchandy and Rao, 1990) and hydroxyl radicals (Pulla Reddy and Lokesh, 1994). However, curcumin may possess pro-oxidant activity as well as antioxidant effects, dependent on dose and chemical environment, e.g. availability of free Cu^{2+} ions (Ahsan, 1999).

Nitric oxide (NO) is a short-lived molecule required for many physiological functions for example, immune defence and intracellular signalling. It is produced from L-arginine by NO synthases (NOS) (Lala and Chakraborty, 2001). NO has an unpaired electron, and therefore produces many reactive intermediates that are capable of damaging DNA or hindering DNA repair (deRojas-Waker *et al.*, 1995; Laval and Wink, 1994; Graziewicz *et al.*, 1996). Peak inducible NOS (iNOS) activity may relate to the progression of colonic adenomas to carcinomas (Ambs *et al.*, 1999). Upregulation of COX-2 via NF-κB or AP-1 pathways, or increasing intracellular concentrations of reduced glutathione, appears to confer resistance to NO-induced apoptosis in malignant

cells *in vitro* (von Knethen and Brune 1997; von Knethen *et al.*, 1999). Curcumin has been shown to inhibit the activity of iNOS in activated macrophages (Brouet and Oshima, 1995). Chan and colleagues (1998) demonstrated that the equivalent of 92 ng curcumin per gram body weight (g bw) curcumin could significantly inhibit murine hepatic iNOS gene induction by lipopolysaccharide as an aqueous alkaline solution mixed into the drinking water. Furthermore the inhibition of iNOS expression by curcumin may be mediated by inhibition of IKK activity and NFκB activation (Pan *et al.*, 2000). Therefore, inhibition of iNOS activity by curcumin may be important in the compound's chemopreventive efficacy.

Curcumin inhibits cell proliferation and induces apoptosis in cancer cells (Jiang *et al.*, 1996). Its mechanisms of action appear diverse, modifying numerous stages of apoptosis. Figure 1.9.3 depicts a simplified diagram of apoptotic pathways. Apoptosis can be initiated through intrinsic (mitochondrial) and extrinsic (death receptor) pathways. In both, induction of apoptosis leads to the activation of an initiator caspase (8 and 9), which activates a cascade of downstream effector caspases (3, 6, and 7). Active effector caspases subsequently cleave numerous substrates, resulting in the biochemical and morphological hallmarks characteristic of apoptosis (Figure 1.9.3). These cascades are not independent, as cross-talk exists between the two pathways. For example, cleavage of the pro-apoptotic BCL-2 family member, BID, by caspase-8 activates the mitochondrial pathway after apoptosis induction through death receptors, thereby amplifying the apoptotic signal (Igney and Krammer, 2002).

Curcumin has been shown to induce apoptosis in acute myelogenous leukaemia cells through activation of caspase-8, BID cleavage and mitochondrial release of cytochrome *c* (Anto *et al.*, 2002). Anto and colleagues (2002) also suggest that BCL-2 and BCL-X_L are critical negative regulators of curcumin-induced apoptosis. In melanoma cells, curcumin activates caspase-3 and –8, but not caspase-9, suggesting that curcumin induces apoptosis though a death receptor pathway (Bush *et al.*, 2001). Activation of caspase-3 by curcumin has also been demonstrated in breast epithelial cells (Kim *et al.*, 2001) and gastric and colon cells (Moragoda *et al.*, 2001). Cleavage of the caspase substrate, poly (ADP-ribose) polymerase (PARP), an enzyme integral to DNA repair, has also been demonstrated in response to curcumin (Cipriani *et al.*, 2001; Moragoda *et*

al., 2001). Furthermore, curcumin may induce apoptosis by the inhibition of cell signalling pathways involving NF-κB, AP-1 or JNK and the downregulation of the expression of survival genes *egr-1*, *c-myc*, and *bcl-x_L* or abnormal tumour suppressor genes such as *p53* (Plummer *et al.*, 1999; Huang *et al.*, 1991; Chen and Tan, 1998; Han *et al.*, 1999). *In vivo,* dietary administration of curcumin during promotion/progression of colon cancer, induced in mice by azoxymethane, produced a significant increase in the apoptotic histological index when compared to controls (Samaha *et al.*, 1997).

Curcumin may also affect the cell cycle. In mouse embryo fibroblast, mouse sarcoma, HT29 human colon carcinoma, human kidney carcinoma, and human hepatocellular carcinoma cell lines grown *in vitro* in the presence of curcumin (9μM), Jiang *et al.*, (1996) observed cell shrinkage, chromatin condensation and DNA fragmentation. In other colon carcinoma cells cultured *in vitro*, curcumin induced apoptotic cell death by cell cycle arrest in the S and G2/M phases (Chen *et al.*, 1999), and in the MCF-7 human breast tumour cell line the same was observed at G2 or M phases (Simon *et al.*, 1998). The presence of the diketone moiety may be essential for such antiproliferative activity (Simon *et al.*, 1998). Furthermore, this cell cycle arrest may be due to the assembly of aberrant, monopolar mitotic spindles that are impaired in their ability to segregate chromosomes. Although one report has claimed that the inhibition of cell proliferation may be non-selective with regard to transformed/non-transformed cell lines *in vitro* (Gautam *et al.*, 1998), comparison in our laboratory of the non-malignant human colon epithelial cell line (HCEC) with the malignant colon adenocarcinoma cell line HT29 showed tumour-specificity, with an IC_{50} for the malignant cells of about 5 μM compared to 14 μM for the non-malignant cells (Plummer *et al.*, 1999).

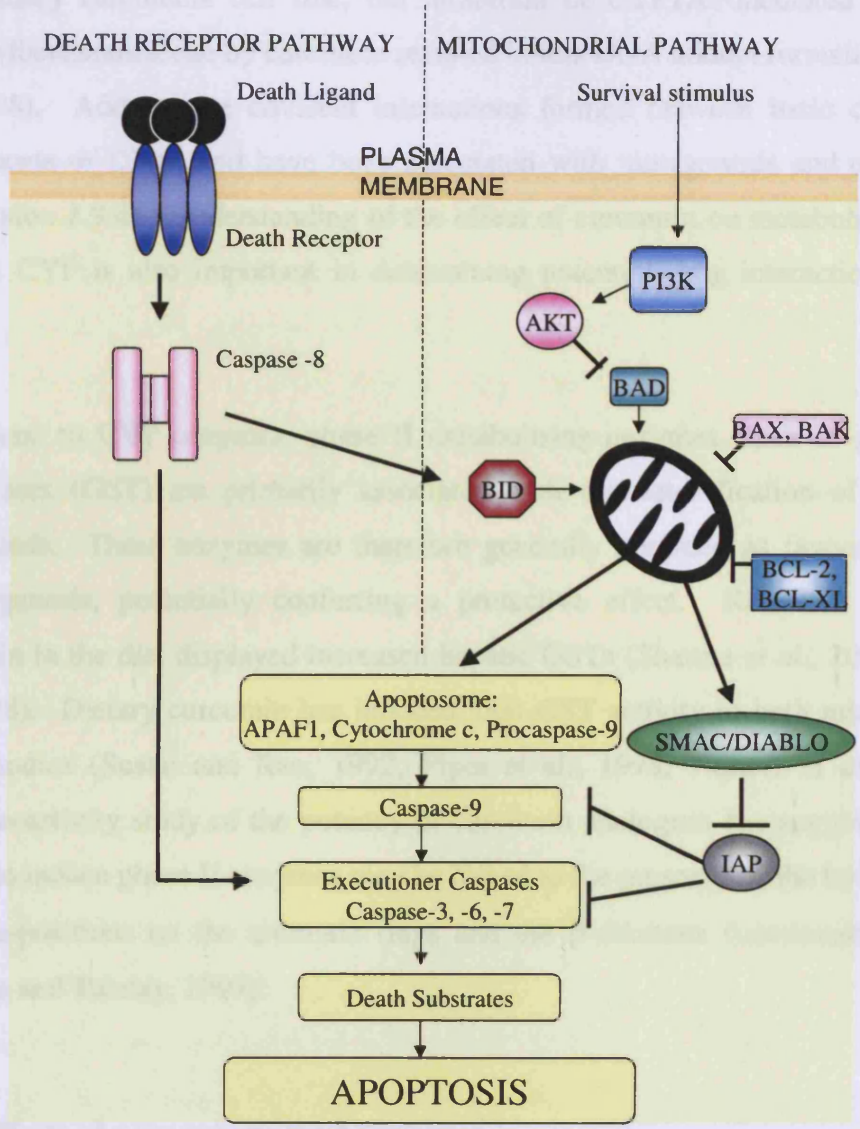

Figure 1.9.3 The two main apoptotic pathways (modified from Igney and Krammer, 2002)

The cytochromes P450 (CYP) enzymes such as CYP1A1 and 1A2 are important in the metabolic conversion and activation of many carcinogens (Timbrell, 1991). For example, the metabolism of the hepatocarcinogen aflatoxin B_1 to form the electrophile carcinogen AFB_1-8, 9-epoxide (Wogan, 1973), and the metabolism of the polycyclic hydrocarbon B [a] P, a procarcinogen found in cigarette smoke to form the carcinogen B [a] P 7,8-dihydrodiol, 9,10-oxide (BPDE), (Timbrell 1991). Inhibition of CYP isoenzymes by curcumin has been demonstrated in cells cultured *in vitro* (Firozi *et al.*, 1996; Oetari *et al.*, 1996) and this may represent one mechanism by which dietary curcumin protects animals against the toxic effects of many chemicals. For example, in

a mammary carcinoma cell line, the inhibition of CYP1A1-mediated activation of dimethylbenzanthracene by curcumin resulted in less DNA adduct formation (Ciolino *et al.*, 1998). Adducts are covalent interactions formed between toxic chemicals and constituents of DNA, and have been associated with mutagenesis and carcinogenesis (see section 1.9.4). Understanding of the effect of curcumin on metabolising enzymes such as CYP is also important in determining potential drug interactions in clinical usage.

In contrast to CYP enzymes, phase II metabolising enzymes, such as glutathione-S-transferases (GST) are primarily associated with the detoxification of carcinogenic compounds. These enzymes are therefore generally regarded as favourable in early carcinogenesis, potentially conferring a protective effect. Rats that received 2% curcumin in the diet displayed increased hepatic GSTs (Sharma *et al.*, 2001b; Singh *et al.*, 1998). Dietary curcumin has induced total GST activity in both mice and rats in other studies (Susan and Rao, 1992; Piper et al., 1998; Nijhoff *et al.*, 1993). A structure-activity study of the potency of curcumin analogues has suggested that their ability to induce phase II enzymes may be linked to the presence of the hydroxyl groups at ortho-positions on the aromatic rings and the β-diketone functionality (Dinkova-Kostova and Talalay, 1999).

1.9.3 Effects of curcumin in the Min/+ mouse

Mahmoud and colleagues (2000) demonstrated that lifetime administration of curcumin (0.1%) reduced intestinal adenomas in Min/+ mice by 64%. The reduction in tumour burden was associated with a decrease in the expression of the oncoprotein β-catenin in the enterocytes, an induction in enterocyte apoptosis, and normalisation of enterocyte proliferation. Collett and colleagues (2001) confirmed that curcumin (0.2%) reduced proximal adenomas in Min/+ mice treated with 2-amino-1-methyl-6-phenylimidazo [4,5b] pyridine (PhIP) (300p.p.m), by 52%. However, administration of 0.2% curcumin was without effect in untreated Min/+ mice. PhIP is a heterocyclic amine found in cooked meat and fish and curcumin was shown to enhance PhIP-induced apoptosis (Collett et al., 2001). Furthermore, curcumin modulates lymphocyte-mediated immune functions by increasing mucosal CD4 (+) T cells and B cells (Churchill et al., 2000).

1.9.4 Biological markers of the efficacy of curcumin in the Min/+ mouse

For biomarkers of carcinogenesis to be utilised in human cancer chemoprevention studies they have to be investigated and validated in preclinical models. As discussed previously (section 1.2), these biomarkers often relate both to the genetic pathways implicated in colorectal carcinogenesis and the possible mechanism of action of the agent being investigated. Strictly, biomarkers should reflect changes in preneoplastic cells prior to the development of neoplasia. However, in this project they will be regarded as indicators of carcinogenesis. The effect of curcumin on these indicators will be investigated. The principles and criteria used for biomarker selection have been previously discussed in section 1.2. The observations from this research will provide useful information on the pharmacodynamic properties of curcumin.

COX-2 over expression is implicated in colorectal carcinogenesis (Eberhart *et al.*, 1994; Kargman *et al.*, 1995). Its involvement in this process and the intervention by curcumin was discussed previously. Importantly, inhibition of COX-2 expression or activity has been shown to decrease adenoma formation in the Min/+ mouse (Jacoby *et al.*, 2000) and a significant decrease in polyp size and number was demonstrated in COX-2 knockout mice when compared to controls with the same $Apc^{\Delta 716}$ knockout background (Oshima *et al.*, 1996). A marked increase in COX-2 expression has also been demonstrated in the adenomas of Min/+ mice (Hull *et al.*, 1999). Increased COX-2 mRNA has been observed in a majority of colon carcinomas compared to normal surrounding mucosa, and in many adenomas (Eberhart *et al.*, 1994; Kargman *et al.*, 1995). Higher levels of eicosanoids, including PGs, have also been found in tumour tissue (Bennett *et al.*, 1977). Therefore, COX-2 levels should be investigated in preclinical chemoprevention studies using curcumin, a known modulator of COX-2 function (see section 1.9.2).

Min/+ mice harbour a defect in the *Apc* gene, a key component of the Wnt signalling pathway and "gatekeeper" of colorectal carcinogenesis (Fearon and Vogelstein, 1990). APC regulates levels of β-catenin (as described previously, section 1.6). Mahmoud and colleagues (2000) have demonstrated that dietary administration of curcumin (0.1%) decreases β-catenin levels in the Min/+ mouse model, therefore maybe a potential biological marker of the efficacy of curcumin in the Min/+ mouse.

Accumulation of DNA damage appears to contribute substantially to the development of colorectal cancer (Fearon and Jones, 1992). The measurement of such damage may be informative in the early stages of carcinogenesis and may in turn act as a biomarker of intervention in chemoprevention studies *in vivo*. Malondialdehyde (MDA) is a product of lipid peroxidation and prostaglandin biosynthesis. MDA is carcinogenic in rodents and considered to be the highly mutagenic product of lipid peroxidation (Marnett, 1999). COX-2 can contribute to the generation of MDA via breakdown of its product, PGH_2 (Diczfalusy *et al.*, 1977). MDA can be found in colorectal cancer tissue at high levels (Ozdemirler *et al.*, 1998) and these levels appear to correlate with levels of PGE_2 (Hendrickse *et al.*, 1994). MDA also reacts with DNA under physiological conditions to form adducts, predominantly with deoxyguanosine to generate M_1G adducts (Marnett, 1999). M_1G adducts have been associated with carcinogenesis in rodents and detected in human liver, pancreas, breast tissues and blood leukocytes at levels ranging from 1 to 120 per 10^8 nucleotides (Marnett, 1999). In one study, median M_1G levels in normal breast tissue from patients with breast cancer were almost 3-fold higher than those from women without cancer (Wang *et al.*, 1996). The formation of M_1G adducts in human cells has also been demonstrated in the absence of MDA, *via* base propenals resulting from oxidative DNA damage (Dedon *et al.*, 1998, Plastaras *et al.*, 2000a). DNA adducts arising directly or indirectly from oxygen radicals may act as biomarkers of mutagenesis and carcinogenesis in humans (Marnett, 2000). Adduct levels in colon mucosa and CRC tissues have not been described in either rodents or humans.

The effects of curcumin on MDA and M_1G levels have not been described previously in the Min/+ mouse. There are at least 3 mechanisms by which curcumin may affect the formation of M_1G adducts (Figure 1.9.4). Curcumin is a potent inhibitor of lipid peroxidation *in vitro* and *in vivo* (Ammon and Wahl, 1991; Ramirez Bosca *et al.*, 1995; Venkatesan and Rao, 2000) and an inhibitor of cyclooxygenase activity (see section 1.9.2), which may decrease MDA levels in adenomas and normal mucosa. Curcumin is also a potent antioxidant under physiological conditions (Jovanovic *et al.*, 1999) therefore even in the absence of MDA, oxidative DNA damage which can contribute to formation of M_1G adducts in vitro, via base propenals (Dedon *et al.*, 1998) may be inhibited.

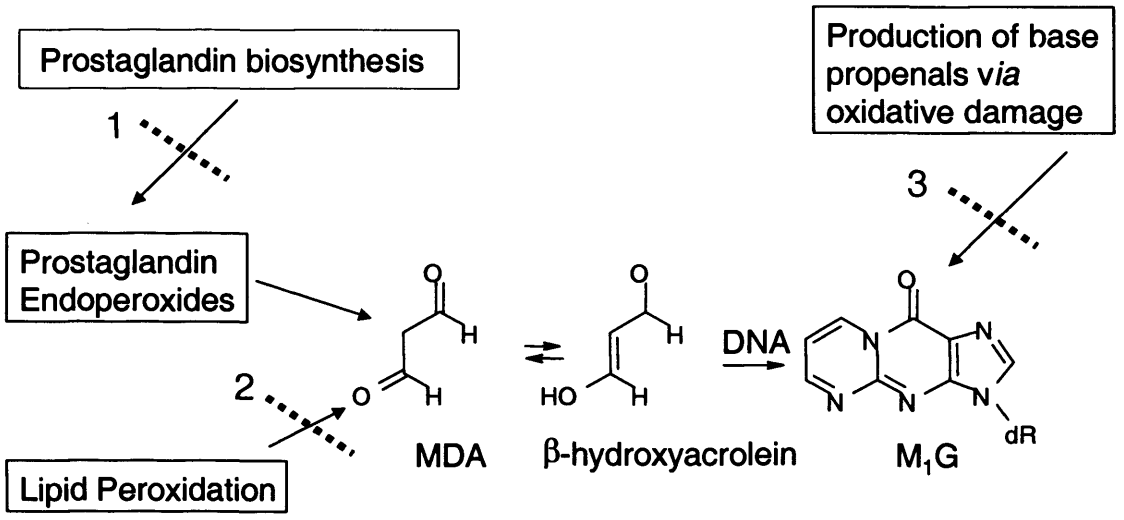

Figure 1.9.4 Potential mechanisms of inhibition of MDA and M_1G adduct formation by curcumin. Curcumin may 1) inhibit the formation of prostaglandins by inhibition of cyclooxygenase, 2) inhibit lipid peroxidation and 3) inhibit oxidative damage by scavenging free radicals.

8-Oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG), the most extensively studied adduct arising from oxidative DNA damage, is generated by reactive oxygen species, particularly the hydroxyl radical, which can bind to, or abstract hydrogen atoms from, DNA bases (Marnett, 1999). 8-Oxo-dG has been shown to cause miscoding by DNA polymerase *in vitro* and is associated with mutagenesis in bacterial and mammalian cells (Shibutani *et al,* 1991; Cheng *et al.,* 1992; Moriya, 1993; Le Page *et al.,* 1995). It induces G to T transversions in human DNA, lesions frequently seen in tumour suppressor genes such as the *Apc* gene (Hussain and Harris, 1998). Reports of levels of 8-oxo-dG in human tissues range from one adduct in 10^7 nucleotides to one adduct in 10^3 nucleotides (Cadet *et al.,* 1997). Administration of the antigenotoxic antioxidant N-acetylcysteine in the diet has recently been shown to reduce 8-oxo-dG levels in the hind leg of mice by 50% (Malins *et al,* 2002). These results suggest that in organs, probably including premalignant target tissues, levels of 8-oxo-dG can be affected by antioxidants. 8-Oxo-dG may thus serve as a marker of the chemopreventive efficacy of antioxidants, such as curcumin in the Min/+ mouse.

1.9.5 Pharmacokinetics of curcumin

Previous studies on the pharmacokinetics in humans and rodents suggest that curcumin is poorly absorbed from the gastrointestinal tract (Shoba *et al.*, 1998; Cheng *et al.*, 1998; Sharma *et al.*, 2001c; Wahlstrom and Blenhow, 1978; Pan *et al.*, 1999; Ireson *et al.* 2001a). Administration of 2 g of pure curcumin powder to fasting volunteers resulted in low curcumin concentrations in the plasma (less than 10 ng/ ml) 1 h post dose (Shoba *et al.*, 1998). Colleagues in this laboratory could not detect curcumin in the plasma of colorectal cancer patients, who received up to 180 mg curcumin for 29 days (Sharma *et al.*, 2001c). In a published abstract, Cheng and colleagues (1998) administered 0.5 g – 8 g curcumin daily for 3 months to patients with precancerous lesions or resected tumours. The 8 g/ day dose resulted in peak serum concentrations of 1.75 ± 0.8 μM. The serum concentrations were found to peak at 1 – 2 h after oral intake, and gradually decline within 12 h. In a recent study of 18 healthy volunteers who received 50 - 200 mg microionised curcumin, curcumin was below that limit of detection (0.63 ng/ml) (Brenner *et al.*, submitted).

Sharma *et al.*, (2001b) showed that dietary administration of curcumin (2% for 14 days) to rats, yielded low drug levels in the plasma, between 0 - 12 nM, whereas tissue concentrations of curcumin in the liver and colon mucosa were 0.1 - 0.9 nmol/ g and 0.2 - 1.8 μmol/ g, respectively. Ravindranath and Chandrasekhara (1980) could not detect curcumin colourimetrically in heart blood and only traces (<5 μg/ ml) were observed in portal vein blood after intragastric administration of curcumin (400 mg) to rats. When curcumin (1 g/ kg) was administered via intragastric intubation in a suspension of arachis oil to rats, only traces were detected in the plasma and 75% of curcumin was excreted in the faeces (Wahlstrom and Blennow, 1978). Three hours post dosing, curcumin could be detected in the plasma of only 25% of the animals. Studies by Ravindranath and Chandrasekhara (1980; 1981) suggest that the bioavailability of curcumin is approximately 60% irrespective of dose; curcumin was administered to rats at a level of 2000 mg/ kg and 40, 320, 2000 mg/ kg of tritiated curcumin. This value may be an over estimation as it was calculated by determination of the amount of unchanged curcumin excreted in the faeces.

It is feasible that the absorption of curcumin, as reflected by plasma levels, may be affected by strain, species, and sex of animal as well as dosing vehicle. Freireich and colleagues (1966) investigated the maximum tolerated dose of anticancer agents in humans, mouse, rat, hamster, dog and monkey. It was observed that the most accurate prediction of the maximum tolerated dose (MTD) in humans could be determined using the surface area (mg/ m^2) as opposed to weight (mg/ kg). The dose according to surface area (mg/ m^2) can be calculated by multiplying the dose measured in mg/ kg by a constant (Km). Km is the appropriate factor for interconversion of mg/ kg and mg/ m^2 for each species.

To improve the absorption of curcumin, Shoba and colleagues (1998) investigated the effect of concomitant administration of piperine (L-piperoylpiperidine), a constituent of black pepper. Piperine has been reported to increase the bioavailability of compounds by inhibition of glucuronidation in the liver (Atal *et al.,* 1985) and intestine (Singh *et al.,* 1986). Higher plasma levels of curcumin were attained in humans and rats when curcumin was co-administered with piperine than curcumin alone. It was hypothesised that increased plasma levels of curcumin were due to decreased glucuronidation of parent compound.

Ravindranath and Chandrasekhara have performed two studies investigating the distribution of curcumin in rats. After oral dosing of approximately 2000 mg/ kg only trace levels of curcumin could be detected in portal blood, liver and kidney between 15 min and 24 h (Ravindranath and Chandrasekhara, 1980). The majority of the curcumin dose accumulated in the gastrointestinal tract. Thirty minutes after administration of curcumin, 90% could be accounted for in the stomach and small intestine. After seven hours, maximum levels were observed in the caecum and small intestine and only small amounts in the stomach and small intestine. Following administration of tritiated curcumin (approximately 40, 320 and 2000 mg/ kg), the levels of total radioactivity in blood, liver and kidney was found to be in the range of 9.6 – 14%, 5.1 – 6.9%, and 14.7 – 23.3% of the administered dose respectively (Ravindranath and Chandrasekhara, 1981; 1982). Distribution of curcumin has also been investigated in mice, which received an intraperitoneal dose (*ip)* of 100 mg/ kg. One hour after the *ip* dose, the concentration of curcumin in the gastrointestinal tract (117 μg/ g) was approximately

six times greater than in the liver and spleen. Only trace levels were detected in the plasma and brain (Pan *et al.*, 1999).

The poor bioavailability of curcumin may be a consequence of avid intestinal and/or hepatic metabolism. Intravenous administration (approximately 50 mg/ kg) of curcumin to rats resulted in the presence of glucuronide conjugates of tetrahydrocurcumin and hexahydrocurcumin in the bile (Holder *et al.*, 1978). Dihydroferulic acid and ferulic acid were also identified in the bile (Holder *et al.*, 1978), the latter being a degradation product of curcumin in phosphate buffer (Wang *et al.*, 1997). Oral administration of curcumin to rats (100 mg/ kg) also resulted in the presence of curcumin glucuronide and curcumin sulphate in plasma. In mice, following an *ip* dose of 100 mg/ kg and intragastric dose of 1000 mg/ kg, curcumin glucuronide and tetrahydrocurcumin glucuronide were identified in plasma (Pan *et al.*, 1999). Ireson and colleagues (2001a) have also demonstrated that the major biotransformation products of curcumin, identified in rat plasma after intravenous and intragastric administration of 40 mg/ kg and 500 mg/ kg respectively, are curcumin glucuronide and curcumin sulphate. Hexahydrocurucmin, hexahydrocurcuminol, hexahydrocurcumin glucuronide were present in rat plasma in trace amounts. In humans, Sharma and colleagues identified curcumin sulphate in the faeces of one patient that received 2200 mg of curcumin extract for 29 days (Sharma *et al.*, 2001c).

Studies of *in vitro* metabolism of curcumin suggest that curcumin undergoes rapid metabolism. Ireson *et al.*, (2002; 2001a; 2001b) have extensively studied the metabolism of curcumin *in vitro.* Curcumin glucuronide was identified in intestinal and hepatic microsomes, and curcumin sulphate, tetrahydrocurcumin, and hexahydrocurcumin were found in intestinal and hepatic cytosol from humans and rats (Ireson *et al.*, 2002). Curcumin sulphate was identified in incubations of curcumin with intact gut sacs (Ireson *et al.*, 2002). Curcumin was sulphated by human phenol sulphotransferase isozymes SULT 1A1 and SULT 1A3 (Ireson *et al.*, 2002). Curcumin incubated with suspensions of human rat hepatocytes resulted in the formation of hexahydrocurcumin and hexahydrocurcuminol (Ireson *et al.*, 2001a). This confirms previous studies by Wahlstrom and Blennow, (1978) who incubated curcumin in suspension of isolated hepatocytes or liver microsomes, which resulted in rapid

metabolic reduction with 90% of substrate disappearing from the media within 30 min. A summary of the metabolism of curcumin is shown in Figure 1.9.5.

It has been established that the main route of excretion is faecal. Curcumin was detected in the faeces of colorectal cancer patients that received up to 2200 mg curcumin per day for 29 days. Curcumin was not detected in urine (Sharma *et al.*, 2001c). Curcumin and its metabolites were undetected in the urine following administration of a single oral dose of 2000 mg/ kg curcumin (Ravindranath and Chandrasekhara, 1980) and tritiated curcumin (Ravindranath and Chandrasekhara, 1981-1982) to rats. When a dose of 1000 mg/ kg curcumin was administrated as an intragastric bolus to rats, only 0.001% of the dose was excreted in the bile (Walhstrom and Blenow, 1978). However, when a dose of 20 mg/ kg of tritiated curcumin was administered intravenously to rats, 85% of the radiolabelled compound was recovered in the bile within 3 h (Holder *et al.*, 1978). Forty percent of the cold curcumin was excreted in the faeces unchanged in 72 h and 33% of the radiolabelled compound was excreted within 72 h. After administration of higher doses of tritiated curcumin (40 or 320 mg/ kg) 80-90% of radiolabelled compound was excreted within 72 h (Ravindranath and Chandrasekhara, 1981; 1982). Holder and colleagues (1978) also found that 90% of an intravenous dose of 20 or 50 mg/ kg tritiated curcumin was excreted within 3 h.

1.10 Summary

Curcumin, the polyphenol derived from the herbal remedy and dietary spice turmeric, possesses wide-ranging anti-inflammatory and anti-cancer properties. Many of these activities are attributable to its potent antioxidant capacity at neutral and acidic pH, its scavenging of reactive oxygen and nitrogen species, its inhibition of cell signalling pathways at multiple levels and its diverse effects on cellular enzymes. However, the low oral bioavailability of curcumin in rodents and humans may limit its useful in the chemoprevention of malignancies remote from the gastrointestinal tract. After evaluation of the current information on curcumin and its pharmacokinetic and pharmacodynamic properties in animal models of colorectal cancer, a discrepancy in assessing the relationship between these pharmacological parameters has emerged. Administration of curcumin in the diet to Min/+ mice has been shown to decrease

tumour number and influence biological markers of neoplasia (Mahmoud *et al,* 2000; Collett *et al.,* 2001) but the pharmacokinetics of such a dosing regime are unknown. Without considering these in parallel, it is difficult to determine whether dietary administration of curcumin has any value in the clinical setting. This needs to be rectified and this anomaly will be addressed in this project.

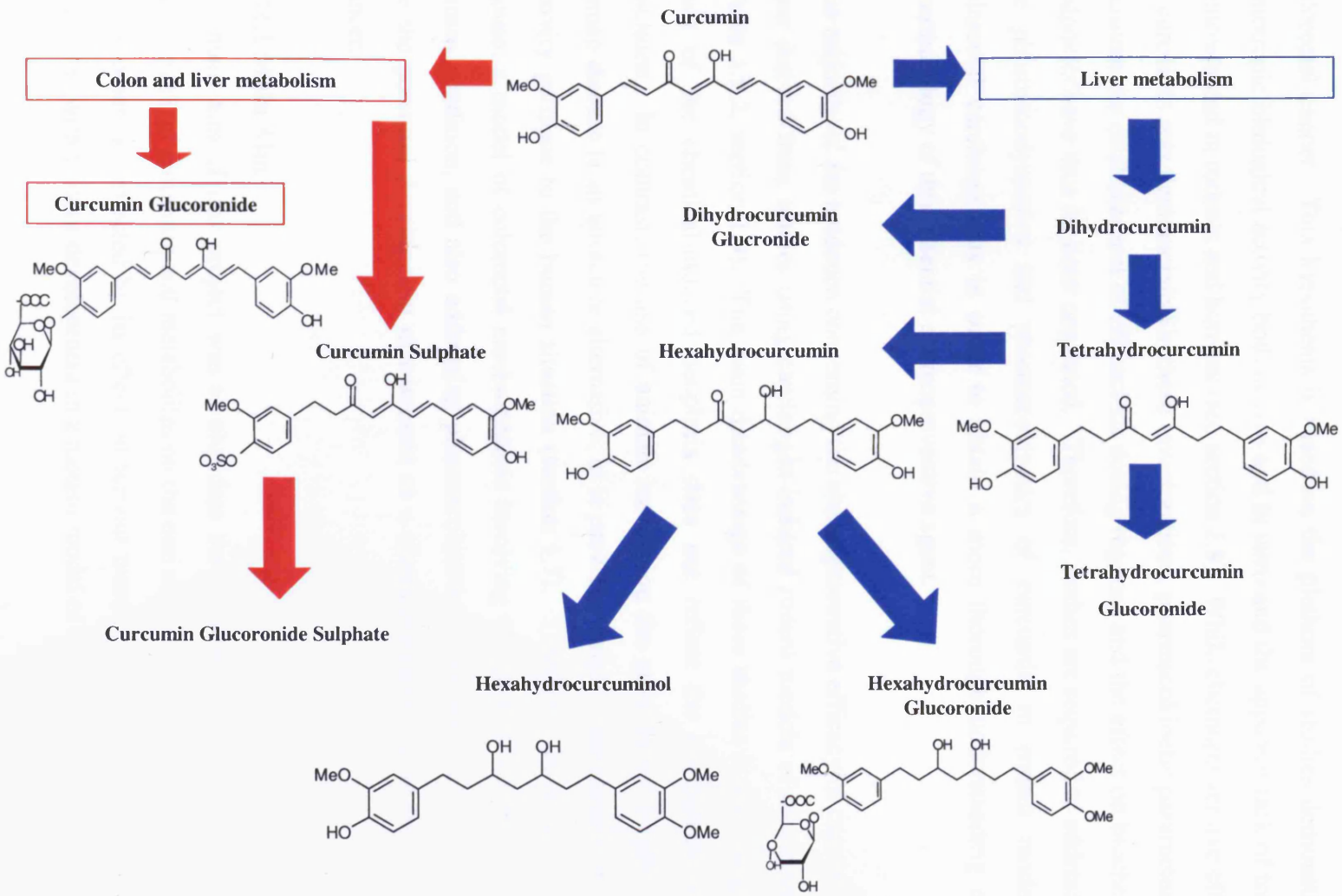

Figure 1.9.5 Metabolic pathways of curcumin

1.11. Aims

Curcumin is potentially a safe, inexpensive and efficacious agent for the prevention of colorectal cancer. This hypothesis is based on the plethora of studies demonstrating pleiotropic biological activity both *in vivo* and *in vitro* and the apparent lack of toxicity demonstrated in rodents and humans (see section 1.9). While chemopreventive efficacy of curcumin was demonstrated in these investigations, pharmacokinetic parameters that facilitate the establishment of efficacious dosing regimes and the effect on biochemical endpoints have thus far been neglected. Therefore, studies are required to address both the pharmacodynamics and pharmacokinetics of curcumin in rodent models of colorectal carcinogenesis in order to obtain a more thorough understanding of the pharmacology of this potential chemopreventive agent.

The majority of the evidence concerning the chemopreventive efficacy of curcumin has been derived from studies using carcinogen-induced rodent models of carcinogenesis (Table 1.9.2, section 1.9). The main disadvantage of these studies is that the genetic basis of the chemical-induced neoplasia does not reflect the comparable human condition. In contrast, the use of animals harbouring the gene defect pivotal to the human disease is an attractive alternative, as it provides insights into chemopreventive activity germane to the human situation (section 1.7). Therefore, utilising the Min/+ mouse, a model of colorectal carcinogenesis involving the gene defect central to the human condition, and also addressing pharmacokinetic issues was considered pivotal for the potential development of curcumin as a chemopreventive agent for colorectal cancer.

1.11.1 Main Aim

The main aim of this project was to elucidate the relationship between plasma and tissue levels of curcumin and metabolites on the one side and chemopreventive efficacy on the other, as reflected by its effect on tumour number and on biochemical events associated with tumour development in a mutant model of colorectal carcinogenesis.

1.11.2 Other objectives

i) Mahmoud *et al.*, (1999) demonstrated that curcumin (0.1%) could reduce tumour multiplicity in the Min/+ mouse by 64%. Curcumin (0.2%) has also been shown to reduce proximal adenomas in Min/+ mice treated with 2-amino-1-methyl-6-phenyl imidazo [4,5*b*]pyridine (PhIP) by 52% (Collett *et al.*, 2001) (section 1.9.3). However, neither the effect of varying the dietary level of curcumin on tumour burden nor a comprehensive study of the effect of curcumin on tumour size and distribution in the intestine has been investigated. Therefore, a primary objective of this project was to determine the effect of different doses of dietary curcumin on tumour burden, size and distribution in the Min/+ mouse.

ii) Varying the time period of dietary administration of NSAIDS has been shown to have differential effects on adenoma burden in Min/+ mice (Jacoby *et al.*, 2000) (chapter 3 section 3.1). Furthermore, *in utero* administration of piroxicam was embryotoxic to Min/+ progeny, favouring the birth of non-tumour bearing wildtype offspring (Jacoby *et al.*, 2000) (section 3.1). In order to determine the effect of dietary curcumin on the different stages of colorectal carcinogenesis, the agent was administered at different time intervals and the efficacy of these dosing regimes was measured. Furthermore, curcumin was administered *in utero* in order to explore its effect - if any - on the expected Mendelian ratio of tumour bearing (Min/+) and non-tumour bearing (wildtype) offspring.

iii) Another objective of the project was to determine the safety of curcumin as a potential chemopreventive agent. Thus the weight and well being of Min/+ mice that had received curcumin in the diet was assessed. Furthermore, post-mortem analysis was used to reveal any abnormal pathology associated with the ingestion of this phytochemical.

iv) A further aim of this work was to study, in a preliminary fashion, the effect of a combination of curcumin with another chemopreventive agent in the Min/+ mouse. Conflicting data exists concerning the chemopreventive efficacy of aspirin in this model (chapter 3, section 3.1). According to an as yet untested hypothesis (Sansom *et al*, 2001) aspirin may be efficacious if administered *in utero,* during the initiation stage of colorectal carcinogenesis. In order to explore this idea further, effects of combined pre-

weaning administration of aspirin with subsequent administration of curcumin on tumour burden was studied in the Min/+ mouse. Furthermore, it was hypothesised that such a combination with curcumin may ameliorate the potentially limiting side effects of aspirin administration, such as gastric erosion and loss of mucosal integrity. Effects of *in utero* administration of aspirin alone on Min/+ mouse embryotoxicity and on growth and survival of Min/+ mice were also determined.

v) Further experiments were designed in order to evaluate potential indicators of carcinogenesis that may be affected by curcumin (section 1.9.4). The aims of these studies were two-fold. The first was to find out about levels of COX-2, MDA, M_1G and 8-oxo-dG in adenomatous versus normal Min/+ mouse intestinal tissue, and the second to determine the effect of curcumin on these indicators. It was considered that this information might aid in the identification of possible biomarkers of putative chemopreventive agents in clinical trials. In order to identify leads to possible novel biomarkers and new mechanistic information regarding curcumin, RNA from intestinal tissue of Min/+ mice, background strain mice and curcumin-treated animals were subjected to gene expression analyses by cDNA microarray.

vi) Finally, in accordance with the main aim of this project, a pharmacokinetic assessment of dietary administration of curcumin was performed. Studies to determine levels of agent and metabolites in plasma and many tissues including the target tissue and the rate of their disappearance were used to facilitate the optimisation of dosing regimes in humans. This information might also provide vital data on the bioavailability of curcumin in neoplasias remote from the gastrointestinal tract. Furthermore, identification of putative metabolites of curcumin in the target tissue was considered to indicate a potential contribution by these species to the chemopreventive efficacy of dietary curcumin.

CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

2.1.1 General chemicals and kits

All chemicals and reagents were purchased from Sigma-Aldrich Company Ltd. (Poole, UK) and all solvents from Fischer Scientific, (Loughborough, UK) unless other wise stated.

2.1.2 Animals

The C57BL/6J Min/+ colony was established from male C57BL/6J Min/+ mice and C57BL/6J +/+ mice purchased from Jackson Laboratories (Bar Harbor, Maine, USA). Subsequent female breeders were purchased from Charles River (Margate, UK). Mice used in the pharmacokinetic studies were purchased from Charles River. Terminal anaesthesia was induced *via* inhalation of halothane (3% induction; 1.5% maintenance, in $1m^3$/ min nitric oxide: 0.5 m^3/ min oxygen) (Astra Zeneca, Macclesfield, UK).

2.1.3 Animal diets

RM1 (pellets) and RM3 (fine ground) mouse diets were purchased from Special Dietary Services (SDS, Witham, UK) and the AIN 76A (fine ground) diet from PMI Nutrition (Nottingham, UK). These were supplemented with aspirin or curcumin supplied by Sigma-Aldrich and Apin Chemicals (Abingdon, UK) respectively and prepared by Biomedical Services, MRC Toxicology Unit, University of Leicester.

2.1.4 Determination of Min/+ Genotype

*Hind*III Restriction Enzyme plus SuRE/Cut Buffer B, was purchased from Roche Diagnostics (Lewes, East Sussex, UK) and the Reddy Load PCR Mix from Advanced Biotechnologies (Epsom, UK). Protein and Nucleic Acid Chemistry Laboratory (University of Leicester) prepared the forward and reverse primers. Gibco (Paisley, UK) supplied the 100bp ladder and Bio-Rad Laboratories (Hemel Hempstead, UK) supplied the bromophenol blue.

2.1.5 Histology: tissue processing

The wax (Difco Polywax) was purchased from Difco Laboratories Ltd (Surrey, UK)

2.1.6 Immunohistochemistry studies

Antibodies against COX-2, goat polyclonal IgG and Bcl-2, rat monoclonal IgG, and biotinylated mouse anti rat IgG were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, USA). β-Catenin, mouse polyclonal IgG, was obtained from Transduction Laboratories (Exeter, UK). The biotinylated mouse anti goat IgG and biotinylated rabbit anti-mouse (whole molecule) horseradish peroxidase (HRP) conjugates were obtained from Dako Laboratories (Ely, UK). The ABC complex and diaminobenzidine (DAB) were purchased from Vector Laboratories (Burlingame, CA, USA) and Dako Laboratories respectively. BDH Laboratories (Poole, UK) supplied DPX mountant.

2.1.7 Western blotting studies

Acrylamide (30% acrylamide: bisacrylamide) was purchased from Anachem Ltd. (Luton, UK). The ECL reagents, hyperfilm and hybond nitrocellulose were supplied by Amersham Pharmacia Biotech Ltd. (Little Chalfont, UK). Antibody against COX-2, goat polyclonal IgG was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). α-Tubulin mouse monoclonal antibody was supplied by Oncogene Research Products (Nottingham, UK). β-Catenin, mouse polyclonal IgG, was obtained from Transduction Laboratories (Exeter, UK). Goat anti-murine and donkey anti-goat horseradish peroxidase antibodies were purchased from Sigma-Aldrich (Poole, UK) and Santa Cruz Biotechnology Inc., respectively. Pre-stained broad range SDS-Page standards were purchased from Bio-Rad Labs (Hemel Hempstead, UK).

2.1.8 Bradford protein assay

Protein assay reagent was purchased from Bio-Rad laboratories (Hemel Hempstead, UK).

2.1.9 Isolation of DNA from mouse tissue

Genomic DNA extraction was performed using reagents and columns obtained from Qiagen (Hilden, Germany).

2.1.10 Determination of MDA and M_1G levels

MDA levels were assessed by the colorimetric Lipid Peroxidation Assay Kit (Calbiochem Corp., San Diego, CA, USA). Murine M_1G monoclonal antibody D10A1 was prepared as previously described (Sevilla *et al.*, 1997) and obtained from Prof. L.J. Marnett, Vanderbilt University, USA. Anti-murine horseradish peroxidase antibody was purchased from Dako (Ely, UK). M_1G standards and NaMDA, the sodium salt of monomeric MDA, were synthesised and characterised by Dr Raj Singh of the University of Leicester, as previously described (Sheng *et al.*, 1997; Chapeau *et al.*, 1991).

2.1.11 Determination of 8-oxo-7, 8-dihydro-2'-deoxyguanosine

Shrimp alkaline phosphatase was purchased from Amersham Biotech. Ltd. (Little Chalfont, Bucks., UK). The centrifugal filter device (MWt. cut off 3000, YM-3) was purchased from Millipore Corp. (Bedford, MA, USA). Dr Raj Singh of the University of Leicester prepared the immunoaffinity column.

2.1.12 Determination of haematocrit

Heparinised 75mm microhaematocrit tubes were purchased from Richardson's of Leicester, (Leicester, UK).

2.1.13.1 cDNA microarray studies

Cy3-dUTP, Cy5-dUTP, and low T dNTP's were purchased from Amersham Pharmacia Biotech Ltd. (Little Chalfont, UK). Superscript II reverse transcriptase, 5X superscript buffer, 0.1M DTT, tRNA and mouse COT DNA were purchased from Gibco (Paisley, UK). RNAsin RNAse was obtained from Promega (Southampton, UK), and microcon columns were purchased from Millipore Corp. (Bedford, MA, USA).

2.1.13.2 Mouse EST clones and printing of the arrays

The arrays comprised 4,246 mouse sequence tag (EST) clones (2, 783 individual Genebank clusters). Two-thirds of the clones were obtained from the IMAGE collections held at the MRC Human Gene Mapping Project (http://www.hgmp.mrc.ac.uk/). The remaining one-third of the EST clones were obtained from Research Genetics (RG9 set, http://www.resgen.com). All clones described in this thesis were verified by sequence analysis. CDNA from the EST was obtained via PCR amplification using plasmid-specific primers. The PCR products were all separated by electrophoresis on agarose gels to make sure that only single product was obtained for each clone. The reactions products were precipitated and prepared for array, using methods described (DeRisi *et al.*, 1997; Eisen and Brown, 1999). Arrays were printed on poly-*L*-lysine-coated slides, UV-crosslinked and blocked prior to use (DeRisi *et al.*, 1997; Eisen and Brown, 1999; Turton *et al*, 2001). The arrays were printed using an arrayer built essentially according to the Standford designs (*cf.* http://www.le.ac.uk/cmht/microarray_lab/Home.htm). This was carried out by members of the Bioinformatics Group, MRC Toxicology Unit, University of Leicester, UK. The centre-to-centre distance of the features was 210μm, and eacxh feature was 90-100μm in diameter.

2.1.14 Pharmacokinetic studies

[^{14}C] Radiolabelled curcumin was a gift from Michael Threadgill, University of Bath. The synthesis of the radio labelled curcumin was performed as previously described (Figure 2.1: Parveen and Threadgill, 2000). The chemical yield (determined by HPLC) and radiochemical yield (determined by HPLC fraction collection followed by scintillation counting) were 81% respectively and the specific activity was 12.7MBq/mMol (Parveen and Threadgill, 2000). OptiSolve and Scintillation Fluid were obtained from Wallac Scintillation Products (Milton Keynes, UK). Prof. Chang-kee Lim of University of Leicester supplied the internal standard, 5,10,15,20-tetra-(m-hydrophenyl)-chlorine (M-THPC).

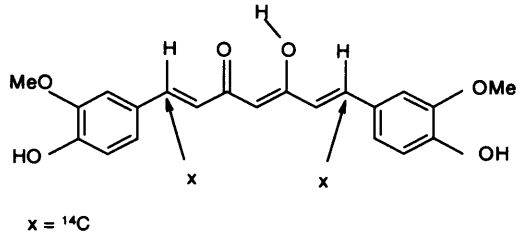

Figure 2.1. Structure of [^{14}C] curcumin

2.2 Methods

2.2.1 Maintenance of C57BL/6J Min/+ mouse colony

Mouse husbandry, handling and dissection were performed under the Project Licence 80/1250 granted to the MRC Toxicology unit by the UK Home Office. University of Leicester Ethical Committee for Animal Experimentation approved all animal studies. C57BL/6J Min/+ mice colony was established from 10 male C57BL/6J Min/+ mice and 30 female C57BL/6J +/+ mice. The colony was maintained on a C57BL/6J background whereby Min/+ males were mated with +/+ C57BL/6J females. The animals were fed on a standard diet, RM1 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. Ear punching at 3 weeks of age permitted identification and the superfluous tissue was retained for genotyping. Biomedical Services, University of Leicester, performed general maintenance of the C57BL/6J Min/+ mice colony.

2.2.2. Determination of Min/+ genotype

The presence of the mutant allele was detected in DNA extracted from ear punch tissue obtained during cage identification of the mice at weaning using an allele-specific polymerase chain reaction (PCR) assay. Ear punch tissue was digested for 5 h at 56 °C in 200 µl PBND buffer (50 mM KCl, 10 mM Tris-HCl, 0.1 mg/ ml gelatin, 0.45% (v/ v) IPEGAL CA 630, 0.45% (v/ v) Tween 20) and 40 µg of proteinase K. After digestion, the enzyme was inactivated by exposure to 95 °C for 15 min. PCR cocktail contained DNA lysate (5µl), Reddy load PCR mix (41 µl) and 10 pmol of both the forward and reverse primers.

Forward primer: 5'-TCTCGTTCTGAGAAAGACAGAAGCT-3'

Reverse primer: 5'-TGATACTTCTTCCAAAGCTTTGGCTAT-3'.

The PCR reaction was performed using Biometra Trio-thermablock (Biometra Inc., Maidstone, UK). PCR conditions were optimised and described below. PCR sample (17 µl) was incubated at 37 °C for 3 h with 40 U of *Hind*III and SuRE/ Cut Buffer B (2 µl). The sample and DNA ladder was loaded onto a 3% agarose gel and run at 110V for 1-2 h. The gel was prepared using 1x tris borate ethylenediaminetetraacetic acid pH 8 (TBE) containing ethidium bromide (0.004%). The DNA ladder was prepared using 100bp ladder (2 µl), distilled water (7 µl) and 10x loading buffer (0.01M EDTA, 0.5% (w/v) sucrose and 0.01% bromophenol blue). The gel was then visualised using a dual intensity UV transilluminator and a Polaroid photograph taken.

STEP	TEMPERATURE (°C)	TIME (min)	CYCLES
1	94	2	1
2	94	1	40
3	60	1	
4	72	1	
5	72	10	1

2.2.3 Curcumin administration regimes in C57BL/6J Min/+ Mice

Min/+ offspring from batch mates were assigned randomly to each study group. The method used encouraged balanced recruitment to all of the treatment sets over time and diminished the chance that treatment effects would be compounded with potential time effects (Jacoby *et al.* 2000). This method increases the likelihood that experiments performed at different times would be comparable, making it possible to compare the magnitude of drug effects in different studies. This is observed when comparing the tumour multiplicity and size in control groups throughout the studies described below.

Powdered RM3 diet was used in all studies unless otherwise stated (see appendix 1 for diets used). RM3 is a high nutrient diet that promotes excellent breeding performance and growth rates. RM3 was used in the studies as it mimics a "Western" style diet that is high in protein. Curcumin was mixed mechanically in the RM3 to ensure uniform distribution. Curcumin concentration in the diet was confirmed using HPLC analysis

using the extraction method described in section 2.2.17.1 and HPLC method described in section 2.2.18. Curcumin diets were replaced in the cages weekly and stored at 4 °C in the dark.

2.2.3.1 Effect of lifetime administration of dietary curcumin on tumour formation in C57BL/6J Min/+ mice

Breeding pairs and offspring were maintained throughout on RM3 diet. Offspring were weaned at 3 weeks and genotyped to determine APC^{MIN} (Min/+) status. At 4 weeks Min/+ littermates were divided following a randomised block design, into control (RM3 diet only) or treatment groups, which constituted curcumin at 0.1%, 0.2% or 0.5% mixed in with RM3 diet. These dose levels represent respectively a dietary intake of approximately 150 mg/ kg per day (*pd*), 300 mg/ kg *pd* or 750 mg/ kg *pd* curcumin. The curcumin was mixed into the high protein RM3 diet mechanically to ensure a uniform feed and curcumin intake. Each group contained 10-12 animals. The animals were continued on their respective diets until 18 weeks of age when the animals were culled by cardiac exsanguination under terminal halothane anaesthesia. Blood samples were taken and intestinal tumours were scored as described below.

2.2.3.2 Effect of short-term administration of dietary curcumin on tumour formation in C57BL/6J Min/+ mice

Breeding pairs were established and fed RM3 diet or RM3 containing 0.2% curcumin (300 mg/ kg *pd*) for 2 weeks or until pregnancy was established. Two weeks after birth, the mothers were removed and the offspring continued on their respective diets until they were 30 days old, during which time the offspring were genotyped. At 30 days of age, the Min/+ mice were divided into 3 treatment groups: 1) Min/+ mice who received 0.2% curcumin perinatally and during days 1 - 30; 2) Min/+ mice fed 0.2% curcumin from day 30 - 75 and 3) Min/+ mice fed 0.2% curcumin from day 75 -120. The animals received a standard powdered RM3 diet when the experimental curcumin diet was stopped. At 120 days the animals were culled by cardiac exsanguination under terminal halothane anaesthesia. Each group contained 10-14 animals. Blood samples were taken and intestinal tumours were scored as described below.

2.2.3.3 Effect of a combination of curcumin and aspirin on tumour formation in C57BL/6J Min/+ mice

Breeding pairs were established and fed RM3 diet or RM3 containing 0.05% aspirin (75 mg/ kg *pd*) for 2 weeks or until pregnancy was established. Two weeks after birth, the mothers were removed and the offspring continued on their respective diets until they were 30 days old, during which time the offspring were genotyped. At 30 days the Min/+ offspring were divided into 3 treatment groups: 1) Min/+ offspring who received 0.05% aspirin in RM3 diet perinatally and during days 1-30; 2) Min/+ mice fed 0.05% aspirin perinatally and during days 1-30 followed by RM3 diet containing 0.2% curcumin during days 30-120 and 3) Min/+ animals fed RM3 control diet throughout. The animals received a standard powdered RM3 diet when the aspirin diet was suspended. At 120 days the animals were culled by cardiac exsanguination under terminal halothane anaesthesia. Each group contained 8-10 animals. Blood samples were taken and intestinal tumours were scored as described below.

2.2.3.4 Effect of different diet on tumour formation in C57BL/6J Min/+ mice

Breeding pairs and offspring were maintained throughout on RM3 diet. Offspring were weaned at 3 weeks and genotyped to determine APC^{MIN} (Min/+) status. At 4 weeks Min/+ littermates were divided following a randomised block design into standard powdered RM3 diet or standard powdered AIN 76A diet. The AIN 76A diet is a lower nutrient diet (see appendix 1 for diet composition). Each group contained 11 - 16 animals. Mice were continued on their respective diets until 18 weeks of age when they were culled by cardiac exsanguination under terminal halothane anaesthesia. Blood samples were taken and intestinal tumours were scored as described below.

2.2.3.5 Effect of curcumin on markers of chemoprevention in C57BL/6J Min/+ mice

This study was performed as for 2.2.3.1 whereby male Min/+ mice (n = 4-8) were fed a 0.2% curcumin for the 8-oxo-7,8-dihydro-2'-deoxyguanosine study, or 0.1% and 0.2% curcumin diet for the MDA and M_1G studies, in RM3 diet from 4 to 18 weeks of age. Control animals were fed RM3 diet from 4-18 weeks of age. Micro dissection of adenomas and intestinal scrapings were performed as described in section 2.2.9.

2.2.3.6 Effect of curcumin on gene expression in C57BL/6J +/+ (wildtype) and C57BL/6J Min/+ mice.

Treatment groups (n = 4) were established, all genotyped at 3 weeks of age as described in section 2.2.2. The mice were segregated so that 4 female mice from identical parents were separated into each group and this was repeated until n = 4 for each group. Groups 1 and 2 were fed RM3 diet from 4-9 weeks of age and consisted of C57BL/6J +/+ and C57BL/6J Min/+ female mice respectively. Group 3 consisted of C57BL/6J Min/+ female mice fed RM3 diet containing 0.2% curcumin and group 4 consisted of C57BL/6J +/+ female mice fed an identical curcumin diet. At 9 weeks of age the animals were culled and the small intestine removed and frozen in liquid nitrogen. Gene expression patterns were then identified by cDNA microarray as described in section 2.2.16.

2.2.4 Tumour enumeration

Mice were killed under licenced procedure by cardiac exsanguination under terminal halothane anaesthesia. Necropsy was performed and the entire gastrointestinal tract was removed for dissection. Approximately 10 ml of phosphate buffered saline (PBS; 0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4) was flushed through the gut using a syringe inserted into the anus to remove intestinal contents. The tissue was placed on blotting paper and opened longitudinally out flat using iris scissors and then rinsed with PBS to remove any residual intestinal contents. The stomach and the caecum were omitted from the analysis due to their low tumour incidence. The small intestine and colon were fixed flat in methacarn (60% (v/ v) methanol, 30% (v/ v) chloroform and 10% (v/ v) acetic acid) for 2-4 h. The tissue was then examined under 3x magnification. The small intestine was divided visually into three segments of approximately equal length (proximal, middle and distal) and the large intestine was left uninterrupted. Tumour number, location, and diameter within these four regions were recorded. Tumour diameter was classified as <1 mm, 1 mm – 3 mm, >3 mm.

2.2.5 Preparation of 3-aminopropyltriethoxy silane (APES) coated slides

The slides were washed overnight in 5% TEEPOL liquid detergent or equivalent and then rinsed in running hot tap water (approximately 45 °C) for 1 h. After rinsing in distilled water, the slides were rinsed in 99% IMS and dried in an oven (37 °C) for approximately 30 min. The slides were then exposed to a series of solutions (as described below) and left to dry in an oven (37 °C) for approximately 30 min. Once dry the slides were ready for histology use. Slides are coated with apes to retain tissue sections on the slides during immunohistochemistry.

1) 2% APES in 99% IMS (1 – 2 min)
2) 99% IMS (Bath 1) (1 - 2 min)
3) 99% IMS (Bath 2) (1 – 2 min)
4) 100% Acetone (1 - 2 min)

} Performed in a fume cupboard

2.2.6 Tissue processing

After tumour scoring, the small intestine and colon were rolled from the proximal to distal ends pinned "in a Swiss roll" using a 25 G x 1" hypodermic needle and placed in 95% IMS prior to embedding in paraffin. Sections were taken from the middle portion of the "Swiss roll" preparations and placed on APES coated slides as described below. Jenny Edwards (Department of Biochemical Mechanisms, MRC Toxicology Unit, Leicester) process the sections using a Shandon Citadel 2000 processor. Sections were cut to a thickness of 5 microns and the dehydrated and stained as described in section 2.2.7 or 2.2.8. The following table describes the tissue processing schedule for embedding the tissue in wax prior to sectioning.

Container	Reagent	Time (Hrs)
1	70% IMS	2
2	90% IMS	2
3	100% IMS	2
4	100% IMS	2
5	100% IMS	2
6	100% IMS	1
7	100% IMS	1
8	Chloroform	2
9	Chloroform	2
10	Chloroform	2
11	Wax	2
12	Wax	2

2.2.7 Haematoxylin and eosin (H&E) staining of intestinal sections

Sections were stained with haematoxylin and eosin for evaluation of the mucosal histology. All haematoxylin and eosin staining was performed on Shandon Varistain 24-4 Staining Machine (Shandon Inc, Pittsburgh, Pennsylvania, USA). The following table describes the processing schedule for H & E staining of the intestinal sections.

Container	Reagent	Time (min)
1	Xylene	2
2	Xylene	3
3	IMS	1
4	IMS	1
5	70% IMS	1
6	Water	1
7	Hematoxylin	15
8	Water	1
9	1% Acid Water	0.25
10	Water	6
11	Water	1
12	Water	2
13	1% Aqueous Eosin	3
14	Water	2
15	70% IMS	1
16	IMS	1
17	IMS	1
18	IMS	1
19	IMS	1
20	Xylene	2
21	Xylene	5

2.2.8 Immunohistochemistry for the detection of COX-2, β-catenin and Bcl-2 protein in the small intestine of C57BL/6J Min/+ mice

Sections were taken from the middle portion of the "Swiss roll" preparations and placed on APES coated slides as previously described. To prepare sections for immunohistochemistry, the paraffin was removed by soaking the sections in histoclear for 5 min followed by rehydration with periodic agitation through a graded alcohol series using the following protocol.

Solvent	Time (minutes)
Histoclear	2
99% IMS	2
99% IMS	2
95% IMS	2

The sections were then washed in running tap water for 5 minutes and rinsed in distilled water. Antigen retrieval was achieved by microwaving at 700 W for 3x 5 min in 10 mM citrate buffer, pH 6. After heating the slides were cooled for 15 min prior to rinsing twice in distilled water and once in tris buffered saline (TBS; 5 mM Tris, 15 mM NaCl) for 5 min. Incubating the slides with 2% H_2O_2 in distilled water for 30 min blocked endogenous peroxidase activity. The slides were then washed in TBS containing 0.1% bovine serum albumin (BSA). Non-specific staining was blocked with normal rabbit serum, 1: 5 in TBS (100 µl per slide) for 10 min. The test sections were then incubated with an appropriately diluted antibody in 20% normal serum (100 µl per slide). Normal serum was left on the negative control sections.

Dilutions:

Antibody	Dilution
Goat polyclonal antibody against COX-2	1: 50
Mouse polyclonal against β-catenin	1: 50
Rat monoclonal against Bcl-2	1: 50

The dilution was determined by an optimisation study using a dose range of 1: 20 to 1: 400. Incubation was for 1 h at room temperature on a rocking platform, or overnight at 4 °C. After the TBS containing 0.1% BSA wash, the slides were incubated with an appropriately diluted secondary antibody in TBS for 30 min on a rocker platform at room temperature.

Dilutions:

Antibody	Dilution
Biotinylated mouse anti-goat horseradish peroxidase	1: 400
Biotinylated rabbit anti-mouse horseradish peroxidase	1: 400
Biotinylated mouse anti-rat horseradish peroxidase	1: 400

An ABC complex containing (0.001%, v/ v) streptavidin, (0.001%, v/ v) biotinylated horseradish peroxidase in TBS was allowed to equilibrate for 30 min. The streptavidin binds to the biotinylated secondary antibody, amplifying the staining. After a TBS wash containing 0.1% BSA, the ABC solution (100 µl) was applied to each section and incubated for a further 30 min. Visualisation by diaminobenzidine (DAB) and counterstaining with haematoxylin was then performed. DAB was prepared according to manufacturer's instructions and applied to each section (100 µl) for 5 min. Sections were rinsed in TBS and then washed for 5 min in running tap water. The slides were counterstained with haematoxylin for 15 sec. The sections were then dehydrated through graded alcohols to histoclear prior to mounting (reverse of above). The slides were transferred to fresh histoclear and mounted in a resinous mountant using appropriately sized cover slips.

2.2.9 Microdissection of intestinal adenomas and mucosa from C57BL/6J Min/+ mice

Male Min/+ mice (n = 8) were culled under licence at 17 weeks of age. The entire gastrointestinal tract was removed for dissection. Approximately 10 ml of phosphate buffer saline (PBS) was flushed through the gut to remove intestinal contents. The tissue was placed on blotting paper and opened longitudinally using iris scissors and then washed extensively with PBS. Intestinal adenomas were identified under 3x magnification and collected by micro dissection using iris scissors. Proximal small bowel and colon epithelial mucosa were collected by gently brushing the epithelial layers with a metal spatula.

2.2.10 Western blotting: detection of COX-2 protein

Male Min/+ mice (n = 4) were culled under licence at 17 weeks of age. Isolation and micro dissection of intestinal adenomas and epithelial scrapings were performed as described in section 2.2.9. The samples were frozen in dry ice and stored at –80 °C. Buffers and gels for western blotting are described below.

Lysis Buffer

NaCl	0.15 M
Tris pH 8	1%
Tween 20	1%
Diethyldithiocarbamic acid	50 mM
Ethylenediaminetetraacetic acid pH 8	1 mM
Complete™ (protease inhibitors)	1 x

Polyacrylamide Running Gel

Acrylamide (30% acrylamide: 0.78% bisacrylamide)	33%
5X Resolving buffer (2.1 M tris pH 8.8)	5%
SDS (sodium dodecyl sulphate)	0.1%
Ammonium persulphate	0.08%
TEMED	0.0004%.

The components were added sequentially (TEMED last), mixed thoroughly and poured into Bio-Rad labs gel-casters. The gel was overlaid with IMS and left to set.

Polyacrylamide Stacking Gel

Acrylamide (30% acrylamide: 0.78% bisacrylamide)	12.5%
5X stacking buffer (1.5 M tris pH 6.8)	5%
SDS	0.1%
Ammonium persulphate	0.08%
TEMED	0.0004%.

The components were added sequentially (TEMED last), mixed thoroughly and poured over the running gel, a comb inserted and left to set.

SDS loading Buffer

5X stacking gel buffer	0.125 M
SDS	4%
Glycerol	20%
Bromophenol blue	0.02%
Dithiothreitol	0.2 M

The samples (~50 mg) were lysed in 300 µl of lysis buffer vortexed and left on ice for 20 min prior to sonication (3x 10 sec, every 20sec). After centrifugation (10,000 g, 5 min), 10µl of the supernatant was diluted 1:5000 for Bradford protein assay (see section 2.2.10.2) or stored at – 80 °C. The samples (200 µg of protein, ~10 µl) were added to and equal volume of loading buffer and heated to 100 °C for 5 min. Samples were then run on 10% SDS Page Maxigel at 100 mV (see above for preparation of polyacrylamide running and stacking gels) with prestained broad range standards and an appropriate marker at 100 mV. The reservoir buffer was prepared using 0.025 M Tris, 0.192 M glycine, 0.1% SDS) The proteins were transferred onto a nitrocellulose membrane at 30 mV overnight in transfer buffer (48 mM Tris, 39 mM glycine, 20% (v/ v) methanol, 0.037% SDS) using a semi-dry transfer apparatus (Bio-Rad Labs). Following transfer, the membranes were blocked in 10% non-fat milk in tris-buffered saline tween (TBST) for 2 h on a shaking platform. After 2x 5 min TBST washes, the membranes were incubated at room temperature for 2 h with primary Cox-2 goat polyclonal antibody at a dilution of 1: 500 in TBST. After 5x 5 min TBST washes, the membranes were incubated at room temperature for 1 h with a HRP donkey anti-goat secondary antibody diluted to 1: 2000 in 5% non-fat milk: TBST solution. The antibody was removed with 5x 5 min TBST washes and the immunoblots were then developed using enhanced chemiluminescence and were exposed to hyperfilm for an appropriate time and then developed using a hyperprocessor.

2.2.10.1 Detection of α-tubulin protein

The COX-2 immunoblots described above were then stripped and re-probed for α-tubulin. This housekeeping protein was selected as an indicator of protein levels loaded on to the gel since its intracellular regulation is considered independent of COX-2 expression. Stripping of the blots required heating at 60 °C in 50 ml of stripping buffer (2% SDS, 7.56% (w/ v) Tris, 0.8% β-mercaptoethanol) for 45 min. After 2x 5 min TBST washes, the immunoblots were reblocked in 10% non-fat milk in TBST overnight on a rocking platform at 4 °C. After 2x 5 min TBST washes, the membranes were incubated at room temperature for 2 h with primary α-tubulin mouse monoclonal antibody at a dilution of 1: 500 in TBST. After 5x 5min TBST washes, the membranes were reincubated at room temperature for 1 h with a HRP rabbit anti-mouse secondary

antibody diluted to 1:2000 in 5% non-fat milk: TBST solution. The antibody was removed with 5x 5 min TBST washes and the immunoblots were then developed using enhanced chemiluminescence (ECL reagents 1ml/ blot for 1 min) and were exposed to hyperfilm for an appropriate time and then developed using a hyperprocessor.

2.2.10.2 Determination of total protein (Bradford protein assay)

Based on the observation that the A_{MAX} for an acidic solution of Coomassie Brilliant Blue G250 shifts from 465 nm to 595 nm when binding to protein occurs, the protein levels of the intestinal epithelial scrapings and adenoma samples were determined. Bovine serum albumin (BSA) standards were prepared in distilled water at a concentration range of 0-20 µg/ml protein. The samples were diluted 1: 5000 in distilled water in duplicate. The standards and samples (800 µl) were placed in a cuvette and 200 µl of protein assay reagent was added to each and left to equilibrate for 10 min. The samples were then analysed at a wavelength of 595 nm and the protein concentration was determined enabling the normalisation of the protein concentration of the intestinal epithelial scrapings and adenoma samples for western blotting.

2.2.11 Procedure for isolation of DNA from intestinal adenomas and mucosa from C57BL/6J Min/+ mice

The samples were weighed and approximately 95 mg of each sample was homogenised using a hand-held glass-Teflon homogeniser on ice in 9.5 ml of G2 digestion buffer (Qiagen kit buffers). The homogenate was transferred to a 15 ml polypropylene tube where 10 mg Proteinase K (0.5 ml of 20 mg/ ml solution) and 0.45 mg RNAse A (15 µl of 30 mg/ ml solution in glycerol) was added and vortexed. The sample was incubated at 45 °C for 2 h. Qiagen genomic-tip (midi tip, 100/g) was equilibrated with buffer QBT (4 ml) and allowed to empty by gravity flow. The sample was vortexed (30 sec), applied to the genomic tips and allowed to enter the column by gravity flow. The Qiagen genomic tip was washed with 2x 7.5 ml washed using wash buffer QC prior to elution of the DNA with elution buffer QF (5 ml; 45 °C) into 50 ml polypropylene tube. Ice-cold propanol (3.5 ml) was added and the tubes were left overnight at –20 °C. After centrifugation at 5000 g at 4 °C for 25 min, the DNA was transferred to a 1.5 ml

Starstedt tube using a small spatula. HPLC grade water (100 µl) was added and the sample centrifuged at 12,000x g for 2 min, vortexed (5 sec) and left to dissolve on a rotating table. After measuring the DNA concentration the sample was aliquoted at 50 µg and stored under nitrogen at – 80 °C. An aliquot (5 µl) was removed and diluted with 100µl HPLC grade water. The UV absorbance was determined at 260 nm and 280 nm (Kontron Uvikon 860 spectrophotometer). The concentration is calculated by assuming that A_{260nm} = 20 is equivalent to 1 mg/ ml for double stranded DNA. The purity of DNA was assessed spectrophotometrically using the 260/ 280 nm ratio (range 1.6 to 1.9). The absorbance of a DNA sample at 280 nm gives an estimate of the protein contamination of the sample. The ratio of the absorbance at 260 nm/ absorbance at 280 nm is a measure of the purity of a DNA sample.

2.2.12 Immunoslotblot assay for the determination of malondialdehyde-deoxyguanosine (M_1G) adduct formation

Male Min/+ mice (n = 8) were culled under licence. Isolation and microdissection of intestinal adenomas and epithelial scrapings was performed as described in section 2.2.9. The samples were frozen in dry ice. The whole livers were removed and snap frozen in liquid nitrogen. Extraction of genomic DNA was performed as described in section 2.2.11. Analysis of M_1G adduct levels by immunoslot blot was performed with assistance from Ricky Sharma, Department of Oncology, University of Leicester, as previously described (Leuratti *et al.*, 1998). M_1G levels were analysed by immunoslot blot, using 1 µg of each sample/standard DNA in triplicate. Each blot consisted of 9 standards and 15 samples, all in triplicate. Discrepancies in the amount of DNA in each slot were corrected for by staining the nitrocellulose filter with propidium iodide and performing UV light densitometry. The detection limit for M_1G was 5 adducts per 10^8 nucleotides.

2.2.13 Colourimetric assay detection of malondialdehyde (MDA) levels

Male Min/+ mice (n = 8) were culled under licence. Microdissection of intestinal adenomas and epithelial mucosa were performed as described in section 2.2.9. MDA levels of fresh tissue homogenates were analysed immediately by colourimetric assay, as previously described (Erdelmeier *et al.*, 1998) with assistance from Ricky Sharma,

Department of Oncology, University of Leicester. The method principally measures the free MDA in homogenised tissue, without detecting base propenals (Erdelmeier *et al.*, 1998). Genomic DNA extraction was performed without addition of antioxidants, since they may give rise to artefactual formation of adducts (Chaudhary *et al.*, 1994). The detection limit was approximately 0.1nmol/mg protein, and results were corrected for protein levels.

2.2.14 Detection of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG) adducts

Male Min/+ mice (n=4) were culled under licence at 17 weeks of age. Intestinal adenomas and mucosa were isolated as described in section 2.2.9 and DNA was extracted essentially as described in section 2.2.11, with the addition of 5 mM deferoxamine mesylate to the G2 buffer. Deferoxamine mesylate is an antioxidant included to prevent artefactual formation of 8-oxo-dG during adduct detection. DNA (50 μg) and internal standard 8-oxo-dG $^{15}N_5$ (2 pmol) were evaporated to dryness using a speed vacuum pump. Buffer (30 μl; 40 mM Tris-HCl, 10 mM $MgCl_2$, pH 8.5) was added prior to the addition of snake venom phosphodiesterase (0.05 U in 15.5 μl), deoxyribonuclease I (10 U in 2.5μl) and shrimp alkaline phosphatase (2 U in 2 μl). Samples were vortexed briefly, incubated (2 h, 37 °C) and centrifuged (10,000 g, 5 min). The digested DNA was transferred to a centrifugal filter device and recentrifuged at 10,000 g for 30 min. The filtrate was then stored at – 20 °C prior to elution through the 8-oxo-dG specific immunoaffinity column. HPLC analysis required 5 μl of each sample. The remainder of the filtrated samples (~45 μl) were diluted with loading buffer (1 ml; 0.05 M sodium phosphate, 0.02% sodium azide (w/ v), pH 7.6) and applied to the immunoaffinity column. The solution was allowed to elute by gravity flow at room temperature. Unbound material was eluted by the application of loading buffer (5 ml) followed by HPLC grade water (5 ml). The 8-oxo-dG was eluted using methanol: HPLC grade water (1: 1; 5 ml). The column was regenerated using HPLC grade water (5 ml) followed by loading buffer (5 ml). The methanol: water fraction was evaporated to dryness using the speed vacuum pump.

HPLC conditions were an isocratic mobile phase 0.1 M tetraethylammonium pH 5 / 4% methanol at a flow rate of 0.2 ml/min using UV detection at 254 nm. The LC-MS/ MS

consisted of a Varian 9012 pump attached to 7125 Rheodyne with a 20µl injection loop connected to a Micromass Quattro-BioQ (Micromass, Manchester, UK) tandem quadrupole mass spectrometer with an electrospray interface. A HyPurity (ThermoQuest Hypersil, Runcorn, UK), C18, (3 µ, 1.0 × 150 mm) column connected to a Uniguard C18 (3 µ, 1.0 × 10 mm) guard cartridge attached to a KrudKatcher disposable pre-column (5 µ) filter (Phenomenex, Macclesfield, UK) was used. The column was eluted isocratically with 0.1% acetic acid/ 10% methanol (v/ v) at a flow rate of 50 µl/min. The collision gas argon was set at 1.0×10^{-3} mbar. The samples were analysed in positive ion multiple reaction mode (MRM) for the $(M+H)^+$ to BH_2^+ transitions of:

8-OHdG ***m/z* 284 to 168**
$[^{15}N_5]$8-OHdG ***m/z* 289 to 173**

2.2.15 Determination of haematocrit

Blood samples were collected by cardiac exsanguination under terminal halothane anaesthesia and placed in heparinised 75 mm microhaematocrit tubes (Richardson's of Leicester, Leicester, UK) by capillary action. The tip of the haematocrit tube was sealed using a Bunsen burner flame and centrifuged in a microhaematocrit centrifuge for 15 min. The haematocrit was then determined as described previously (Strumia *et al.,* 1954) using the ratio between erythrocytes and plasma and expressed as a percentage.

2.2.16 Gene expression analysis using DNA microarrays

cDNA microarrays are capable of profiling gene expression of thousands of genes in a single experiment. Templates for genes of interest are amplified by PCR and purified before being robotically printed on coated glass microscope slides. Total RNA from test and control samples is labelled with either Cy3- or Cy5-dUTP using a single round of reverse transcription. The labelled RNA is allowed to hybridise, under stringent conditions, to the DNA on the array. Laser excitation of the targets yields an emission with characteristic spectra, which is measured by a scanning confocal laser microscope.

Monochrome images from a single hybridisation are viewed as a normalised ratio (Cy3/Cy5) in which significant deviations from 1 (no change) indicate overexpression (>1) or underexpression (<1) of genes, relative to the control sample (adapted from Duggan *et al.*, 1999).

2.2.16.1 Extraction of RNA from intestinal mucosa from C57BL/6J +/+ and C57BL/6J Min/+ mice.

Two samples (30 mg) were cut at 4 °C from a cross section of each small intestine, approximately 10 cm from the distal end using a sterile scalpel. Each sample was ground in liquid nitrogen using a cold pestle and mortar and transferred to a cold 50 ml tube and stored on dry ice. TRI Reagent (300 µl) was added and the samples homogenised. The lysate was then homogenised at room temperature with a further addition of TRI Reagent (400 µl) and transferred to a 1.5 ml eppendorffs. The original 50ml tube was rinsed with TRI Reagent (300 µl) and this was added to the lysate and gently mixed. After allowing the lysate to stand for 5 min at room temperature, chloroform (200 µl) was added to the samples and vortexed for 15 sec. The samples were then left for a further 10 min prior to centrifugation at 11,000 g at 4 °C for 10 min. The upper colourless aqueous phase containing the RNA was transferred to a 1.5 ml eppendorff containing an equal volume of isopropanol, vortexed and allowed to stand for 10 min at room temperature. The centrifugation procedure was repeated (11,000 g at 4 °C for 10 min) and the sample was then washed twice with 70% ethanol (2x 750 µl). After removing the ethanol, RNAse-free water (100 µl) was added to each pellet and the duplicate samples combined. The entire extraction procedure was then repeated as described above, initially adding 1 ml TRI Reagent. After obtaining a RNA pellet this was washed twice with 70% ethanol, and dissolved in RNAse-free water (200 µl). To reprecipitate the RNA, sodium acetate (3 M, pH 5.2) (20 µl) and 100% ethanol (600 µl) was added and left to strand at room temperature for 10 min. The sample was centrifuged (11,000 g at 4 °C for 10 min) and the RNA pellet washed once with 70% ethanol (1 ml) and then left to air dry. Depending on the size of the RNA pellet, RNAse-free water was added to dissolve the RNA (5-40 µl) and then quantitated using spectrophotometry.

2.2.16.2 Quantitation of RNA by UV absorbance

The concentration of RNA in aqueous solution was determined by measuring the absorbance of a dilution of the solution at 260 nm. The samples were diluted 1 in 500 with sterile water, spectrophotometer was zeroed using sterile water and the absorbance measured at 260 nm and 280 nm.

OD260: [RNA]µg/ml = OD260 X dilution X 40

Comparisons of absorbance at 260nm and 280 nm of a nucleic acid solution provide an estimate of the purity of the preparation. A 260/280 ratio of approximately 1.6-1.9, indicates a protein free preparation, ratios lower than these indicate protein contamination.

2.2.16.3 Confirmation of RNA quality by gel electrophoresis.

A 1.4% agarose gel was prepared essentially as described in 2.2.2 using 1x TBE (50 ml), ethidium bromide (2.5 µl) and agarose (0.7 g). RNA was run at 2 µg/ lane (2µl sample, 3 µl running buffer). RNA samples should have two clear rRNA bands when separated on an agarose gel, with the 28S band appearing more intense than the 18S band. There should be little or no smear between the 28S and 18S bands. The gel was visualised using a dual intensity UV transilluminator and a Polaroid photograph taken.

2.2.16.4 Annealing, labelling and hydrolysation of RNA

To anneal the RNA, the samples (50 µg in 10 µl) were incubated (70 °C, 8 min) with 8 µg/µl Oligo dT_{25} (0.5 µl) and then the temperature was reduced to 42 °C for 30 min. A master mix of first strand buffer (4 µl), 0.1 M DTT nucleotides (2 µl) and dNTP (0.5 µl) was added to a 2 µl aliquot of 1 M Cy dUTP (Cy3 or Cy5 cyanine dyes). RNAsin (0.5 µl) and superscript II (0.5 µl) were then added. The master mix was then added to the RNA sample while it was maintained at 42 °C. After incubation (1 h) a further 0.5 µl of superscript reverse transcriptase was added. After a further hour 20.5 µl of water was added. The RNA was then hydrolysed by sequential additions of 0.5 M EDTA (1 µl), 10% w/v SDS (1 µl) and 3 M NaOH (3 µl). Residual RNA was

hydrolysed by incubation (70 °C, 10 min), prior to the addition of 2 M HCl (3 µl), 1 M Tris/ HCl pH 7.5 (10 µl) and 4 µg/ml tRNA (1 µl).

2.2.16.5 Purification of RNA

The samples were purified using a microcon YM-30 micro-concentrator method. Tris-EDTA buffer (140 µl) was added to each dye tube and then these probes were combined into one centricon prior to microfugation (10,000g, 7 min) and the flow-through discarded. Tris-EDTA buffer (450 µl) was then added to each sample, microfuged (as previously) and the flow-through discarded. The process was repeated with the addition of Mouse Cot1 DNA (10 µg), poly A (1 µg) and 4 µg tRNA. The samples were microfuged (10,000g, 2 min) until the filter was dry in the middle and the probe formed a liquid ring. The flow-through was discarded. The columns were then inverted in a fresh tube and microfuged (10,000g, 2 min), prior to drying.

2.2.16.6 Preparation for hybridisation to microarray slides

Probes were resuspended in hybridisation buffer (14 µl) (deionised formamide: 50x Denhardts solution: water: 10% SDS; 10:1:2:1 filtered through a 0.45 µ syringe filter), added to 20x SSPE (6µl; 3 M NaCl/ 1 mM NaH_2PO_4/ 20 mM EDTA) and denatured (100 °C, 2 min) prior to incubation (42 °C, 30-60 min). The total probe volume for hybridisation was 20 µl.

2.2.16.7 Microarray slide blocking and prescan

Slides were heated (100 °C, 2 min), washed in double distilled water to remove salts and centrifuged to dryness. Slides were prescanned to discard badly printed slides.

2.2.16.8 Washing of array coverslips.

It is crucial that all coverslips and slides are completely dust free. Contamination may lead to artefacts during analysis and poor RNA hybridisation. All coverslips were placed in racks and then washed in 1% SDS for 30 min with occasional agitation prior

to 5 rinses in fresh changes of pure water for 5 min per wash. The coverslips were then centrifuged (1000 g, 5 min) to remove residual water and stored in a dust-free environment.

2.2.16.9 Prehybridisation of the arrays and wash step

To reduce non-specific binding of material to the arrays and the inherent green fluorescence, the slides were prehybridised under a coverslip (42 °C, 45-60 min) using 20 µl prehybridisation solution (55% H_2O, 30% 20x SSPE, 10% Denhardts, 0.5% SDS and 1% w/v BSA, filtered through 0.45 µ membrane). Slides were washed in 1x SSC, 0.03% SDS for 10 min, allowing the coverslip to gently slide off, transferred to 0.2x SSC for 5 min and then 0.05x SSC for 5 min. The slides were dried by centrifugation.

2.2.16.10 Hybridisation.

Using an array template slide as a guide, 20 µl of probe was place on the left of the array and a coverslip gently placed over the array, enabling the probe to cover the array. The slides were placed in a humid airtight hybridisation chamber in a 42 °C water bath overnight. The slides were then washed in 1x SSC, 0.03% SDS for 10 min, 0.2x SSC for 5 min and then 0.05x SSC for 5 min. The slides were dried by centrifugation and then analysed.

2.2.16.11 Analysis of fluorescence and data processing.

Fluorescence was measured using the GenePix 3.0 (version 3.0.0.85) software (Axon Instruments, Union City, USA). Feature sizes were determined using the inbuilt automated parameters in the first instance and then adjusted manually where appropriate. The fluorescence of each pixel within the feature was determined and the median fluorescence of these pixel measurements taken as the measure of fluorescence for the whole feature. The local background fluorescence was measured using the default parameters of GenePix 3.0. The raw feature data for each channel were globally centred by reference to the median fluorescence of the whole feature set for that

channel. To analyse the gene changes from the replicates of each of the three experiments, the absorbance data for both the red and green channels were normalised. The mean and standard deviation of the four replicates for both channels including any replicate clones was calculated and a t-test was applied. The $\log_2$ ratios were calculated when the significance was reached using six degrees of freedom. Data processing was carried out using ConvertData version 3.2.3a. These values were fed into cluster 3 (http://www-genome.Stanford.edu/) and clustered hierarchically using complete linkage. Prior to viewing, the weighted significance data were displayed using Treeview http://www.rana.lbl.gov. The density of colour indicates the ratio as indicated in the figure key.

2.2.17 Pharmacokinetic of curcumin in C57BL/6J mice

2.2.17.1 Determination of steady–state levels of curcumin in target tissues and excreta of C57BL/6J mice

Male C57BL/ 6J mice aged 8 weeks were allocated to 3 groups (n = 3) and fed an RM3 diet containing 0.1%, 0.2% or 0.5% curcumin for 8 days. These dose levels represent respectively a dietary intake of approximately 150 mg/ kg *pd*, 300 mg/ kg *pd* or 750 mg/ kg *pd* curcumin The animals were placed into metabolism cages 24 hours prior to culling by cardiac exsanguination under terminal halothane anaesthesia. The small intestine and colon were isolated as described in 2.2.9 and tissue epithelial scrapings were collected by gently brushing the epithelial layers with a metal spatula. Whole liver samples were also collected as well as faeces and urine samples from the metabolism cages. Plasma was separated from the blood samples, where stated, by centrifugation (10,000 g, 5 min). The tissues were frozen immediately and stored at –80 °C until analysis. The tissue (100 mg-1.5 g) samples were homogenised in 2 ml of 1 M acetate buffer (pH 4.5) and then extracted with 10-15 ml of ethyl acetate. The mixtures were centrifuged (2800 g, 4 °C, 15 min) and the supernatant removed and the organic layer evaporated under nitrogen. Aliquots of plasma and blood were extracted directly or with the addition of an equal volume of 1 M acetate buffer pH 4.5 with twice the volume of ethyl acetate. The mixtures were centrifuged (2800 g, 4 °C, 15 min) and the supernatant removed and the organic layer was evaporated under nitrogen. Faecal

samples (200 mg) were homogenised with 2 ml of 1 M acetate buffer (pH 4.5) and extracted as described above in 10x volume of ethyl acetate.

2.2.17.2 Determination of disappearance of curcumin after administration of 0.2% curcumin in RM3 diet

Male C57BL/6J +/+ mice aged 8 weeks were allocated to 9 groups of (n=3) and fed an RM3 diet containing 0.2% curcumin for 8 days. This is equivalent to approximately 300 mg/ kg *pd.* The curcumin was mixed into the powdered RM3 diet mechanically to ensure uniform mixing and curcumin intake. The animals were culled at 0, 3, 6, 24, 48, 72, 96, 192 and 384 h post termination of the curcumin diet. The curcumin diet was replaced by RM3 diet and the animals were placed into metabolism cages 24 h prior to culling by terminal anaesthesia with halothane. (Animals culled 3 and 6 h post termination of the curcumin diet were exempt from this procedure as no faecal samples were collected for these time points). Blood and liver samples plus small bowel and colon epithelial scrapings were taken as described previously. Faecal samples were collected from the metabolism cages. All samples were frozen immediately and stored at –80 °C until analysis. Curcumin extractions were performed as described in section 2.2.17.2.

2.2.18 HPLC analysis of curcumin and its metabolites

HPLC was performed with a Varian chromatograph (Walton on Thames, UK) equipped with a Pro-Star 230 solvent delivery system and a Pro-star 310 UV-Vis detector and 410 Varian autosampler. A Waters Symmetry-Shield RP_{18} column (3.9 mm x 150 mm, 5 μm) (Elstree, UK) was used. The mobile phase consisted of acetonitrile and 0.01 M ammonium acetate, pH 4.5, using a linear gradient of 5 - 45% acetonitrile over 30 min and further increased to 95% over 20 min. The flow rate was 1ml/min and the total run time was 60 min. Detection of curcumin, curcumin sulphate and curcumin glucuronide was achieved at 420 nm. For the detection of products of curcumin reduction, the detector was switched to 280 nm. 5,10,15,20-Tetra-(m-hydrophenyl)-chlorin was used as an internal standard. Samples were reconstituted in acetonitrile: water (1: 1), and the injection volume was 50 μl.

2.2.19 Mass spectrometry of curcumin and its metabolites

Mass spectrometry was performed on a Quattro Bio-Q tandem mass spectrometer upgraded to Quattro MK II specifications (Micromass, Altrincham, Cheshire UK) with a pneumatically assisted electrospray interface. Samples were analysed in negative ion mode. The temperature was maintained at 120 °C, the operating voltage of the electrospray capillary was 3.88 V and the cone voltage 32 V. Tandem mass spectrometric experiments were conducted using argon as the collision gas and collision energy of 25 eV. Samples were prepared either by extraction procedures described in section 2.2.17.2 or by collection of HPLC eluent fractions of the desired retention time, which were subsequently evaporated to dryness under a stream of nitrogen. Samples were reconstituted in water: methanol (1: 1) and introduced into the mass spectrometer via continuous infusion using a Harvard apparatus model 22 syringe pump (Harvard Apparatus, South Natick, MA) pumped at a flow rate of 10 µl/ min.

2.2.20 Determination of radioactivity levels in tissues after administration of 100 mg/kg intraperitoneal (*ip*). [^{14}C]-labelled curcumin

C57BL/6J Male mice (7 weeks) were divided into 7 groups of 3 animals. The mice were killed by cardiac exsanguination under terminal halothane anaesthesia 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, and 24 h after an *ip.* injection of 100 mg/ kg curcumin dissolved in DMSO. The [^{14}C] radiolabelled curcumin constituted 20 mg/ kg of this dose. The production of the radio labelled curcumin was performed as previously described (Parveen and Threadgill., 2000). Brain, heart, lung, liver, spleen, kidney, small intestine and blood samples were taken from each animal. The tissue samples were placed in preweighed scintillation vials, which were then weighed to establish the weight of the tissue, prior to dissolving by the addition of 1.5 ml Optisolve. Up to 200 mg of wet tissue could be dissolved in 1 ml Optisolve, therefore the maximum mass of tissue was approximately 300 mg in a final volume of 15 ml scintillant. These were dissolved at 37 ° C for 48 h. Scintillation fluid (15 ml) was added to each sample and vortexed for 30 sec. Plasma was obtained from the blood samples by centrifugation (10,000g, 5 min). Known volumes of plasma were placed in scintillation vials and the scintillation fluid (15 ml) was added directly and vortexed for 30 sec. All the samples were then liquid scintillation counted using a 10 min ^{14}C count (Wallac 1410 liquid

scintillation counter, PerkinElmer Life Sciences, Cambridge UK). The counts were then evaluated using Excel and the subsequent data analysed using WinNonLin.

2.2.21 Statistical analyses

Data was analysed using Excel (Office 2000) and Graphpad Prism. All results were subjected to statistical analysis using Minitab (version 13) software package. Appropriate analysis and *post hoc* comparisons were performed as stated in Figure legends. Dr. M. Festing, in house bio-statistician, MRC Toxicology Unit, University of Leicester, provided advice.

CHAPTER 3
EFFECT OF CURCUMIN ON TUMOUR DEVELOPMENT IN THE MIN/+ MOUSE

3.1 Introduction

Many potential chemopreventive agents have been investigated in mutated *Apc* mouse models of colorectal cancer, since the discovery of the Min/+ mouse over ten years ago. These include several non-steroidal anti-inflammatory drugs (NSAIDS), including piroxicam (Halberg *et al.,* 2000; Quesada *et al.,* 1998; Jacoby *et al.,* 1996; Ritland and Gendler, 1999; Jacoby *et al.,* 2000), sulindac (Oshima *et al.,* 1996; Chiu *et al.,* 1997; Mahmoud *et al.,* 1999), indomethacin (Chiu *et al.,* 2000) aspirin (Barnes and Lee, 1998; Mahmoud *et al.,* 1998) and COX-2 specific inhibitors such as celecoxib (Jacoby *et al.,* 2000b) and rofecoxib (Oshima *et al.,* 2001). The effects of compounds including dietary fats (Hioki *et al.,* 1997), polyunsaturated fatty acids (Oshima *et al.,* 1995) and plant derived phenolics (Mahmoud *et al.,* 2000) have also been investigated in *Apc* mouse models. As the Min/+ mutant strain develops scores of adenomas, which grow to a detectable size within a few months, it is an excellent model with which to screen for potentially effective agents.

Curcumin prevents carcinogen-induced intestinal pre-malignancies and malignancies in rats and in the Min/+ mouse (Kelloff *et al.,* 2000; Reddy and Rao, 2002). Mahmoud and colleagues (2000) demonstrated that lifetime administration of curcumin (0.1%) reduced intestinal adenomas in Min/+ mice by 64%. Collett and colleagues (2001) confirmed that curcumin (0.2%) reduced proximal adenomas in Min/+ mice treated with 2-amino-1-methyl-6-phenylimidazo [4,5*b*] pyridine (PhIP) (300p.p.m), by 52%. However, administration of 0.2% curcumin was without effect in Min/+ mice that were not treated with PhIP. PhIP is a heterocyclic amine found in cooked meat and fish. The effect of differing dose levels of curcumin has not been investigated. The effects of curcumin on growth and survival of Min/+ mouse are also unknown.

Shoemaker *et al.,* (1995) and Reitmair *et al,* (1996) suggest that early irreversible changes, which lead to the appearance of adenomas in Min/+ mice, have already taken place by 6 days of age. Therefore, investigations were performed to determine the effect of curcumin exposure throughout embyrogenesis and weaning. The NSAID, piroxicam, which is functionally related to curcumin, has been shown to inhibit adenoma development when administered at this time (Ritand and Gendler 1999). The effect of curcumin administered during embryogenesis and weaning has, up to now, not been investigated. It has also been shown that piroxicam may be embryotoxic to Min/+

animals, favouring a majority of wildtype (+/+) to Min/+ animals in the progeny of parents exposed to 0.02% piroxicam (Jacoby *et al.*, 2000). Similar studies using curcumin have not been performed.

Conflicting data exists concerning the chemopreventive efficacy of aspirin in the Min/+ mouse (Mahmoud *et al.*, 1998; Barnes and Lee, 1998; Sansom *et al.*, 2001; Chiu *et al.*, 2000). It has been hypothesised by Sansom *et al.*, (2001) that aspirin may be efficacious if administered *in utero* or early on in carcinogenesis. Aspirin (0.04%) exposure throughout embryogenesis and weaning prolonged Min/+ mouse survival (Sansom *et al.*, 2001). However the effect of *in utero* administration of aspirin has not been investigated. Aspirin and curcumin may have a synergistic action on the retardation of adenoma development as both agents inhibit COX-2 activity: curcumin by inhibition of NFκB activation via the NIK/IKK signalling complex (Plummer *et al.*, 1999) and aspirin as a well documented inhibitor of both COX isozymes (Husain *et al.*, 2002). Therefore, combination studies with curcumin and aspirin have been performed as well as determining the effect of *in utero* exposure to aspirin.

As the main aim of these studies is to aid the rational development of curcumin as a chemopreventive agent in the Western world where the incidence of colorectal cancer is more prevalent than in the Developing world (Parkin *et al.*, 1999), it seemed suitable to include a Western style high nutrient basal diet, referred to as RM3, in these studies. Previous chemoprevention studies using Min/+ mice have often used standardised AIN 76 or the more recent version, AIN 93 diets. These diets are experimental formulations prepared to exact specifications. They are lower nutrients, in particular protein than RM3 (see appendix 1). Therefore an assessment of the effect of feeding Min/+ mice different diets has also been made.

3.2 Determination of Min/+ status

Genotyping of the experimental weaners was performed essentially as described previously (Luongo *et al.,* 1994). However, in accordance with Home Office stipulations on the repeated use of animals, tissue samples were obtained from the weaners by ear-punch, as a by-product of the method used to identify the animals that is not considered a procedure. The use of ear punch tissue subsequently required modifications to the Polymerase Chain Reaction (PCR) method (described in section 2.2.2). An example of the agarose electrophoresis gel of the restriction digest of the PCR product is shown below (Figure 3.2.1).

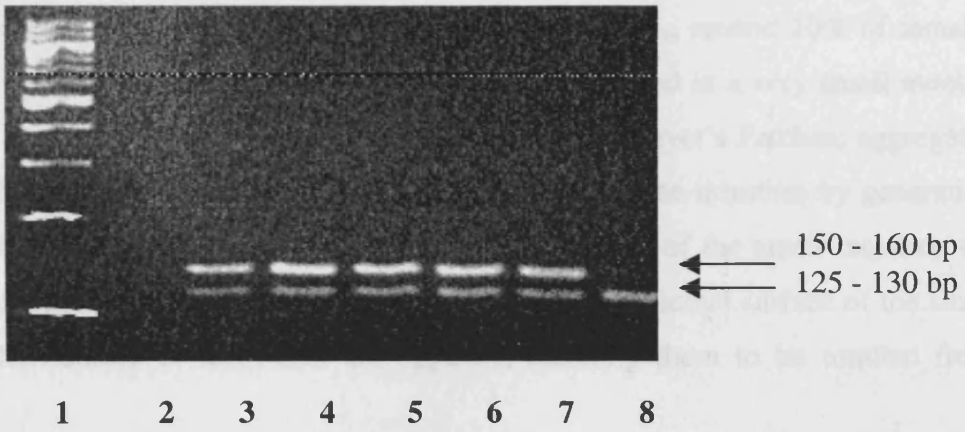

Figure 3.2.1 Representative electrophoresis gel determining the genotype of 6 offspring from a mating of male C57BL/6J Min/+ and female C57BL/6J (+/+) mice. Lane 1 is a 100bp DNA ladder; lane 2 empty, lanes 3-7 represent the Min /+ genotype and lane 8 shows a wildtype +/+ genotype. (For details of the Min/+ genotyping method see section 2.2.2)

Without the use of PCR, the genotype of Min/+ mice can only be determined from the phenotype from around 8 weeks of age. Symptoms include anaemia, white paws, starry coat, enlarged spleen and the presence of intestinal adenomas. The number of PCR cycles was optimised to 40 and the samples were run on a 3% agarose gel, enabling the visualisation of two clear bands. The uncut wildtype band appeared at 150 -160 base pairs whilst the Min band consisted of 125 - 130 base pairs. The use of ear punch tissue, as the source of DNA is novel in terms of Min/+ genotyping, yet the results were highly reliable, > 95% of all mice genotyped were correctly identified as revealed by post-mortem.

3.3 Gross tumour pathology of the Min/+ mouse

In order to accurately assess the chemopreventive efficacy of a compound, it is necessary to compare the pathology of the bowel to that in an untreated C57BL/6J Min/+ mouse. At 18 weeks of age, Min/+ mice fed RM3 diet possess more than 100 adenomas in the small intestine (Figure 3.4.1.1). The adenomas were located mainly in the distal region, nearest the caecum, and middle regions and measure <3 mm in diameter. Larger, but less numerous adenomas were present in the proximal region, nearest the stomach. Very few adenomas were present in the caecum, and are, therefore, omitted from the analysis of tumour burden. There were a small number of large colonic adenomas, which often caused the animals to become moribund, via anal prolapse or severe anaemia. In addition to intestinal tumours, around 10% of females developed mammary tumours. Liver metastases also occurred in a very small number of mice. Present within the small intestine are numerous Peyer's Patches; aggregated unencapsulated lymph nodules that respond to antigens in the intestine by generating plasma cells that secrete antibodies. From the luminal side of the small intestine the Peyer's Patches may resemble tumours, although from the external surface of the small intestine the nodules of the Patch are apparent, enabling them to be omitted from analysis.

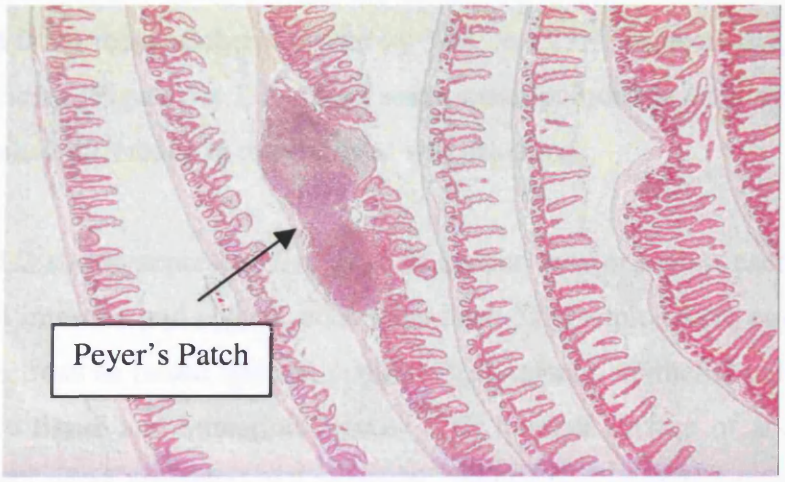

Figure 3.3.1 Representative H & E section (x50 magnification) depicting a Peyer's patch. This section was taken from a control Min/+ mouse fed RM3 diet. These nodules were eliminated from analysis. (For details of immunohistochemistry see section 2.2.8)

3.4 Effect of lifetime administration of dietary curcumin on adenoma multiplicity in C57BL/6J Min/ + mice

3.4.1 Effect of curcumin on total tumour multiplicity in the small intestine and colon of Min/+ mice

Min/+ mice received curcumin in the diet for 14 weeks, commencing one week *post-*weaning, at three concentrations, 0.1%, 0.2% and 0.5% curcumin. These levels represent a dose of approximately 150 mg/ kg, 300 mg/ kg and 750 mg/ kg. Mice were assigned to each group following a randomised block design. Animals were culled at 18 weeks of age and tumour number, distribution and size were recorded *post-mortem.* Figure 3.4.1.1 shows that dietary curcumin at 0.1% was without effect on total intestinal tumour burden (0.1% curcumin 119.1 ± 8.4 adenomas, control 116.4 ± 16 adenomas). However, at 0.2% and 0.5% curcumin, small intestinal tumour number was reduced significantly by 39% and 40% respectively ($p<0.05$), (0.2% curcumin 73.5 ± 21.4 adenomas, control 116.4 ± 16 adenomas; 0.5% curcumin 69.9 ± 33.8 adenomas, control 116.6 ± 40.3 adenomas). Inspection of adenomas revealed a flattened morphology in the case of mice, which received 0.5% curcumin when compared to untreated mice, or mice, which received a lower dose of curcumin. Untreated Min/+ mice possessed only a few colonic adenomas (3.5 ± 3.8, n = 22). Although dietary curcumin at a level of 0.2% and 0.5% reduced their number by 30% and 27%, respectively, this decrease was not significant (Figure 3.4.1.1). Both sexes were included in each treatment group, and no sex-linked difference in tumour load was observed.

Fig. 3.4.1.2 shows representative H & E stained sections from each treatment group. The small intestine and colonic adenomas from Min/+ mice were easily identifiable on H & E sections as raised lesions, containing dysplastic epithelial cells above a base of connective tissue and interstitial tissue. The luminal surface of adenomas was often eroded with some epithelial cell necrosis. Neighbouring Min/+ intestinal mucosa had normal morphology with intact crypts and villus architecture. The reduction in number and size of tumours in the presence of increasing doses of curcumin are clearly visible.

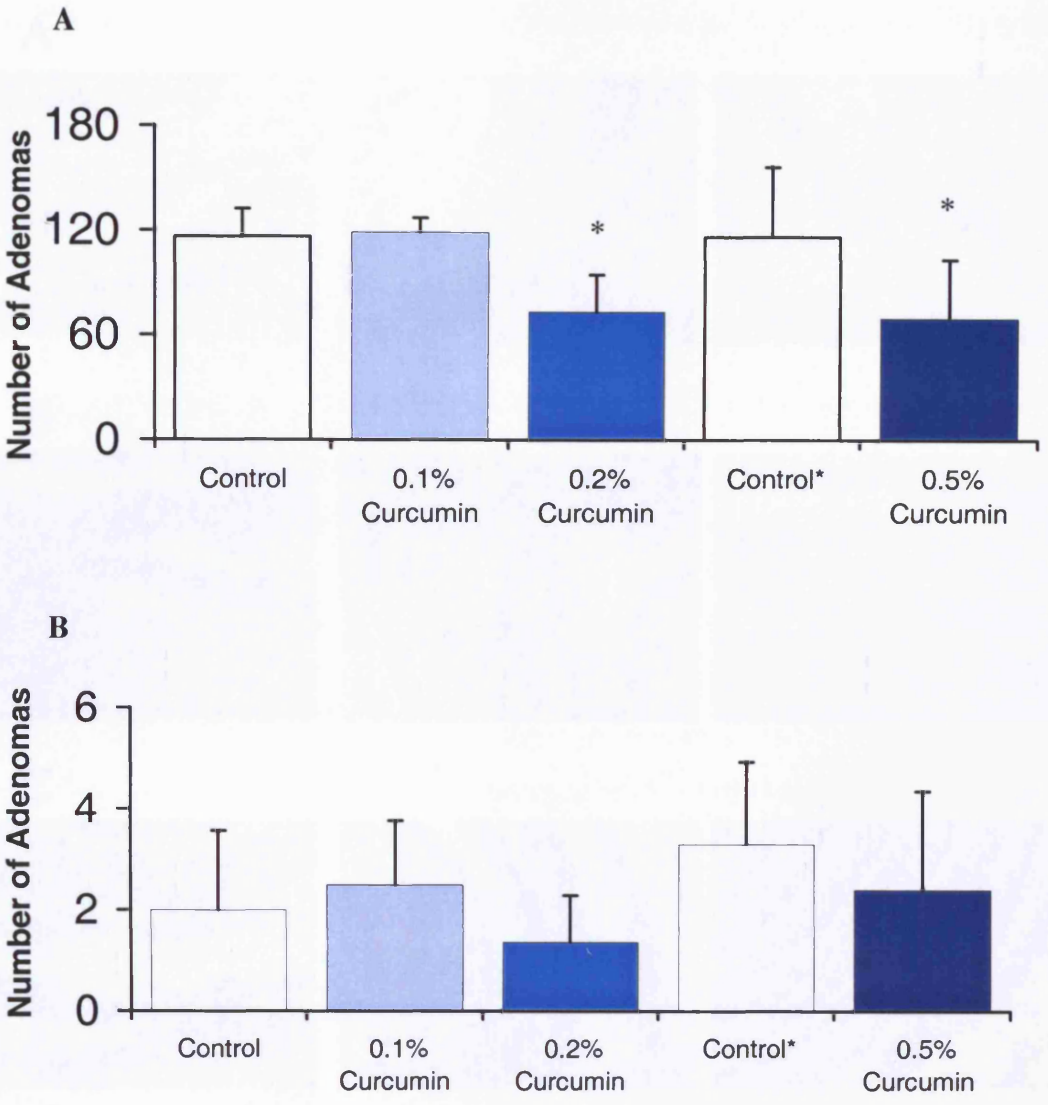

Figure 3.4.1.1 Effect of dietary curcumin on adenoma multiplicity in the small intestine (A) and colon (B) of Min/+ mice. Numbers of mice per group were between 10 and 12; asterisk indicates that the number of adenomas is significantly different from that in control animals ($p<0.05$). Note that there were two control groups (untreated Min/+ mice), one for the study of curcumin 0.1% and 0.2% and the other for the mice on curcumin 0.5% (control*). Values are mean ± SD.

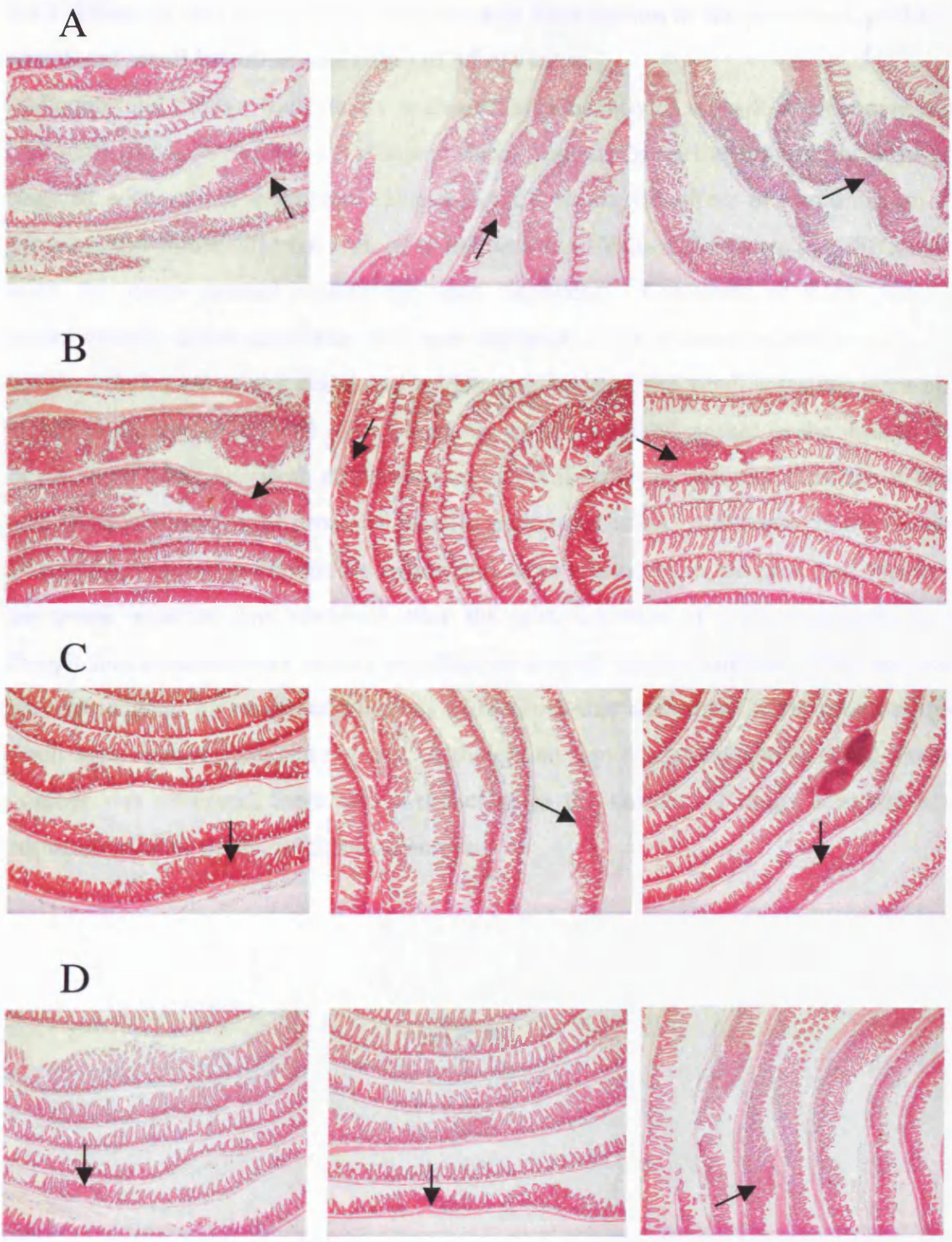

Figure 3.4.1.2 Representative H & E section (X50 magnification) depicting relative adenoma size and number in the small intestine in the presence of A) RM3 diet, B) RM3 diet containing 0.1% curcumin, C) RM3 diet containing 0.2% curcumin, D) RM3 diet containing 0.5% curcumin. Arrows indicate adenomas. (Details of H & E immunohistochemistry as described in section 2.2.8)

3.4.2 Effect of curcumin on tumour size and distribution in the proximal, middle and distal small intestine and colon of Min/+ mice

Although total tumour multiplicity decreased significantly as a result of treatment with curcumin (0.2% or 0.5%), its efficacy varied depending on the tumour location and stage of adenoma development. Figure 3.4.2.1 shows the effect of curcumin on the adenoma size in the different regions of the intestine. Values shown are as a differential from the mean control values for each treatment. Curcumin at 0.2% affected predominantly small adenomas (<1 mm diameter). To a lesser extent, middle-size tumours (1-3 mm) in the distal and middle segments of the small intestine were also affected. Curcumin at 0.5% reduced mainly middle-sized adenomas in the middle part of the small intestine. Both efficacious curcumin dose levels reduced numbers of large adenomas (>3 mm) in the proximal small intestine and colon. A decrease in the number of small adenomas in the middle region and large adenomas in the proximal region of the small intestine was observed after the administration of 0.1% curcumin, even though this concentration lacked an effect on overall tumour number. This decrease was outweighed by increased numbers of medium-size adenomas in the proximal and small adenomas in the distal regions. Although no significant decrease in colon tumour number was observed, there was a reduction in the number of larger tumours after administration of 0.2% and 0.5% curcumin.

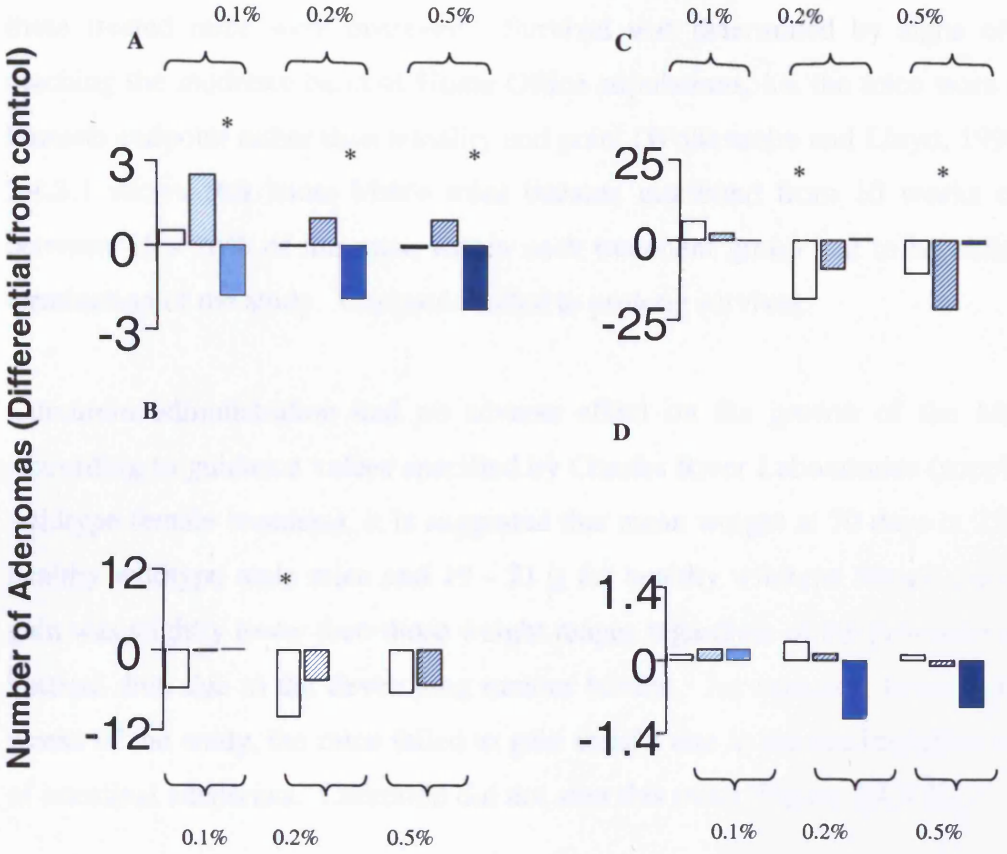

Figure 3.4.2.1 Effect of dietary curcumin on multiplicity of small (<1 mm diameter, □) medium size (1-3 mm, hatched bars) or large (>3 mm, ■) adenomas on the proximal (A), middle (B), distal (C) or colonic (D) sections of the intestine of Min/+ mice. Results are expressed as mean number of adenomas over or below mean adenoma numbers in untreated (control) Min/+ mice. Numbers of mice per group were 10 – 12. Asterisk indicates that the number of adenomas was significantly different from that in control animals ($p<0.05$). The effect of 0.2 and 0.5% dietary curcumin on numbers of small and middle-sized adenomas in the middle portion of the small intestine and of the large adenomas in the colon approached significance ($p<0.1$, but >0.05). Values are mean ± SD.

3.4.3 Effect of curcumin on growth and survival

To assess the toxicity of curcumin at the three dose levels, the growth and survival of these treated mice were observed. Survival was determined by signs of ill health reaching the moderate band of Home Office stipulations, i.e. the mice were culled at a humane endpoint rather than lethality end point (Wolfensohn and Lloyd, 1998). Figure 3.4.3.1 shows that some Min/+ mice became moribund from 10 weeks of age and between 15 - 20% of the mice within each treatment group had to be culled prior to termination of the study. Curcumin failed to prolong survival.

Curcumin administration had no adverse effect on the growth of the Min/+ mice. According to guidance values specified by Charles River Laboratories (suppliers of the wildtype female breeders), it is suggested that mean weight at 70 days is 23 - 25 g for healthy wildtype male mice and 19 - 21 g for healthy wildtype female mice. Weight gain was slightly lower than those weight ranges regardless of the provision of the high nutrient diet, due to the developing tumour burden. As expected, towards the latter 3 weeks of the study, the mice failed to gain weight due to the accumulation and growth of intestinal adenomas. Curcumin did not alter this trend (Figure 3.4.3.2).

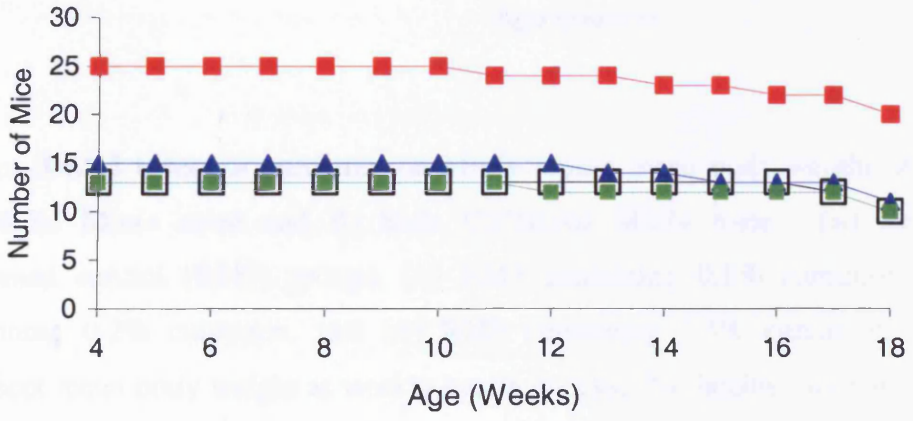

Figure 3.4.3.1 Effect of curcumin on Min/+ mouse survival. (■) Represent the combined control (RM3) groups, (□) RM3 containing 0.1% curcumin, (▲) RM3 containing 0.2% curcumin, and (■) RM3 containing 0.5% curcumin. The results represent survival as defined by signs of ill health equivalent to the moderate band of Home office guidelines.

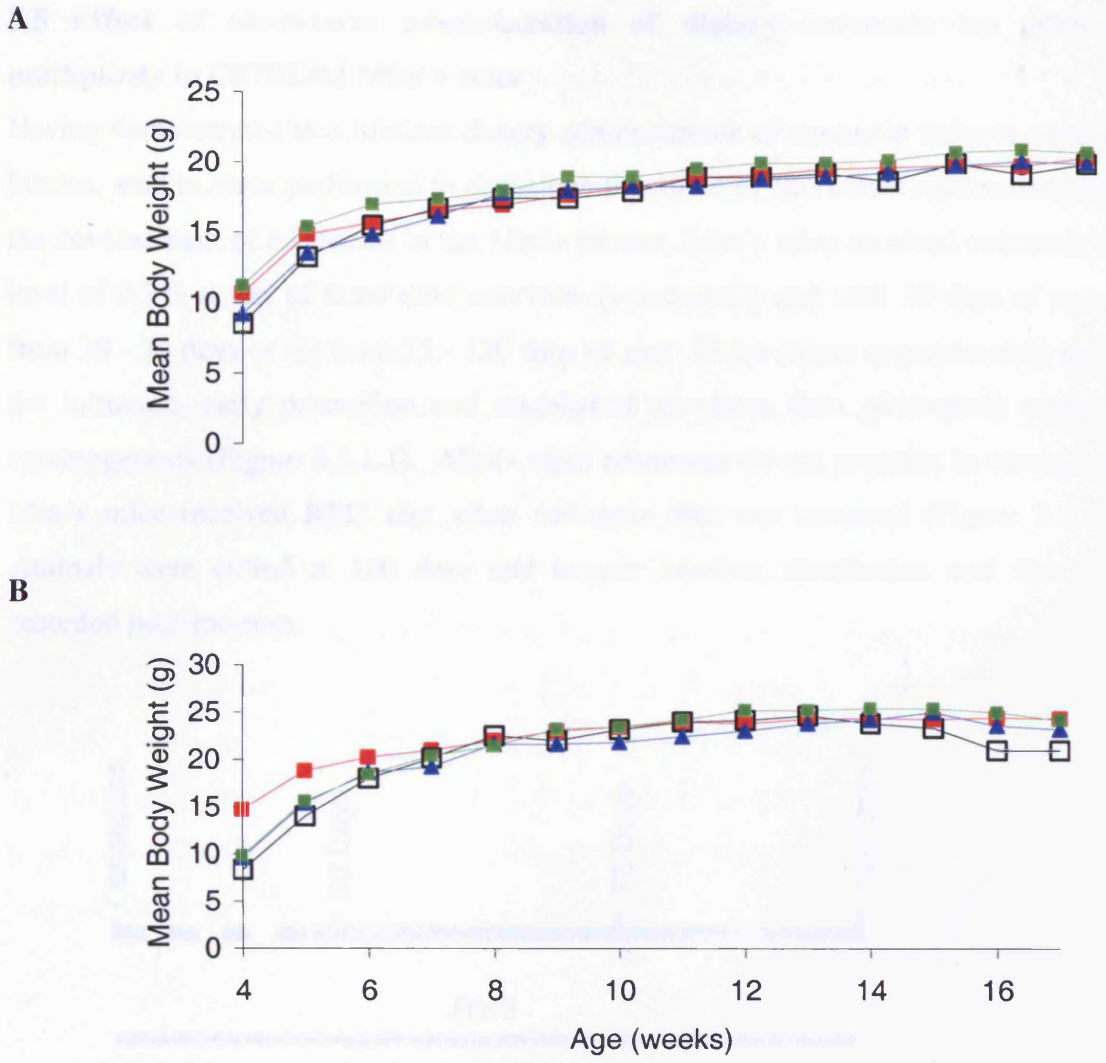

Figure 3.4.3.2 Effect of curcumin on Min/+ mouse mean body weight of A) Female C57BL/6J Min/+ mice and B) Male C57BL/6J Min/+ mice. (■) Represent the combined control (RM3) groups, (□) RM3 containing 0.1% curcumin, (▲) RM3 containing 0.2% curcumin, and (■) RM3 containing 0.5% curcumin. The results represent mean body weight at weekly health checks. No health check was performed during the concluding week of the study.

3.5 Effect of short-term administration of dietary curcumin on adenoma multiplicity in C57BL/6J Min/ + mice

Having demonstrated that lifetime dietary administration of curcumin reduces adenoma burden, studies were performed to determine the effect of short-term administration on the development of adenomas in the Min/+ mouse. Min/+ mice received curcumin at a level of 0.2% at one of three time intervals- i) perinatally and until 30 days of age, ii) from 30 - 75 days or iii) from 75 - 120 days of age. These stages approximately reflect the initiation, early promotion and established neoplasm (late promotion) stages of carcinogenesis (Figure 3.5.1.1). Min/+ mice adenomas do not progress to carcinomas. Min/+ mice received RM3 diet when curcumin diet was removed (Figure 3.5.1.1). Animals were culled at 120 days and tumour number, distribution and size were recorded *post*-mortem.

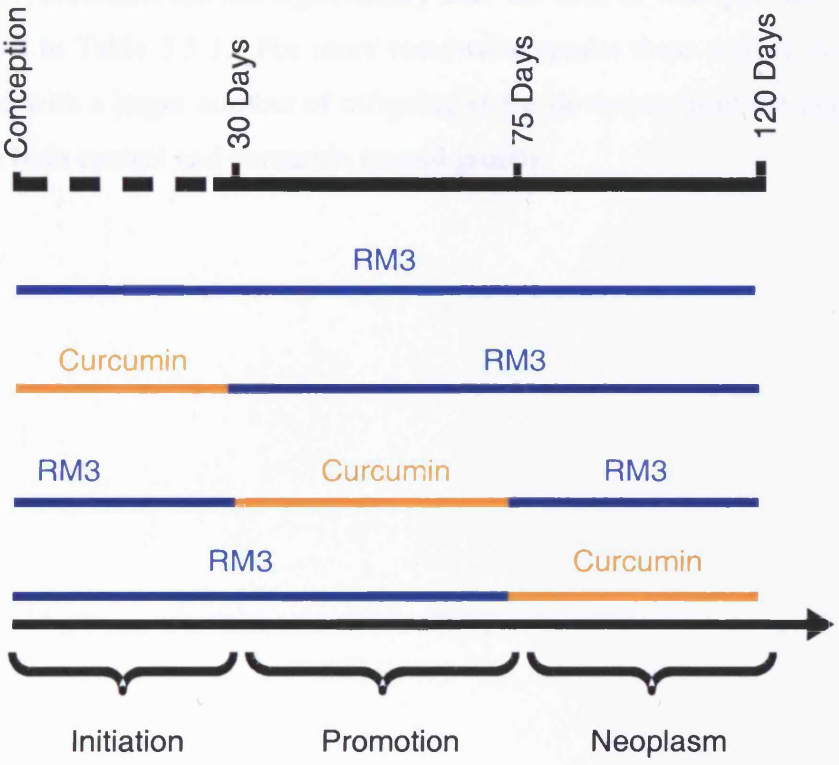

Figure 3.5.1.1 Experimental design to determine the effect of short-term administration of dietary curcumin on adenoma multiplicity in C57BL/6J Min/+ mice. Min/+ mice received curcumin (0.2%) at one of three time points approximately reflecting initiation, and early and late promotion stages of carcinogenesis.

Figure 3.5.1.2 shows that dietary curcumin administered during these time intervals had no significant effect on total intestinal tumour burden; control values were 96.4 ± 20.7 or 97.7 ± 17.2 adenomas, peri-natal administration 107.5 ± 16 adenomas, early administration 93.3 ± 33.8 adenomas, and late administration of curcumin 85.6 ± 27.4 adenomas. Late administration of curcumin did appear to have a small effect, which lacked significance. Curcumin appeared to reduce the number of smaller adenomas in the distal region and the larger adenomas in the proximal region of the small intestine (Figure 3.5.1.3). Early and late curcumin administration reduced small (<1 mm) adenomas by 8 and 13% respectively and reduced middle-sized adenomas by 17 and 22% respectively. However, this was not significant (Figure 3.5.1.3).

Previous studies have suggested that NSAIDS administered perinatally may alter the expected Mendelian ratio of 1:1 (+/+, Min/+) in the progeny (Jacoby *et al.*, 2000). However, curcumin did not significantly alter the ratio of wildtype and Min/+ offspring as shown in Table 3.5.1. For more conclusive results these studies would have to be repeated with a larger number of offspring as the deviation from the expected 50% was large in both control and curcumin treated groups.

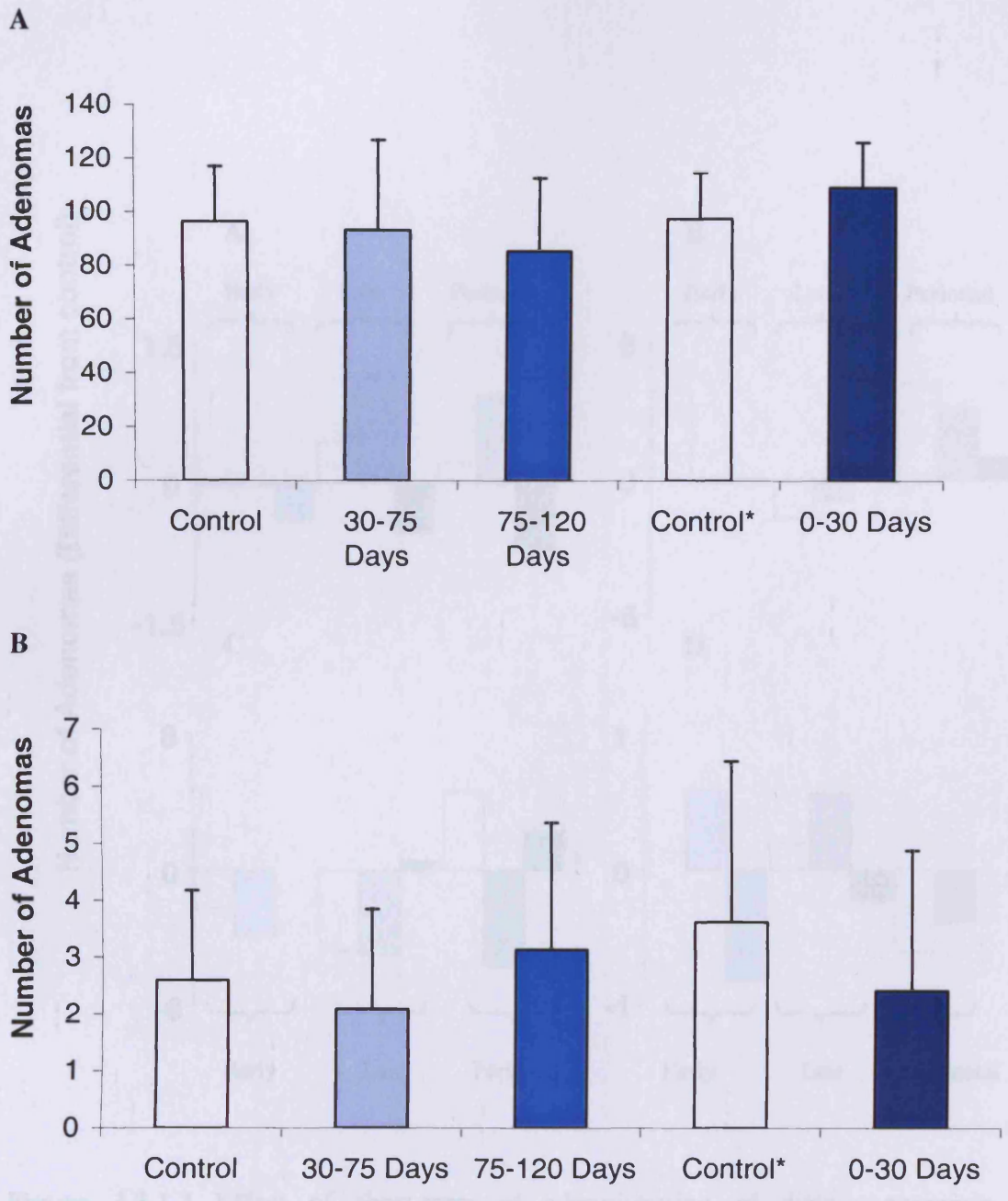

Figure 3.5.1.2 Effect of short-term administration of dietary curcumin (0.2%) on adenoma multiplicity in the small intestine (A) and colon (B) of Min/+ mice. Numbers of mice per group were between 10 and 14. Note that there were two control groups (untreated Min/+ mice), one for the study of curcumin administered post-weaning and the other for the mice fed perinatally (control *). Values are mean ± SD. Dietary curcumin was administered at a level of 0.2% and RM3 diet was administered when the curcumin diet was removed.

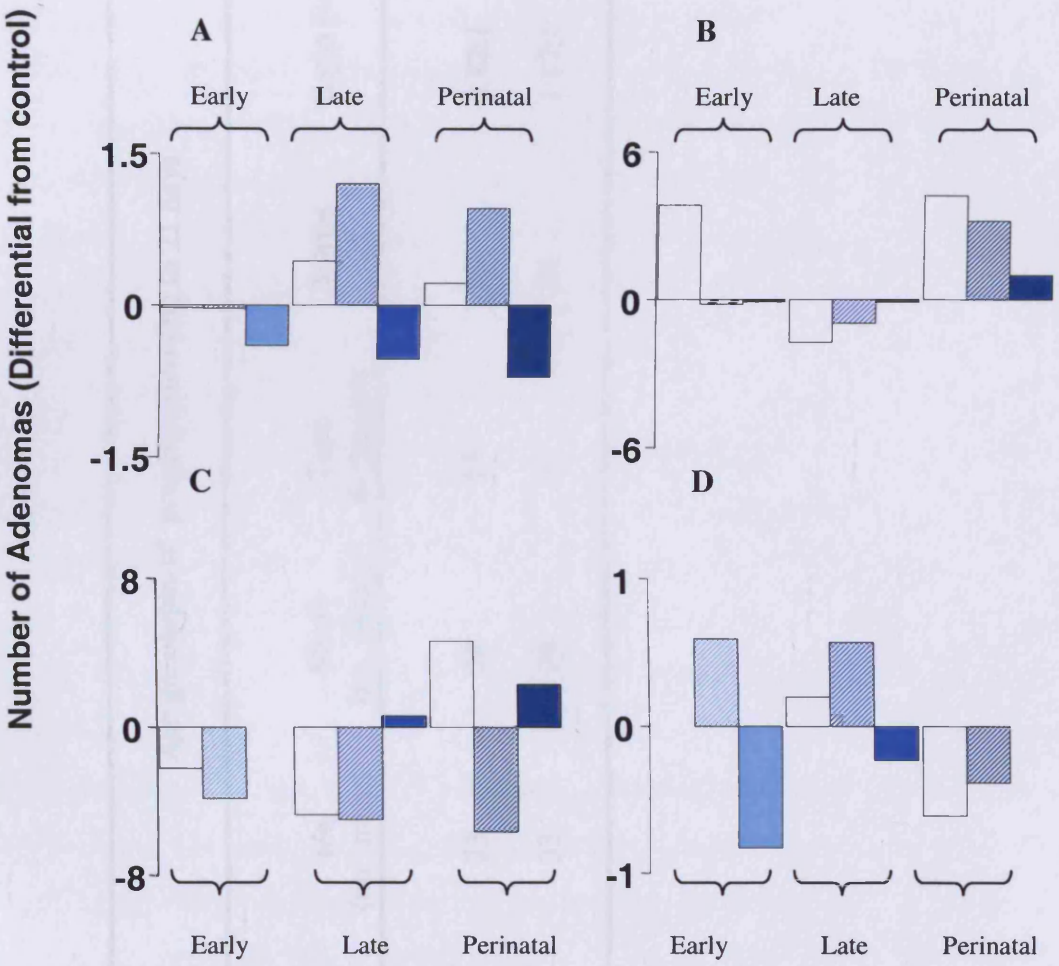

Figure 3.5.1.3 Effect of short-term of administration of dietary curcumin on multiplicity of small (<1 mm diameter, □) medium size (1 – 3 mm, hatched bars) or large (> 3mm, ■) adenomas on the proximal (A), middle (B), distal (C) or colonic (D) sections of the intestine of Min/+ mice. Results are expressed as mean number of adenomas over or below mean adenoma numbers in untreated (control) Min/+ mice. Numbers of mice per group were 10 –14.

Table 3.5.1 Lack of embryotoxicity in C57BL/6J Min/+ mice after administration of curcumin

	Apc genotypes of progeny surviving to 21 days					
Treatment	Pups born per litter	+/+ (no. mice)	Min/+ (no. .mice)	Total in progeny	% Min	Ratio (+/+:Min/+)
RM3 control diet	5.5	23	28	51	45	0.82:1
RM3 + 0.2% Curcumin	6.1	33	28	61	54	1.17:1

3.6 Effect of a combination of curcumin and aspirin on adenoma multiplicity in C57BL/6J Min/ + mice

3.6.1 Effect of pre-weaning aspirin and long-term curcumin on total tumour multiplicity in the small intestine and colon of Min/+ mice

As conflicting data exists concerning the chemopreventive efficacy of aspirin in the Min/+ mouse (Mahmoud *et al.*, 1998; Barnes and Lee, 1998; Sansom *et al.*, 2001; Chiu *et al.*, 2000), preliminary studies were performed to assess the effect of aspirin (0.05%) administered *in utero* until weaning at 28 days, and in combination with sequential administration of an efficacious dose of curcumin (0.2%). It was suggested by Sansom and colleagues (2001) that aspirin delays adenoma formation when administered early, during the initiation period, which occurs *in utero* or during the first few days after birth of the Min/+ mouse (Shoemaker *et al.*, 1995). Such a combination study design has not been implemented with any agent in the Min/+ mouse. Animals were culled at 17 weeks and tumour number, distribution and size were recorded *post-mortem*. Figure 3.6.1.1 shows that pre-weaning aspirin (0.05%) significantly reduced total small intestinal tumour burden by 21%, ($p<0.05$). The aspirin and curcumin combination significantly reduced tumour burden by 35% (control value 132.1 ± 14.8 adenomas, aspirin administration, 104.9 ± 15.9 adenomas and combination administration 84.3 ± 22.4 adenomas, n = 8-10). Both aspirin alone and in combination with curcumin failed to significantly alter colon tumour burden, although values were reduced from 4.4 ± 1.6 adenomas (control) to 3.8 ± 3.1 adenomas (aspirin), and 2.7 ± 1.7 adenomas (combination). This is a preliminary study, as a long-term dietary administration of curcumin group would also have to be included in the experimental design as well increasing total numbers within each group.

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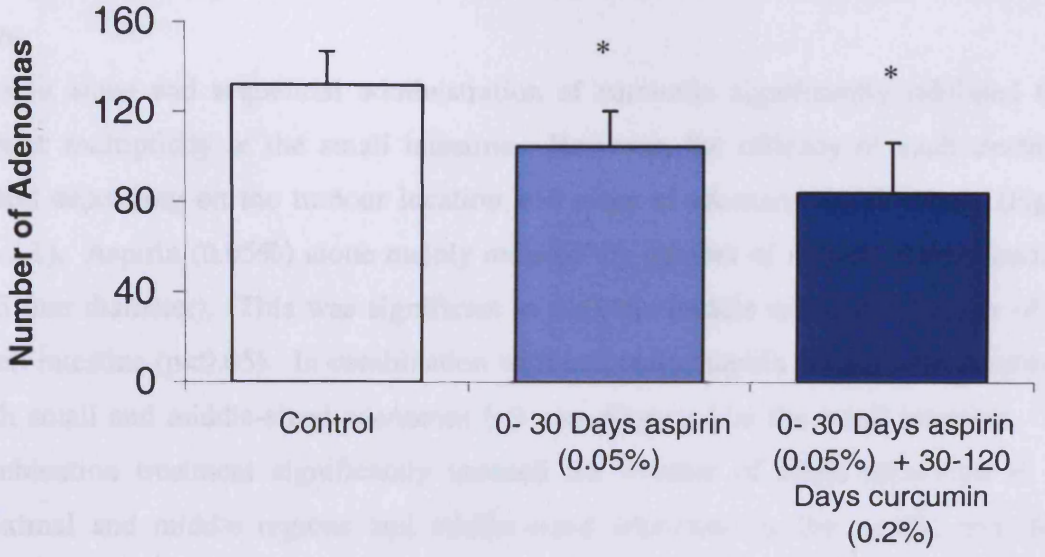

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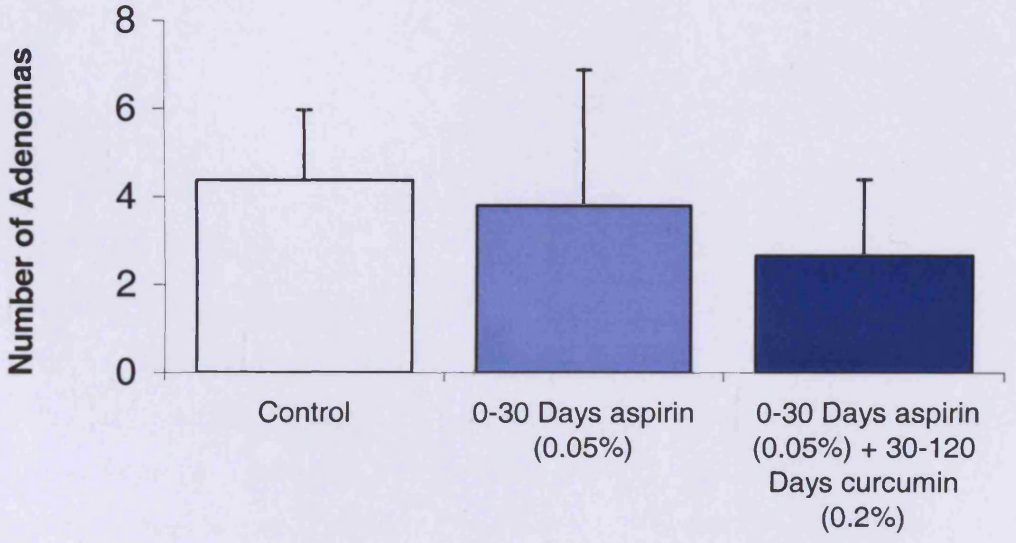

Figure 3.6.1.1 Effect of a combination of aspirin and dietary curcumin on adenoma multiplicity in the small intestine (A) and colon (B) of Min/+ mice. Numbers of mice per group were between 8 and 10; asterisk indicates that the number of adenomas is significantly different from that in control animals ($p<0.05$). Values are mean ± SD.

3.6.2 Effect of pre-weaning aspirin and long term curcumin on tumour size and distribution in the proximal, middle and distal small intestine and colon of Min/+ mice

Aspirin alone and sequential administration of curcumin significantly inhibited total tumour multiplicity in the small intestine. However, the efficacy of each treatment varied depending on the tumour location and stage of adenoma development (Figure 3.6.2.1). Aspirin (0.05%) alone mainly reduced the number of middle-sized adenomas (1-3 mm diameter). This was significant in both the middle and distal regions of the small intestine ($p<0.05$). In combination with curcumin, aspirin reduced the number of both small and middle-sized adenomas (<3 mm diameter) in the small intestine. The combination treatment significantly reduced the number of small adenomas in the proximal and middle regions and middle-sized adenomas in the middle and distal regions. Although the combination treatment reduced the number of adenomas in the colon, none of the size categories were significantly affected.

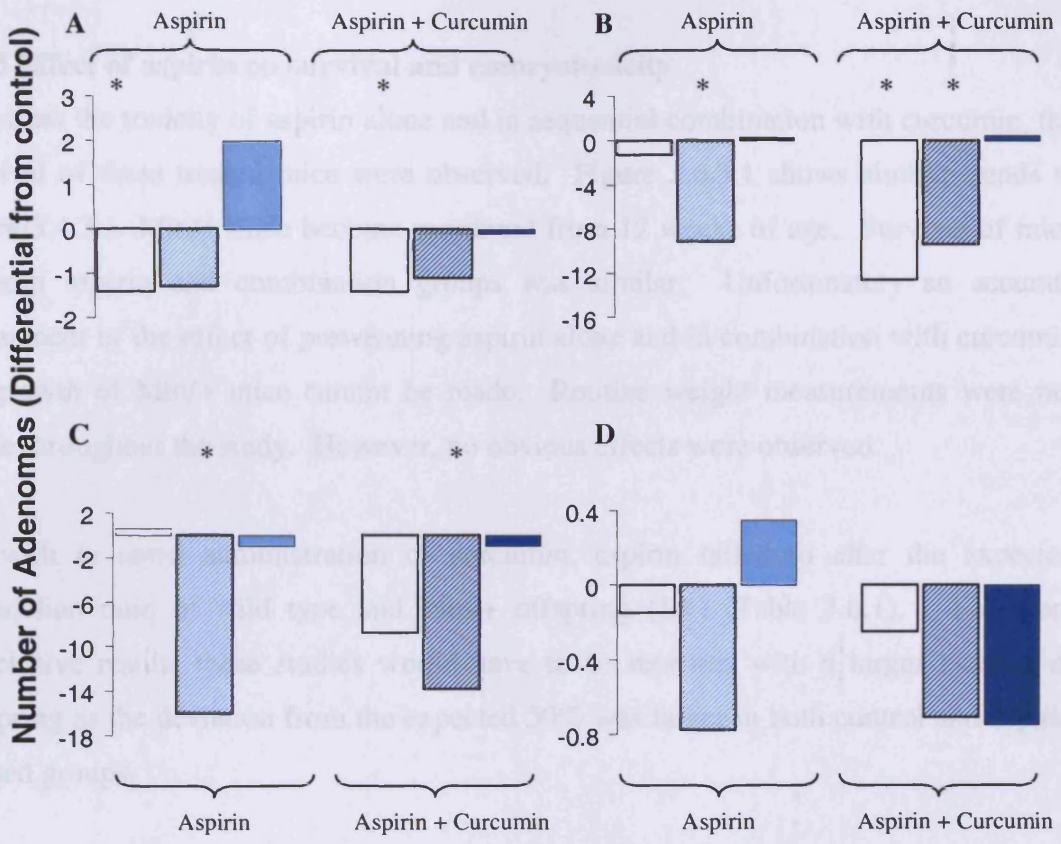

Figure 3.6.2.1 Effect of aspirin and dietary curcumin on multiplicity of small (<1 mm diameter, □) medium size (1-3 mm, hatched bars) or large (>3 mm, ■) adenomas on the proximal (A), middle (B), distal (C) or colonic (D) sections of the intestine of Min/+ mice. Results are expressed as mean number of adenomas over or below mean adenoma numbers in untreated (control) Min/+ mice. Numbers of mice per group were 8 - 10. Asterisk indicates that the number of adenomas was significantly different from that in control animals ($p<0.05$). Values are mean ± SD.

3.6.3 Effect of aspirin on survival and embryotoxicity

To assess the toxicity of aspirin alone and in sequential combination with curcumin, the survival of these treated mice were observed. Figure 3.6.3.1 shows similar trends to figure 3.4.3.1. Min/+ mice become moribund from 12 weeks of age. Survival of mice in both aspirin and combination groups was similar. Unfortunately an accurate assessment of the effect of preweaning aspirin alone and in combination with curcumin on growth of Min/+ mice cannot be made. Routine weight measurements were not made throughout the study. However, no obvious effects were observed.

As with *in utero* administration of curcumin, aspirin failed to alter the expected Mendelian ratio of wild type and Min/+ offspring (1:1) (Table 3.6.1). For more conclusive results these studies would have to be repeated with a larger number of offspring as the deviation from the expected 50% was larger in both control and aspirin treated groups.

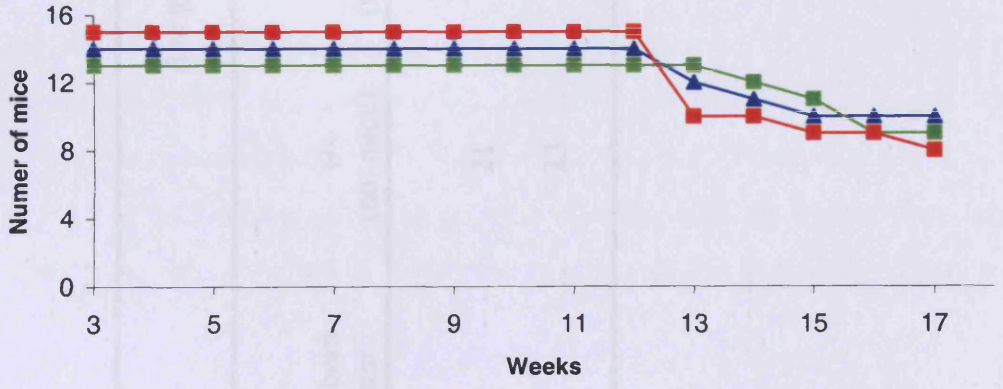

Figure 3.6.3.1 Effect of aspirin and sequential administration of curcumin on Min/+ mouse survival. (■) Represent the combined control (RM3) groups, (▲) aspirin (0.05%) group, and (■) combination group. The results represent survival as termed by signs of ill health equivalent to the moderate band of Home office guidelines.

Table 3.6.1 Lack of embryotoxicity in C57BL/6J Min/+ mice after administration of aspirin

	Apc genotypes of progeny surviving to 21 days					
Treatment	Pups born per litter	+/+ (no. mice)	Min/+ (no. mice)	Total in progeny	% Min	Ratio (+/+: Min/+)
RM3 control diet	7.2	21	15	36	42	1.4:1
RM3 + 0.05% Aspirin	7.1	23	27	50	56	0.85:1

3.7 Effect of different diets on adenoma multiplicity in C57BL/6J Min/ + mice

3.7.1 Effect of basal diet on total tumour multiplicity in the small intestine and colon of Min/+ mice

Colorectal cancer incidence is significantly higher in the "Western World" (Parkin *et al.*, 1999). Therefore it was considered useful to administer a "Western style" diet to the Min/+ mice. This high nutrient diet (RM3) formed the basal diet in all of the previously described studies. The composition of the RM3 diet is described in appendix 1. However, it was therefore necessary to determine whether RM3 diet itself was affecting tumour development. Therefore, from 4 - 18 weeks of age Min/+ mice were administered either RM3 " high nutrient diet" or standard AIN 76A diet. Figure 3.7.1.1 shows that administration of the AIN 76A diet compared to the RM3 diet reduced tumour number by 45% (RM3 diet, 110.5 ± 26.7 adenomas; AIN 76A diet 60.7 ± 13.2 adenomas). Colon adenomas, although less numerous in the AIN 76A group, were not significantly altered (RM3 diet, 2.8 ± 2.4 adenomas; AIN 76A diet 2.3 ± 1.2 adenomas). No sex-linked difference in tumour burden was observed.

The reduction in tumour number after AIN 76A administration was significant for both the small sized distal and middle small adenomas (<1 mm). The number of adenomas (<1 mm) was reduced from 41.3 ± 10.6 to 23.2 ± 9.8 in the distal region and 21.9 ± 9 to 11.7 ± 4.7 in the middle region respectively. Reduction of the middle-sized adenomas (1-3 mm) was also observed in these regions although this lacked significance (Figure 3.7.1.2).

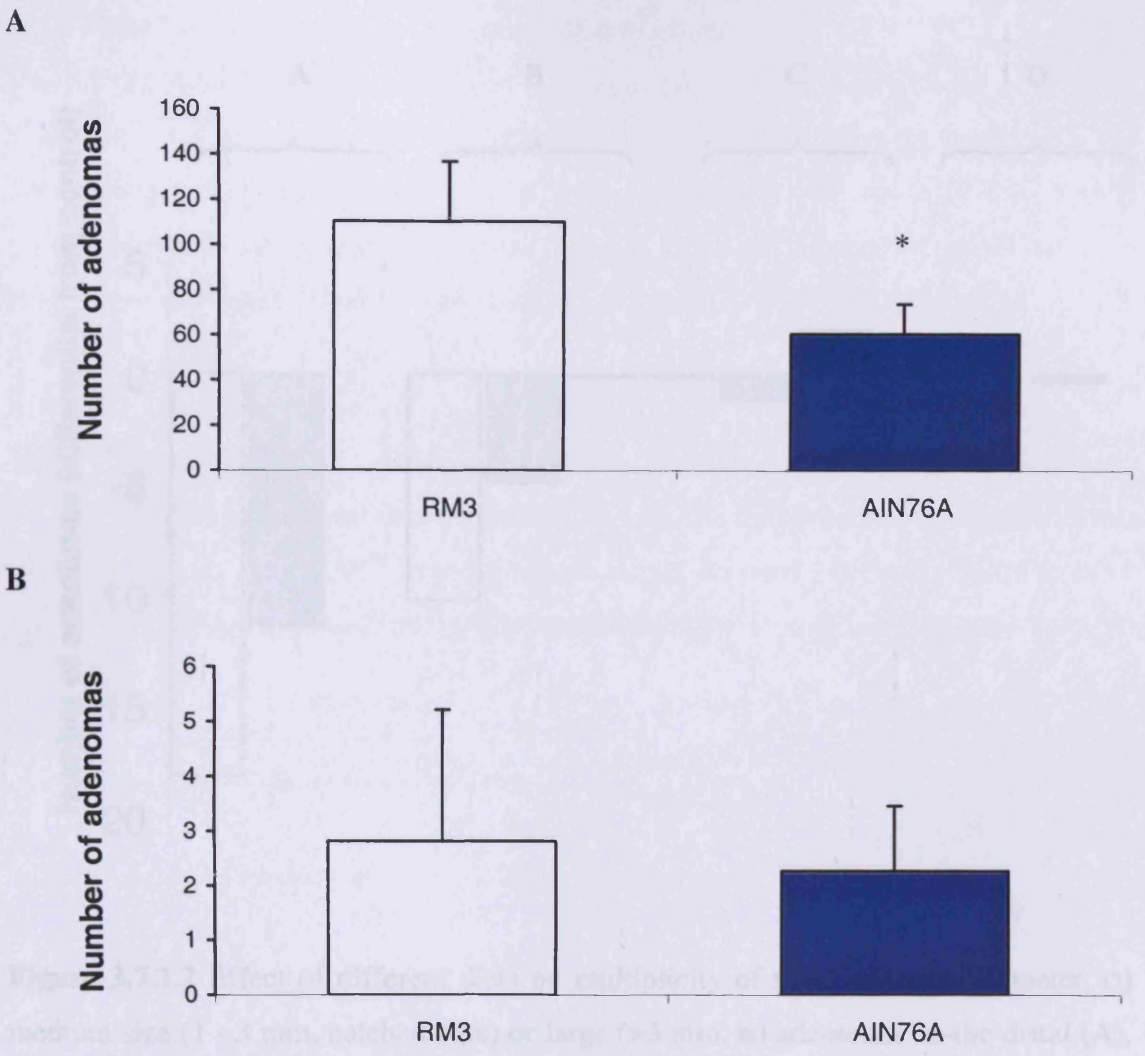

Figure 3.7.1.1 Effect of different diets on adenoma multiplicity in the small intestine (A) and colon (B) of Min/+ mice. (□) Represent RM3 mice and (■) AIN 76A fed mice. Numbers of mice per group were between 11 and 16. Asterisk indicates that the number of adenomas was significantly different from that in control animals ($p<0.05$). Values are mean ± SD.

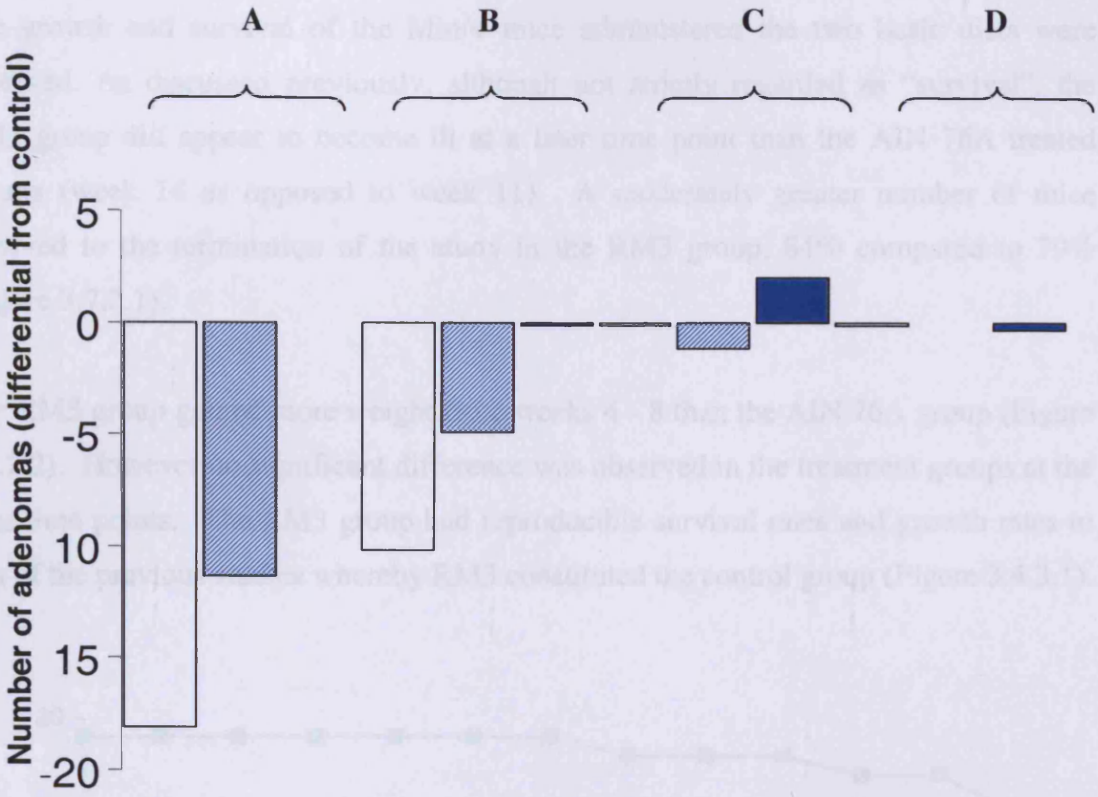

Figure 3.7.1.2 Effect of different diets on multiplicity of small (<1 mm diameter, □) medium size (1 - 3 mm, hatched bars) or large (>3 mm, ■) adenomas on the distal (A), middle (B), proximal (C) or colonic (D) sections of the intestine of Min/+ mice. Results are expressed as mean number of adenomas in Min/+ mice fed AIN 76A diet over or below mean adenoma numbers in Min/+ mice fed RM3 diet. Numbers of mice per group were 11–16. Asterisk indicates that the number of adenomas was significantly different from that in control animals ($p < 0.05$).

3.7.2 Effect of different diets on growth and survival

The growth and survival of the Min/+ mice administered the two basic diets were observed. As discussed previously, although not strictly regarded as "survival", the RM3 group did appear to become ill at a later time point than the AIN 76A treated groups (week 14 as opposed to week 11). A moderately greater number of mice survived to the termination of the study in the RM3 group, 84% compared to 79% (Figure 3.7.2.1).

The RM3 group gained more weight from weeks 4 - 8 than the AIN 76A group (Figure 3.7.2.2). However no significant difference was observed in the treatment groups at the later time points. The RM3 group had reproducible survival rates and growth rates to that of the previous studies whereby RM3 constituted the control group (Figure 3.4.3.1).

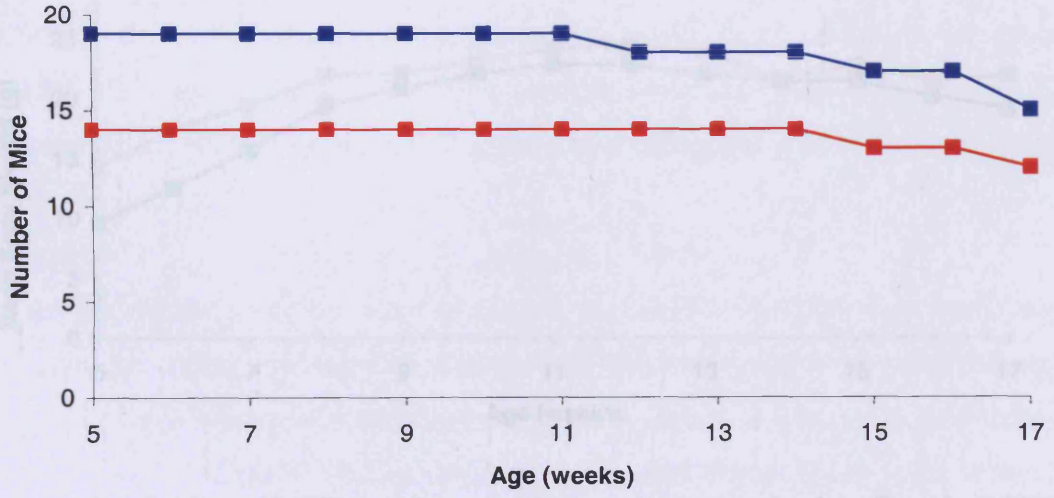

Figure 3.7.2.1 Effect of different diets on Min/+ mouse survival. (■) Represent the control (RM3) groups, and (■) represent the AIN 76A group. The results represent survival as termed by signs of ill-health equivalent to the moderate band of Home office guidelines.

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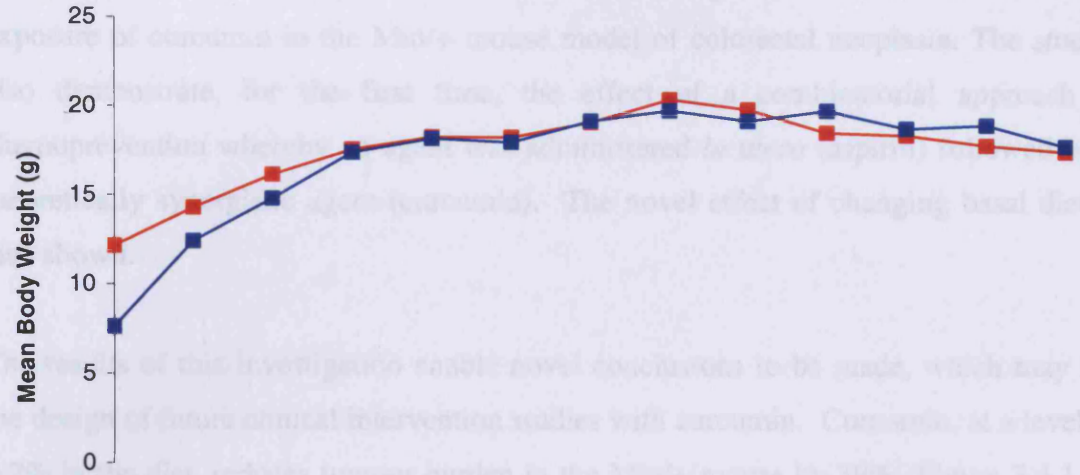

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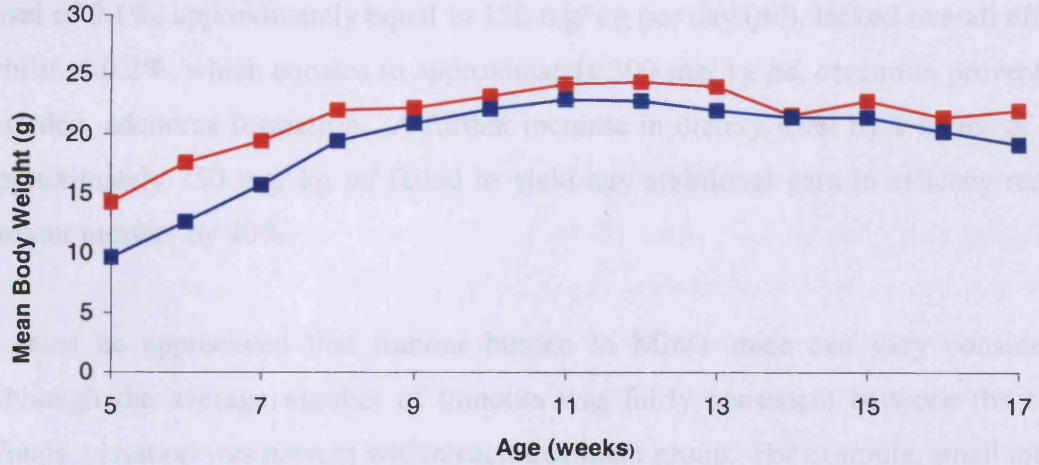


Figure 3.7.2.2 Effect of different diets on Min/+ mouse growth of A) Female C57BL/6J Min/+ mice and B) Male C57BL/6J Min/+ mice. (■) Represent the RM3 diet group and (■) represent the AIN 76A diet group. Numbers of mice per group were 11 – 16. No health check was performed during the commencing or concluding week of the study.

3.8 Discussion

This study defines for the first time the effect of altering both the dose and duration of exposure of curcumin in the Min/+ mouse model of colorectal neoplasia. The studies also demonstrate, for the first time, the effect of a combinatorial approach to chemoprevention whereby an agent was administered *in utero* (aspirin) followed by a theoretically synergistic agent (curcumin). The novel effect of changing basal diet is also shown.

The results of this investigation enable novel conclusions to be made, which may aid the design of future clinical intervention studies with curcumin. Curcumin, at a level of 0.2% in the diet, reduces tumour burden in the Min/+ mouse by 39% (Figure 3.4.1.1). However, the chemopreventive activity of curcumin in the Min/+ mouse model seems to be governed by a rather narrow therapeutic window. Intake of curcumin at a dietary level of 0.1%, approximately equal to 150 mg/ kg per day (*pd*), lacked overall efficacy, whilst at 0.2%, which equates to approximately 300 mg/ kg *pd*, curcumin prevented, or retarded, adenoma formation. A further increase in dietary level by a factor of 2.5 to approximately 750 mg/ kg *pd* failed to yield any additional gain in efficacy reducing tumour number by 40%.

It must be appreciated that tumour burden in Min/+ mice can vary considerably. Although the average number of tumours was fairly consistent between the control groups, variation was present within each treatment group. For example, small intestine tumour number in the 0.5% treatment group varied from 32 to 112. Therefore, assessing trends in tumour retardation must also be noted.

The effect of curcumin on multiplicity of tumours varied depending on their location along the intestinal tract. It was the population of small and medium-size adenomas, which was most susceptible to the preventive efficacy of curcumin, and reduction in adenoma number was most prominent in the middle and distal regions of the intestinal tract, the areas where the majority of tumours occurred. This finding is consistent with previous experience in the Min/+ mouse model using, for example, piroxicam (Jacoby *et al.*, 1996), and the selective COX-2 inhibitor nimesulide (Nakatsugi *et al.*, 1997) or celecoxib (Jacoby *et al.*, 2000b). These small, more numerous tumours are less advanced, suggesting that curcumin retards tumour growth rather than totally inhibiting

it. The H & E slides depict this reduction in tumour size (Figure 3.4.1.2) and it can be seen that the overall surface area occupied by tumours is reduced in the curcumin treated groups. It has been suggested that some adenomas, mainly located in the proximal region are resistant to chemopreventive agents such as piroxicam irrespective of dose and agent (Jacoby *et al.*, 2000). As curcumin appeared to reduce the size of adenomas in this region, at a higher dose curcumin may be unable to totally inhibit their development.

Although curcumin significantly reduced tumour number in the small intestine (0.2%, 0.5%), this agent failed to significantly alter colonic tumours. This may be due to the small number of very large tumours, in which it was difficult statistically to demonstrate size reduction. An appreciable decrease in tumour size was observed (both 0.2% and 0.5% reduced the >3 mm tumours, although not significantly) and therefore curcumin may retard the formation of tumours in both the small intestine and colon of Min/+ mice.

Mahmoud and colleagues (2000) demonstrated that 0.1% curcumin in the diet could reduce tumour formation in the Min/+ mouse by 64%. Collett and colleagues (2001) showed that curcumin (0.2%) reduced proximal adenomas in Min/+ mice treated with PhIP (300 p.p.m), by 52%. In this present study, it has been shown that curcumin (0.2%, 0.5%) can reduce tumour formation in the Min/+ mouse by approximately 40%. Therefore, these studies all confirm the chemopreventive effect of curcumin and the efficacy of this agent in the Min/+ mouse. The high doses required suggest that curcumin is not as effective as the NSAIDS such as piroxicam (Jacoby et *al* 2000). Although, in a clinical setting these traditional NSAIDS agents may induce gastrointestinal side effects, such as peptic ulcers. Curcumin, however, did not induce any side effects in the Min/+ mouse and therefore should be considered as a potential chemopreventive agent for colorectal cancer, possibly in conjunction with other synergistic agents.

In order to achieve chemopreventive activity, exposure to curcumin needs to persist for the whole *post*-weaning lifetime of Min/+ mice. Restriction of exposure to either the first half of this time period, the preneoplastic phase, during which adenomas are usually not yet observed, or the second half, when adenomas are established, was

insufficient to decrease adenoma formation (Figure 3.5.1.2). This is in contrast to previous studies. For example, Ritand and Gendler (1999) demonstrated that piroxicam (0.02%) in AIN 93G administered either from weaning to 100 days or 100 – 200 days reduced adenoma formation by 68% and 96.2% respectively. This data suggested that piroxicam is effective in the regression of established polyps as well as preventing the development of nascent polyps. Early (30 – 80 days) and late (55 – 80 days) treatment with celecoxib (0.05% - 0.15%) also showed a reduction in both the multiplicity and size of tumours in a dose dependant manner (Jacoby *et al.*, 2000b).

Previous studies using curcumin have omitted to investigate both short-term administration of curcumin and the effect of curcumin administered prior to weaning even though the majority of Min/+ mouse adenomas are fixed before 6 days of age (Shoemaker *et al.*, 1995; Reitmair *et al.*, 1996). Therefore, it is possible that potentially the predisposition to tumour development could be modified by *in utero* or preweaning exposure to curcumin. However, it is already recognised that dietary curcumin possesses a poor oral bioavailability (Sharma *et al.*, 2001b), therefore lower levels would be crossing the placenta or be present in the mother's milk and so reduce its chemopreventive potential. However, these studies showed that curcumin failed to modify tumour development when administered at this time (Figure 3.5.1.2). Curcumin also failed to alter the ratio of Min/+ to wildtype offspring (Table 3.5.1).

Conflicting reports exist regarding the chemopreventive efficacy of aspirin in the Min/+ mouse. This may be due to when the aspirin diet is commenced. Further to work by Sansom and colleagues (2001), who demonstrated an increase survival rate when aspirin (0.04%) was administered throughout embryogenesis and lifetime, aspirin was administered *in utero* and prior to weaning. Aspirin (0.05%) *in utero* and prior to weaning reduced tumour formation by 21% ($p<0.05$). A significant reduction in the number of middle-sized adenomas in the middle and distal regions of the small intestine as well as the small adenomas in the proximal region was observed. The decrease in tumour size suggests that aspirin retards tumour development rather than inhibiting tumour formation. In combination with curcumin (0.2%) these effects of aspirin are further enhanced to 36% inhibition, while inhibition of middle sized tumours in the middle region was also significantly increased ($p<0.05$). The degree of attenuation is similar to the effect of long-term administration of curcumin (0.2%) as a single agent

(Figure 3.2.1.1); therefore this combination approach with these agents offers no significant advantage. In contrast, combination studies using sulindac and EKI-569, an irreversible inhibitor of the epidermal growth factor receptor kinase (Torrance *et al.*, 2000), or a combination of piroxicam and difluoromethylornithine (Jacoby *et al.*, 2000) proved more efficacious than single administration of each agent. In both studies a significant proportion of Min/+ mice developed no intestinal tumours. The work described in this chapter utilises agents not previously used in combination as a treatment for colorectal cancer. Moreover, this novel study design of pre-weaning administration of one agent followed by life-time administration of another agent may prove an important strategy when studying combinations of agents such as piroxicam, which have already demonstrated chemopreventive efficacy when administered *in utero* (Jacoby *et al.*, 2000) but may cause side effects if administered long-term.

As with pre weaning administration of curcumin, aspirin did not alter the Mendelian ratio of wild type and Min/+ offspring, neither did it alter survival of Min/+ mice. However, a notable lack of gastrointestinal side effects was observed after aspirin administration. Further studies not reported here suggest that *in utero* exposure to aspirin at doses of 0.1% and 0.3% significantly effected birth rates and the number of surviving weanlings. For example 6 out of 17 litters were lost after birth, and 5 out of 17 failed to produce litters. It has been previously reported that aspirin can cause foetal abnormalities in rodents (Shapiro *et al.*, 1976; Tuchmann-Duplessis *et al.*, 1975) at doses toxic to humans (Wilson *et al.*, 1973). Therefore, increasing the dose of aspirin would not be feasible in this mouse model.

In contrast to work by Mahmoud and colleagues (2000) who showed that curcumin (0.1%) reduced tumour burden in Min/+ mice by 64%, a dietary level of 0.1 % curcumin was insufficient to cause a significant decrease in intestinal adenoma numbers (Figure 3.4.1.1). It is conceivable that this discrepancy is rooted in differences in the dietary matrix employed. In the study described here, a nutrient-rich RM3 rodent breeders diet was utilized, guided by the rationale of mimicking a Western-style diet in humans. In contrast, Mahmoud *et al* (2000) used the AIN 76A chow diet. To explore potential causes of this difference in tumour load and susceptibility to curcumin, an experiment was carried out in which adenoma number was compared between animals on RM3 or AIN-76A diets. Mice on the nutrient-rich RM3 diet bore a significantly

higher adenoma load compared to those on AIN 76A (Figure 3.7.1.1). A similar tentative conclusion as to the role of dietary composition on tumour multiplicity was suggested in a report on the effect of sulindac in Min/+ mice by Boolbol *et al.*, (1996). This interpretation of the influence of diet on tumour burden is also consistent with the finding that beef extract induced, and rye bran decreased, intestinal adenoma multiplicity in Min/+ mice (Mutanen *et al.*, 2000). Yu and colleagues (2001) also showed that fat predisposes to and fibre protects against small intestinal tumours in Min/+ mice. Furthermore, treatment with resistant starch significantly increased the number of intestinal adenomas in $Apc^{1638N/+}$ mice (Williamson *et al.*, 1999). Overall these considerations suggest that a chemopreventive agent like curcumin may be less potent in mice, which are fed an intestinal tumour-promoting diet, predisposing for a high tumour load, than in animals, which receive a non-tumour promoting diet, eliciting fewer tumours.

In an initial study not reported here Min/+ and wild type mice were fed 2% curcumin in AIN 76A diet from weaning. However, approximately 40% of animals died prior to the termination of the study. No abnormalities were observed at autopsy in any of the animals although observations were made that many of the animals appeared to have fits prior to death. These deaths were not linked to either the presence of curcumin or phenotype. However, control Min/+ animals did possess fewer tumours than those reported in RM3 groups described in these studies. Curcumin (2%) also appeared to reduce tumour burden in these mice, although accurate assessment cannot be made due to truncation of the study and the large number of premature deaths.

In conclusion, a number of important observations were made in these studies. Most notably, long-term dietary administration of 0.2% curcumin reduces tumour burden in the Min/+ mouse by 39% without any side effects. Long-term administration of curcumin at a level of 0.5% in the diet fails to further reduce tumour burden. Preweaning administration of aspirin reduced tumour formation in Min/+ mice by 21%. However, sequential administration of curcumin (0.2%) offers no significant advantage over long-term administration of curcumin (0.2%) alone. Furthermore, administration of a high nutrient diet appears to have a tumour promoting effect in Min/+ mice. Having established that curcumin is efficacious in this model, it is necessary to understand potential mechanisms of action of curcumin in the Min/+ mouse.

CHAPTER 4
MARKERS OF CARCINOGENESIS IN THE MIN/+ MOUSE

4.1 Introduction

Studies reported in the previous chapter established an optimal efficacious dose regime of 0.2% curcumin administered from weaning *ad libitum* which significantly reduced tumour burden in the Min/+ mouse by 40%. As described in section 1.9.2, curcumin has been shown to increase enterocyte apoptosis and proliferation (Mahmoud *et al.*, 2000), enhance PhIP induced apoptosis (Collett *et al.*, 2001), decrease the expression of the oncoprotein β-catenin (Mahmoud *et al.*, 2000), and modulate lymphocyte mediated immune functions (Churchill *et al.*, 2000) in Min/+ mice. Curcumin possesses a broad spectrum of activities relevant to chemoprevention, most notably antioxidant properties (as described in section 1.9), but these have not been investigated in the Min/+ mouse. The aims of the work reported here are three fold, 1) determination of a possible mechanism of action of curcumin, 2) determination of indicators of carcinogenesis and 3) determination of the effect of curcumin on these indicators of carcinogenesis (as described in section 1.11).

The use of *in vivo* models in preclinical research is not only to elucidate toxicity and pharmacology efficacy of agents, but also to investigate molecular markers of disease states, which can then be implemented in clinical investigations. Levels of COX-2, and two DNA adducts derived from oxidative stress in intestinal adenomas and surrounding normal mucosa of Min/+ mice were investigated, and an assessment was made of the effect of curcumin on these biomarkers of carcinogenesis. The study of COX-2 expression and DNA adducts is pivotal to the understanding of the early carcinogenic process (Prescott and Fitzpatrick, 2000; De Flora *et al.*, 2001). The work described in this chapter is novel, in terms of the determination of levels in Min/+ mouse tissues and how the levels might be modified by curcumin. Furthermore, the effect of curcumin on haematocrit was also assessed. The degree of anaemia, characterised by the haematocrit, seems to correlate with the ill health of tumour bearing mice. The effect of curcumin on the haematocrit of Min/+ mice has not previously been assessed.

4.2 Levels of COX-2 protein in intestinal adenomas and surrounding mucosa of Min/+ mice and modulation by curcumin

Min/+ mice received curcumin for 14 weeks commencing one week *post*-weaning at three concentrations, 0.1%, 0.2% and 0.5% curcumin. These levels represent a dose of approximately 150 mg/ kg, 300 mg/ kg and 750 mg/ kg. Mice were assigned to each group following a randomised block design. Min/+ mice were culled at 18 weeks of age and localisation of COX-2 protein in the small intestine was determined by immunohistochemistry. Compatible with previous studies (Hull *et al.*, 1999), the Min/+ mice expressed COX-2 in the lamina propria and macrophages of normal tissue. No significant differences could be observed using this method between the adenomas and surrounding epithelial tissue. An increase in the levels of COX-2 could be observed at the luminal surface of the adenomas (as shown below) although this was associated with the epithelial cell necrosis. Curcumin failed to modulate staining. A representative slide showing adenomas and surrounding normal epithelium is shown below (Figure 4.2.1).

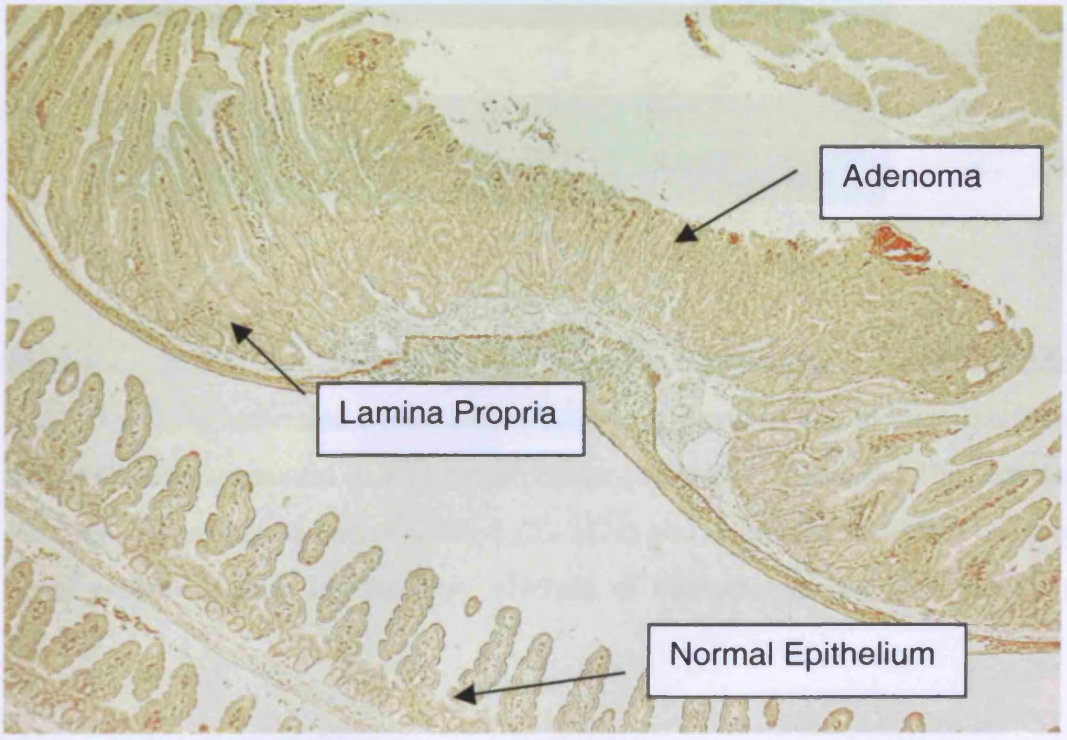


Figure 4.2.1 COX-2 localisation in Min/+ mouse intestine. A representative section showing adenomatous tissue and surrounding normal epithelium. COX-2 (stained brown) was localised predominantly to the macrophages, lamina propria and luminal side of the adenoma. (Details of the methodology are described in section 2.2.8)

The immunohistochemical method proved inconclusive with regard to the up-regulation of COX-2 in Min/+ mice adenomas. Therefore, intestinal adenomas and surrounding mucosa were microdissected from animals under identical experimental conditions and protein levels of COX-2 were studied using Western blot analysis (details of methodology are described in section 2.2.10). A representative blot is shown below (Figure 4.2.2). These results show that COX-2 is up regulated in the adenomas of Min/+ mice compared to the surrounding mucosa.

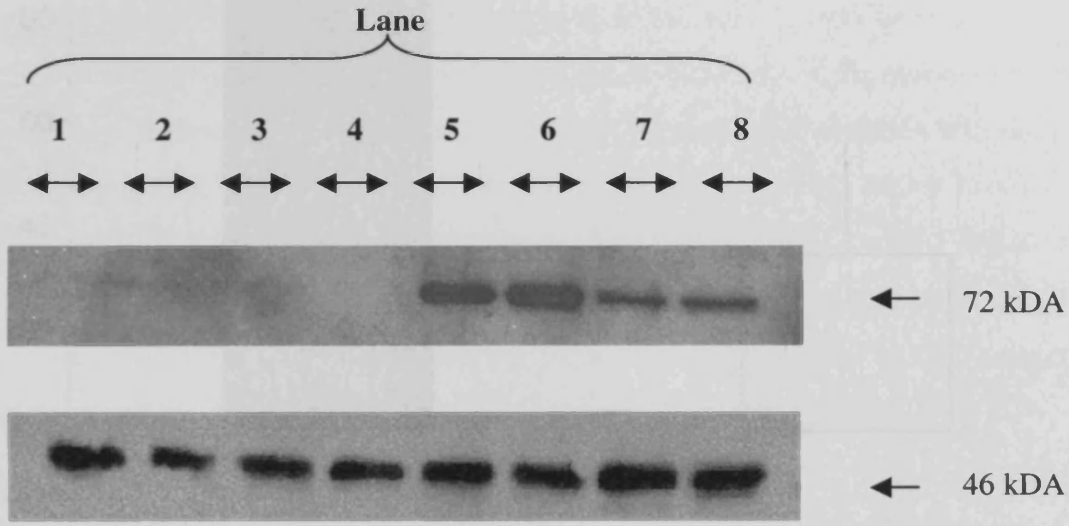


Figure 4.2.2 COX-2 expression in small intestinal mucosa (lanes 1 to 4) and adenomas (lanes 5 to 8) of untreated Min/+ mice (lanes 1, 2, 5 and 6) and of mice that received a diet containing curcumin (0.2%) for 17 weeks (lanes 3, 4, 7 and 8). The representative Western blots show levels of COX-2 (72 kDa) and α-tubulin (46 kDa) to control for equal protein loading and transfer. (Details of methodology are described in section 2.2.10)

Study of the densitometry of the Western blot analysis (depicted in figure 4.2.2) show that curcumin decreased adenoma COX-2 protein levels by 66 ± 19 % compared to adenoma levels in untreated mice. Figure 4.2.3 shows the relative levels of COX-2 in intestinal adenomas from untreated Min/+ mice and Min/+ mice that received curcumin (0.2%).

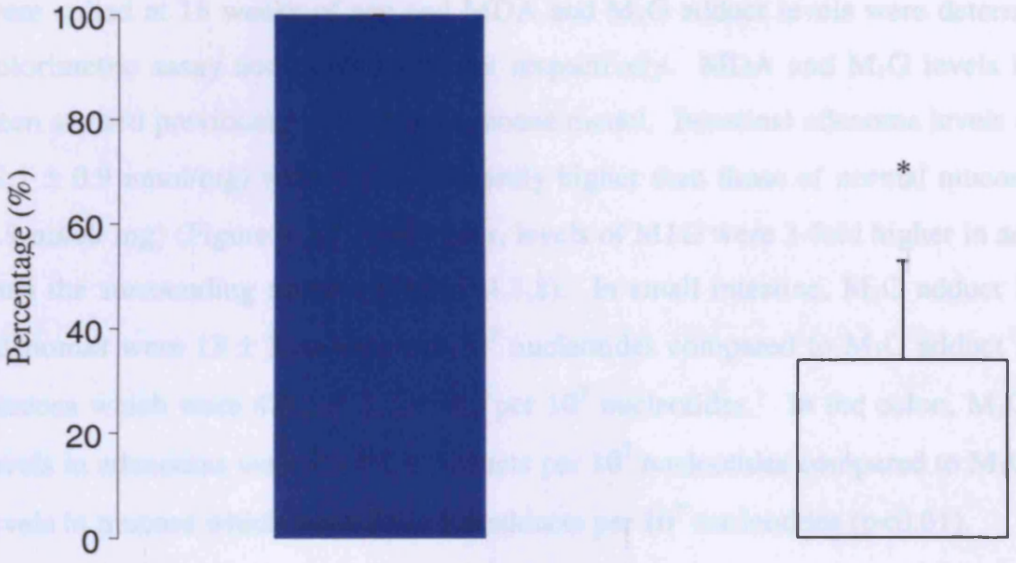


Figure 4.2.3 Effect of curcumin on COX-2 protein levels in adenomas from control (■) and curcumin (0.2%) treated (□) Min/+ mice. Results, which are presented as the percentage of the control COX-2 protein levels, are the mean ± SD of 3 independent determinations performed in duplicate. Asterisk indicates that the value is significantly different from the control mice ($p = 0.001$ by 2-sided t-test; $df = 17$).

4.3 MDA and M_1G adduct levels in intestinal mucosa and adenomas of Min/+ mice and modulation by curcumin

Min/+ mice received curcumin for 14 weeks commencing one week *post*-weaning at two concentrations, 0.1%, 0.2% curcumin. The doses of 0.2% and 0.5% curcumin reduced tumour burden by an equal amount, therefore the higher dose level was not studied. These levels represent a dose of approximately 150 mg/ kg and 300 mg/ kg. Mice were assigned to each group following a randomised block design. Min/+ mice were culled at 18 weeks of age and MDA and M_1G adduct levels were determined by colorimetric assay and immunoslotblot respectively. MDA and M_1G levels have not been studied previously in the Min/+ mouse model. Intestinal adenoma levels of MDA (2.1 ± 0.9 nmol/mg) were not significantly higher than those of normal mucosa (0.9 ± 0.9 nmol/ mg) (Figure 4.3.1). However, levels of M1G were 3-fold higher in adenomas than the surrounding mucosa (Figure 4.3.2). In small intestine, M_1G adduct levels in adenomas were 13 ± 3 adducts per 10^7 nucleotides compared to M_1G adduct levels in mucosa which were 4.0 ± 1.5 adducts per 10^7 nucleotides. In the colon, M_1G adduct levels in adenomas were 9.8 ± 2.1 adducts per 10^7 nucleotides compared to M_1G adduct levels in mucosa which were 2.8 ± 1.4 adducts per 10^7 nucleotides ($p<0.01$).

These M_1G adduct levels in adenomas could also be modulated by the administration of dietary curcumin (Figure 4.3.2). At a low dietary concentration (0.1%), curcumin decreased small intestinal adenoma M_1G levels by 43%, from 13 ± 3 adducts per 10^7 nucleotides in untreated mice to 7.5 ± 2.1 adducts per 10^7 nucleotides in mice on curcumin (p = 0.006 by ANOVA). In the colon of Min/+ mice, 0.1% curcumin decreased adenoma M_1G levels by 35%, from 9.8 ± 2.1 adducts per 10^7 nucleotides in untreated mice to 6.4 ± 3.3 adducts per 10^7 nucleotides in mice on curcumin (p = 0.006 by ANOVA). This dose did not affect adenoma number (as shown in Figure 3.4.1.1). At the higher dose level (0.2%), curcumin decreased small intestinal adenoma M_1G levels by 42%, from 13 ± 3 adducts per 10^7 nucleotides in untreated mice to 7.6 ± 2.9 adducts per 10^7 nucleotides in mice on curcumin (p = 0.006 by ANOVA). In the colon of Min/+ mice, 0.2% curcumin decreased adenoma M_1G levels by 42%, from 9.8 ± 2.1 adducts per 10^7 nucleotides in untreated mice to 5.7 ± 3.2 adducts per 10^7 nucleotides in mice on curcumin (p = 0.006 by ANOVA).

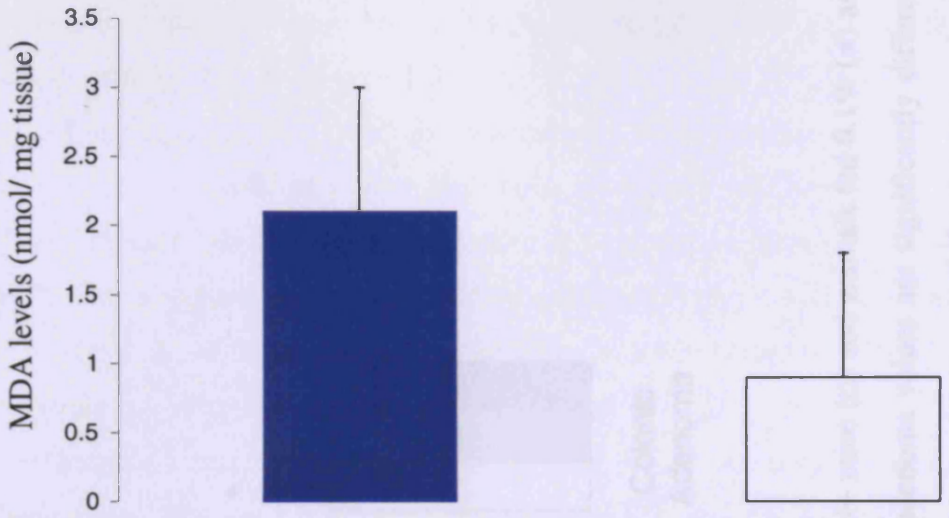


Figure 4.3.1 Levels of MDA in small intestinal mucosa (□) and intestinal adenomas in control Min/+ mice (■) Values are mean ± SD of at least 8 animals. (Details of methodology are described in section 2.2.13)

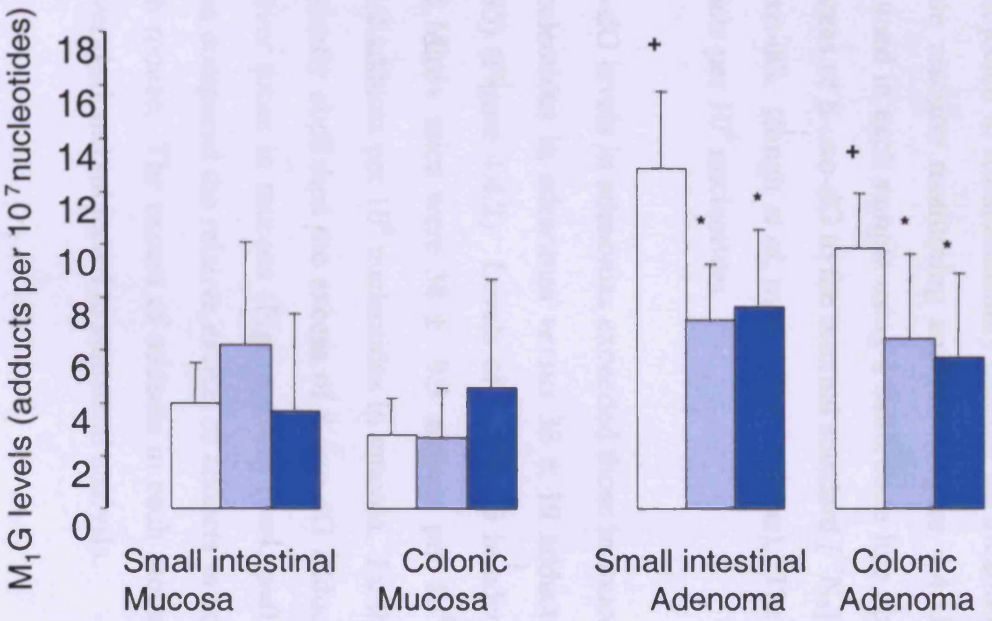


Figure. 4.3.2 Levels of M_1G adduct in normal mucosa and intestinal adenomas in control Min/+ mice (□) and animals fed 0.1% (▪) and 0.2% (■) dietary curcumin. Values are mean ± SD of at least 8 animals. Crosses indicate that adenoma values are significantly different from mucosal values for control animals (by ANOVA, $P < 0.01$). Stars indicate that adenoma values of curcumin-fed animals are significantly different from adenoma values for control animals (by ANOVA, $P < 0.01$).

4.4 Determination of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG) levels in small intestinal mucosa and adenomas of Min/+ mice and modulation by curcumin

8-Oxo-dG can be measured using a variety of techniques including capillary electrophoresis (Inagaki *et al.*, 2001), ^{32}P postlabelling HPLC (Zeisig *et al.*, 1999), liquid chromatography - mass spectrometry (LC-MS) (Renner *et al.*, 2000). The work described here utilised a new technique developed by Raj Singh and colleagues (Department of Biochemistry, University of Leicester). Digested DNA was analysed by HPLC to determine the level of deoxyguanosine (Figure 4.4.1A). Digested DNA was also subjected to immunoaffinity column purification prior to positive ion LC-MS/MS multiple reaction monitoring analysis (Figure 4.4.1B). The level of 8-oxo-dG was determined in each sample using a calibration line obtained by plotting the ratio of the peak areas of 8-oxo-dG to the internal standard [$^{15}N_5$]8-oxo-dG against known amounts of 8-oxo-dG. (Singh *et al*, manuscript in press). The detection limit for 8-oxo-dG was 8 adducts per 10^6 nucleotides.

8-Oxo-dG levels in adenomas exceeded those in mucosa by 32 % (50 ± 38 adducts per 10^6 nucleotides in adenomas *versus* 38 ± 19 adducts per 10^6 nucleotides in mucosa, p=0.185) (Figure 4.4.2). Levels of 8-oxo-dG in adenoma and mucosa from curcumin treated Min/+ mice were 38 ± 9.3 adducts per 10^6 nucleotides in adenomas *versus* 43 ± 9.2 adducts per 10^6 nucleotides in mucosa. Further analysis showed that curcumin significantly abolished the excess of 8-oxo-dG adducts seen in adenomas of untreated mice over those in mucosa (Figure 4.4.2) (n=4, p=0.04 by balanced ANOVA). This analysis compared the relative excess of adducts in adenomas to adducts in the mucosa of each mouse. The excess of adducts in each mouse within the treatment group was then averaged and subjected to statistical analysis.

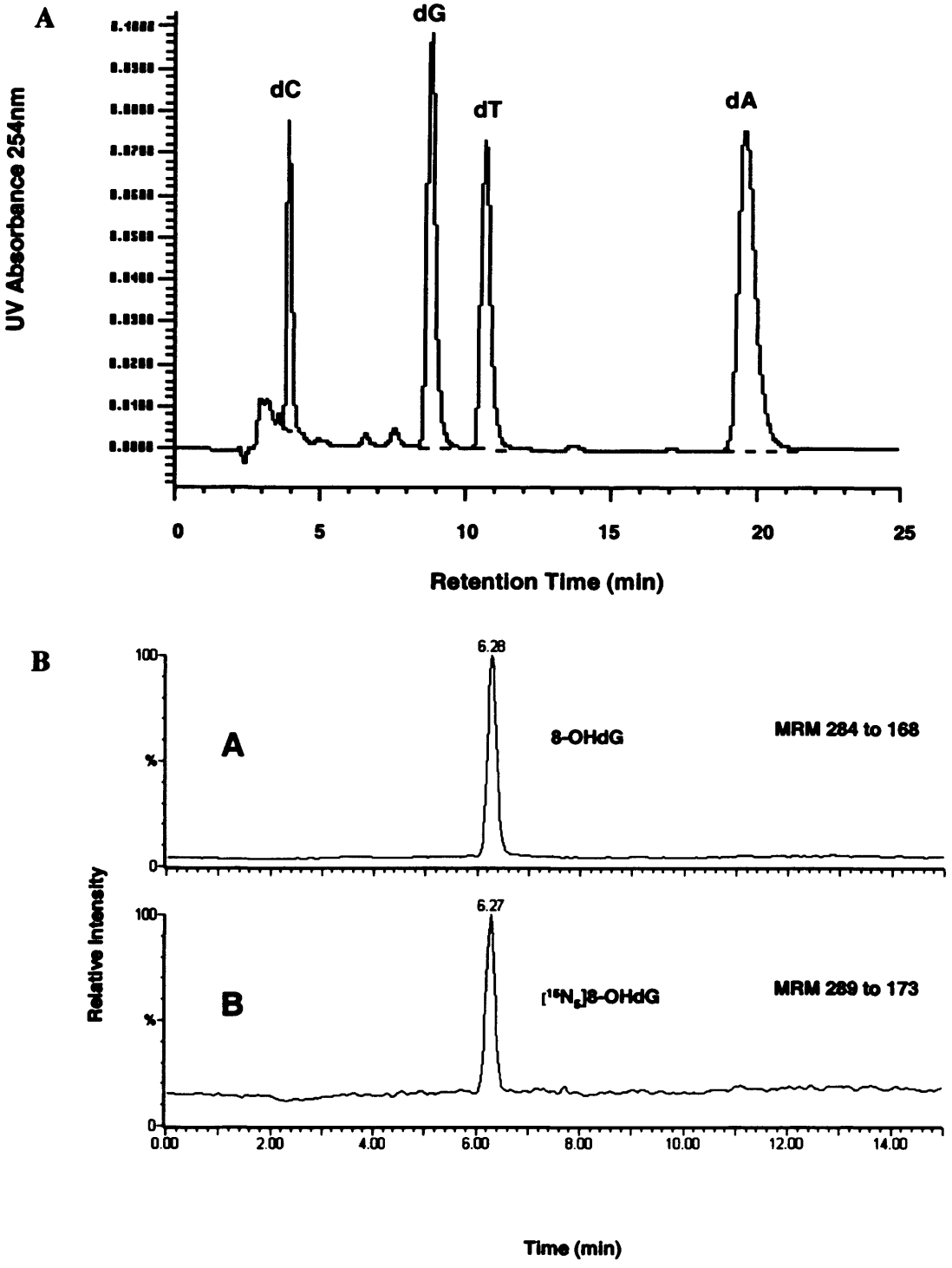


Figure 4.4.1 **A)** Representative HPLC analysis of digested DNA extracted from Min/+ mouse intestinal adenomas. **B)** Representative LC-MS/MS analysis of immunoaffinity purification of digested DNA extracted from Min/+ mouse intestinal adenoma. The internal standard was [$^{15}N_5$]8-oxo-dG. against known amounts of 8-oxo-dG. (Singh *et al*, manuscript in press). The detection limit for 8-oxo-dG was 8 adducts per 10^6 nucleotides. (Details of methodology are described in section 2.2.14)

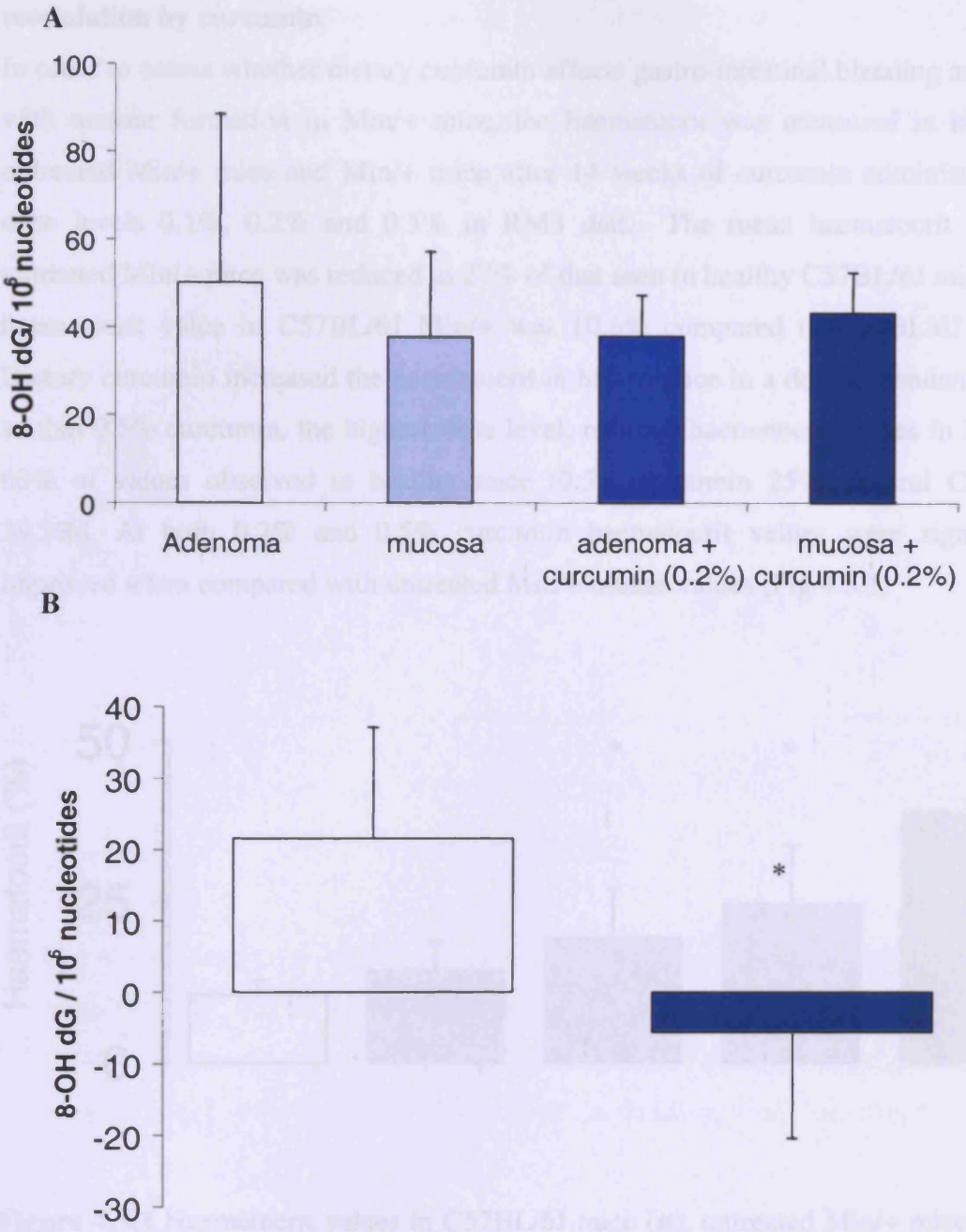


Figure 4.4.2 A) 8-Oxo-dG levels in intestinal mucosa and adenomas of control Min/+ mice and Min/+ mice which received a diet containing curcumin (0.2%) (n = 4, mean ± SD). **B)** Differential 8-oxo-dG levels between adenomatous and normal intestinal mucosa in control Min/+ mice (□) and Min/+ mice that received a diet containing curcumin (0.2%) (■). Curcumin significantly abolished the excess 8-oxo-dG levels in adenomatous tissue (n = 4, mean ± SD, p=0.04 by balanced ANOVA).

4.5 Determination of the relationship between tumour number and haematocrit modulation by curcumin

In order to assess whether dietary curcumin affects gastro-intestinal bleeding associated with tumour formation in Min/+ mice, the haematocrit was measured in individual untreated Min/+ mice and Min/+ mice after 14 weeks of curcumin administration at dose levels 0.1%, 0.2% and 0.5% in RM3 diet. The mean haematocrit value in untreated Min/+ mice was reduced to 27% of that seen in healthy C57BL/6J mice. (The haematocrit value in C57BL/6J Min/+ was 10.6% compared to C57BL/6J 39.3%). Dietary curcumin increased the haematocrit in Min/+ mice in a dose-dependant fashion so that 0.5% curcumin, the highest dose level, restored haematocrit values in Min/+ to 63% of values observed in healthy mice (0.5% curcumin 25%, control C57BL/6J 39.3%). At both 0.2% and 0.5% curcumin haematocrit values were significantly improved when compared with untreated Min/+ mouse values (Fig 4.5.1)

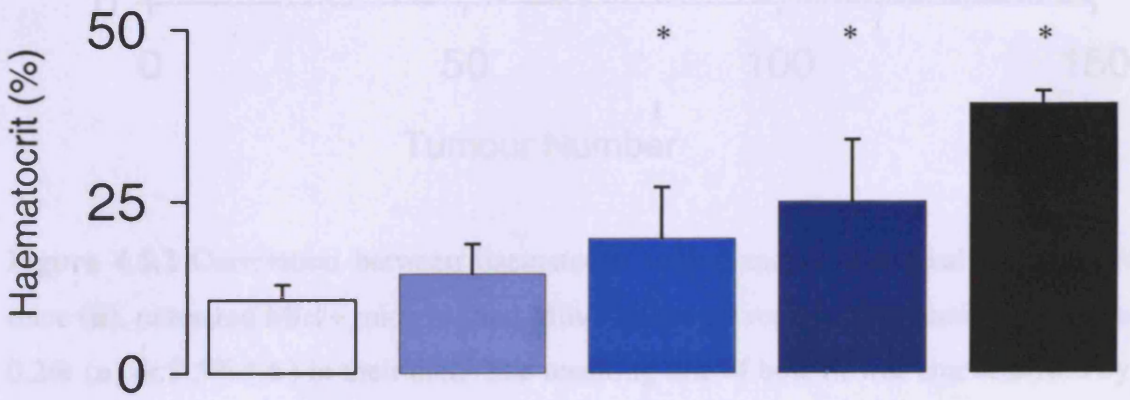


Figure 4.5.1 Haematocrit values in C57BL/6J mice (■), untreated Min/+ mice (□) and Min/+ mice that received a diet containing curcumin at 0.1% (■), 0.2% (■) or 0.5% (■). Values represent the percentage of the blood volume occupied by the packed erythrocytes and are the mean ± SD of 10-12 mice. Asterisk indicates that values are significantly different from those in untreated Min/+ mice ($p<0.05$) (Details of methodology are described in section 2.2.15)

When haematocrit values were plotted against adenoma number irrespective of Min/+ status or diet, a high negative correlation was observed (Figure 4.5.2). The correlation coefficient was -0.84. Very little variation was observed with healthy wildtype mice (37% - 42%) and although the haematocrit values varied within each treatment group, a clear trend between tumour number and haematocrit value was observed.

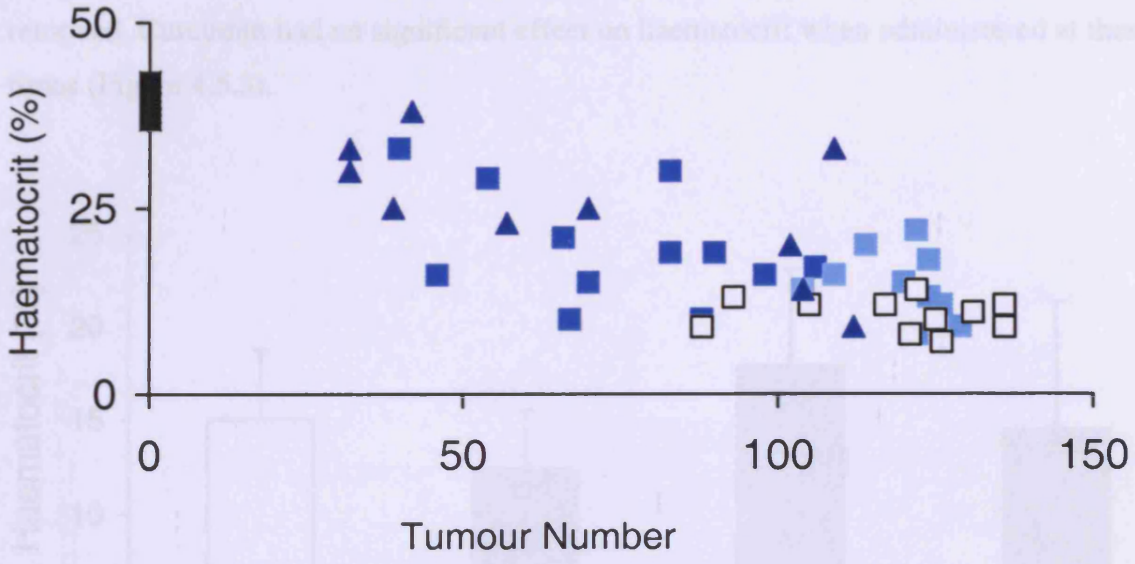


Figure 4.5.2 Correlation between haematocrit values and tumour load in C57BL/6J mice (■), untreated Min/+ mice (□) and Min/+ mice that received curcumin at 0.1% (■), 0.2% (■) or 0.5% (▲) in their diet. The resulting line of best fit was characterised by a correlation coefficient of –0.84. (Details of methodology are described in section 2.2.15)

In order to assess whether dietary curcumin administered at different time intervals affects gastro-intestinal bleeding associated with tumour formation in Min/+ mice, the haematocrit was measured in individual animals after short-term administration of curcumin in RM3 (experimental details are described in section 2.2.5). Min/+ mice received curcumin (0.2%) for one of the following three time intervals, perinatally and until 30 days of age, from 30-75 days of age or from 75-120 days of age. These stages approximately reflect the initiation, promotion and established neoplasm stages of carcinogenesis. Min/+ mice received control (RM3) diet after the curcumin diet was removed. Curcumin had no significant effect on haematocrit when administered at these times (Figure 4.5.3).

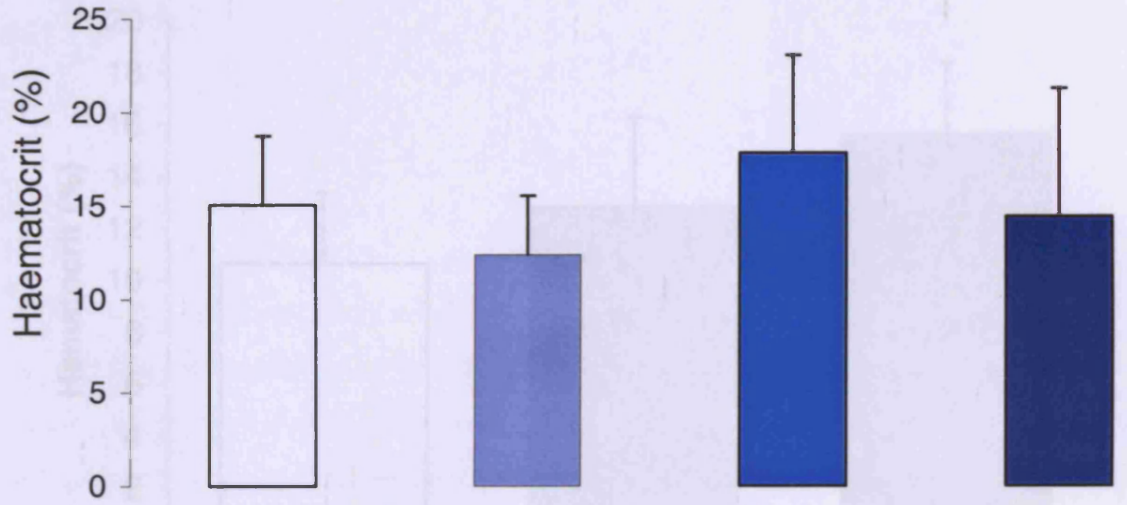


Figure 4.5.3 Haematocrit values in untreated Min/+ mice (□) and Min/+ mice that received curcumin (0.2%) perinatally (■), from 30-75 days (■) or from 75-120 days (■) in their diet. Values represent the percentage of the blood volume occupied by packed erythrocytes and are the mean ± SD of 10-20 mice. (Details of methodology are described in section 2.2.15)

Curcumin (0.2%) when administered from weaning to Min/+ mice increased their haematocrit from 10.6% (untreated Min/+ mice) to 19.6% (Figure 4.5.1). When curcumin treatment was preceded by perinatal exposure to aspirin (0.05%) this value was not significantly different (15.6%, $p > 0.05$) from Min/+ mice that received curcumin post weaning (0.2%). However, the haematocrit of Min/+ that received the combination diet was significantly different from the haematocrit of control untreated Min/+ mice (10.6%) (Figure 4.5.4). Perinatal exposure to aspirin (0.05%) alone raised the haematocrit to 12.8%, although this was not significantly different from the haematocrit of Min/+ mice that received control (RM3) diet (Figure 4.5.4).

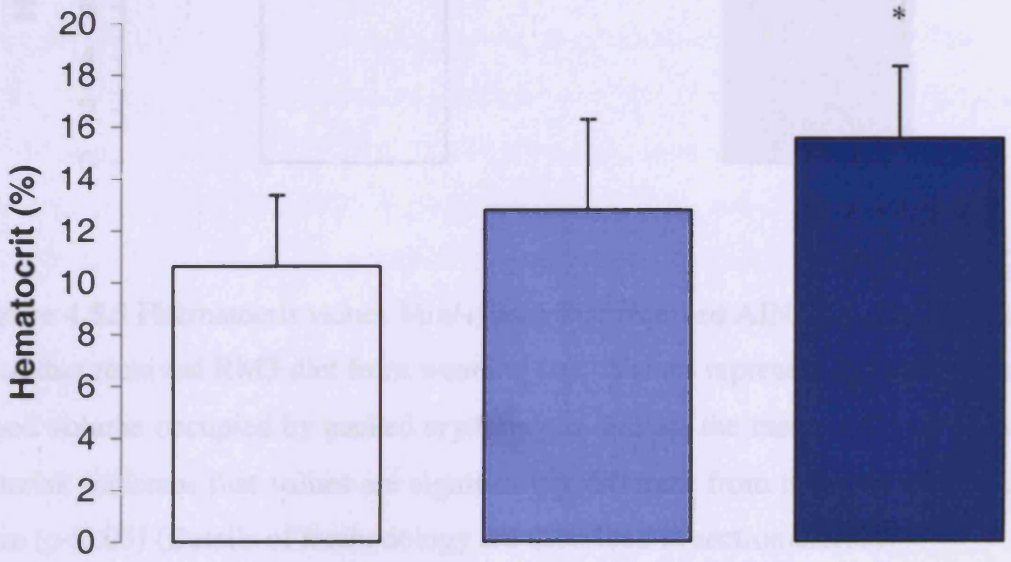


Figure 4.5.4 Haematocrit values in untreated Min/+ mice (□) and Min/+ mice that received a diet containing aspirin perinatally (0.05%) (■), or aspirin perinatally followed by curcumin (0.2%) (■). Values represent the percentage of the blood volume occupied by packed erythrocytes and are the mean ± SD of 8 – 10 mice. Asterisk indicates that values are significantly different from those in untreated Min/+ mice ($p < 0.01$). (Details of methodology are described in section 2.2.15)

In chapter 3, it was shown that changing the basal diet from RM3 to AIN 76A significantly reduced tumour burden in Min/+ mice. The reduction in tumour burden was not accompanied by a significant decrease in haematocrit (RM3 13 ± 4.8; AIN 76A 9.3 ± 2.9).

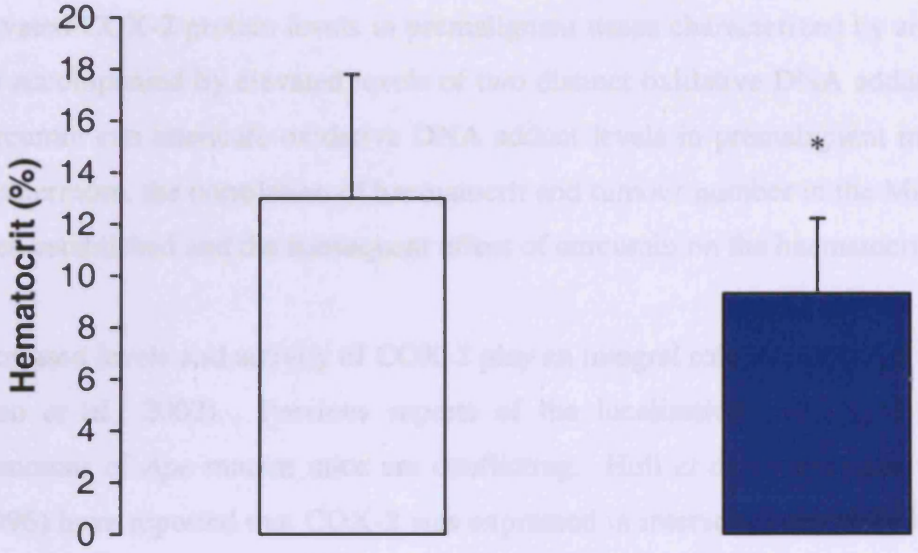


Figure 4.5.5 Haematocrit values Min/+ mice that received AIN 76A diet (■) and Min/+ mice that received RM3 diet from weaning (□). Values represent the percentage of the blood volume occupied by packed erythrocytes and are the mean ± SD of 12–16 mice. Asterisk indicates that values are significantly different from those in untreated Min/+ mice ($p<0.05$) (Details of methodology are described in section 2.2.15)

4.6 Discussion

The aim of the work described here was to identify possible markers of carcinogenesis and the effect of curcumin on these markers, thus identifying possible mechanisms for the chemopreventive efficacy of curcumin. A number of conclusions can be drawn which are potentially relevant to the chemoprevention of intestinal cancer. Firstly, elevated COX-2 protein levels in premalignant tissue characterized by an *Apc* mutation are accompanied by elevated levels of two distinct oxidative DNA adducts. Secondly, curcumin can attenuate oxidative DNA adduct levels in premalignant intestinal tissue. Furthermore, the correlation of haematocrit and tumour number in the Min/+ mouse has been established and the subsequent effect of curcumin on the haematocrit determined.

Increased levels and activity of COX-2 play an integral role in colorectal carcinogenesis (Cao *et al.*, 2002). Previous reports of the localisation of COX-2 protein in the adenomas of *Apc* mutant mice are conflicting. Hull *et al.*, (1999) and Oshima *et al.*, (1996) have reported that COX-2 was expressed in interstitial cells within adenomas of *Apc* mutant mice. However, Williams *et al.*, (1996) described immunostaining for COX-2 in the adenomatous epithelium of a small number of polyps from B6 x 129 Min/+ mice. The work described here shows COX-2 staining in macrophages and lamina propria of normal mucosa (Figure 4.2.1). In accordance with Hull *et al,* (1999), staining was also observed in interstitial cells of adenomas and in cells in close proximity to areas of eroded adenoma surface.

Western blot analysis shows that the expression of COX-2 is significantly upregulated in intestinal adenomas compared to normal surrounding mucosa (Figure 4.2.2). This finding is in agreement with Lal *et al.*, (2001) who demonstrated, by Western blot analysis, increased COX-2 levels in both small and large intestinal adenomas of Min/+ mice. The low levels of COX-2 in surrounding normal mucosa (Figure 4.2.2) is similar to previous studies by Oshima *et al.*, (1996) and Williams *et al.*, (1996) who failed to detect COX-2 in normal small intestinal mucosa of *Apc* mutant mice by Western blot analysis and imunohistochemistry respectively. Large amounts of protein (~400 μg) had to be loaded onto the gel to observe the presence of any COX-2 so the levels present in the adenomas are low which may explain the equivocal result observed when analysing this protein using immunohistochemistry. However, it may be concluded that

COX-2 protein levels are increased in adenomas compared to normal mucosa in the intestine of Min/+ mice.

Dietary administration of curcumin reduced the expression of COX-2 protein in the adenomas of Min/+ mice. This novel result may indicate the contribution that COX-2 inhibition has on the chemopreventive efficacy of curcumin in the Min/+ mouse. The reduction in COX-2 expression by curcumin in Min/+ mice is in accordance with *in vitro* studies, in which curcumin decreased the expression of COX-2 in intestinal cell lines (Zhang *et al.*, 1999). The inhibition of COX-2 by curcumin has been shown to involve the inhibition of NFκB activation via the NIK/IKK signalling complex (as described in section 1.9; Plummer *et al.*, 1999).

DNA adducts formed by endogenous products of oxidative stress have been implicated in the early stages of carcinogenesis in a number of tissues (reviewed in Marnett, 2000), but their detection in premalignant tissue has thus far not been reported. In conjunction with elevated levels of COX-2, M_1G adduct levels were also raised in adenomas compared to normal mucosa of Min/+ mice. M_1G adducts have been associated with carcinogenesis in rodents and detected in human liver, pancreas, and breast tissues and in blood leukocytes (reviewed in Marnett, 1999). M_1G adducts can be generated by MDA, an intracellular product of lipid peroxidation (Marnett, 1999) or indirectly *via* base propenals resulting from oxidative DNA damage (Dedon *et al.*, 1998; Plastaras *et al.*, 2000a). MDA levels in adenomas and normal mucosa of Min/+ mice were not significantly different (Figure 4.3.1). The lack of reproducible data, borne out by the high standard deviation, with regard to MDA levels may be a consequence of the short half-life and the potential for artifactual generation of MDA *ex vivo*. The analysis of DNA adducts produced by MDA and reactive oxygen species appears to be less problematic since the DNA adducts are more stable and theoretical concerns about production of artifacts can be alleviated by careful method development and experimental design (Marnett, 1999; Marnett 2000). The failure to show a correlation between MDA and M_1G levels also suggests that M_1G adducts may be formed indirectly *via* oxidative damage in the adenomas of Min/+ mice.

8-Oxo-dG levels were 32 % higher in intestinal adenomas compared to surrounding normal mucosa of Min/+ mice (50 ± 38 adducts per 10^6 nucleotides in adenomas *versus* 38 ± 19 adducts per 10^6 nucleotides in mucosa, p=0.185) (Figure 4.4.2). 8-Oxo-dG, the most extensively studied adduct arising from oxidative DNA damage, is generated by reactive oxygen species, particularly the hydroxyl radical, which can bind to, or abstract hydrogen atoms from, DNA bases (Marnett 2000). The raised 8-oxo-dG levels in adenomatous tissue, in conjunction with M_1G levels, highlight the importance of oxidative damage in the development of tumours in the Min/+ mouse.

Curcumin was shown to decrease levels of M_1G and 8-oxo-dG in adenomas of Min/+ mice (Figures 4.3.2 and 4.4.2). The results presented here suggest the chemopreventive efficacy of curcumin may be partly due to its antioxidant properties (as described in section 1.9). Manipulation of rodent and human diets has previously been shown to alter colon mucosal and blood leukocyte levels of M_1G adducts (Plastaras *et al.*, 2000a; Sharma *et al.*, 2001b), and dietary administration of the antioxidant N-acetylcysteine has recently been shown to reduce 8-oxo-dG levels in the hind leg of mice by 50% (Malins *et al.*, 2002). These results suggest that levels of M_1G and 8-oxo-dG can be affected by antioxidants *in vivo*, probably including premalignant target tissues. Thus, M_1G and 8-oxo-dG could serve as biomarkers of the chemopreventive efficacy of antioxidants administered in a clinical setting.

One of the phenotypic signs of adenoma maturation in the Min/+ mouse is pale feet and tail tip, which is associated with anaemia. The morbidity is a consequence of the intraluminal haemorrhage and adenoma ulceration associated with tumour development. Figure 4.5.2 shows the high negative correlation between the haematocrit, a measure of anaemia, and tumour load regardless of the diet formulation administered to the Min/+ mice. Long-term administration of curcumin elevated the haematocrit (Figure 4.5.1) in a dose dependant manner. Short-term administration of curcumin was without effect. The rise was in accordance with decreased tumour burden. The finding that dietary curcumin administration is accompanied by raised haematocrit of Min/+ mice is especially intriguing when the advantages and disadvantages of treatment with curcumin are juxtaposed with those reported for cancer chemopreventive NSAIDs such as aspirin, sulindac or piroxicam. Non-selective

COX-2 inhibitors have the notorious drawback that they can elicit severe adverse effects in the gastro-intestinal tract. In the case of, for example, piroxicam, one in five humans on 20 mg (approximately 0.3 mg/ kg) per day experienced adverse gastro-intestinal side effects ranging in severity from dyspepsia to intestinal perforation (Henry *et al.*, 1996). Consistent with the clinical picture, more than 90 % of Min/+ mice which received piroxicam in the diet at a dose corresponding to approximately 33 mg/ kg daily presented with grossly detectable intestinal ulceration after six or more days of treatment, with the most severe lesions occurring in the duodenum (Ritland and Gendler, 1999). In addition to intestinal ulceration, more than 50 % of these mice also displayed regions of benign polypoid hyperplasia in the colon. In contrast to the experience with non-selective COX-2 inhibitors, curcumin not only failed to exacerbate adenoma-induced intestinal bleeding, it actually ameliorated it significantly.

No significant difference in haematocrit was observed between control and Min/+ mice that received pre-weaning administration of aspirin. The short scale of administration and the route of administration may explain this. Aspirin would be reaching the systemic circulation of the foetus directly, or in small quantities in the mother's milk. Therefore, the short term administration of small doses which would reach the intestine may be below concentrations which would elicit long term gastrointestinal side effects. A dosing regime whereby traditional NSAIDS, such as aspirin are administered to individuals prior to the development of polyps may hold promise for the alleviation of traditional NSAID-associated side effects. However, the chemopreventive benefit of such a regime would undoubtedly have to be explored.

The change in basal diet, from RM3 to AIN 76A decreased haematocrit, even though the tumour burden was reduced. The paradoxical result may be explained by the decrease in nutrient consumption, which would limit the potential for erythrocyte production. The decrease in erythrocyte production would augment the anaemia, which would be manifest as a lower haematocrit.

Further work was performed to determine possible markers of carcinogenesis and the subsequent effect of curcumin on these indicators. The Min/+ bears a mutant in the *Apc* gene, which leads to translocation of β-catenin to the nucleus and increased

transcription of a number of target genes (as described in section 1.6). Following on from the work of Mahmoud *et al.*, (2000), levels and localisation of β-catenin were studied in the adenomas of Min/+ mice that received curcumin at three dietary dose levels (0.1%, 0.2% and 0.5%) for 14 weeks which commenced one week post weaning. As expected, elevated levels of β-catenin were observed in the adenomas of Min/+ mice although no modification of these levels was observed by curcumin (Results not shown). The work was followed by western blot analysis of β-catenin expression, although this proved inconclusive due to time and methodological constraints. Further immunohistochemical analysis was performed to study Bcl-2 and Caspase3 although no staining was observed on the methacarn fixed, paraffin embedded sections and this was validated by the Pathology Section, MRC toxicology Unit, University of Leicester.

These studies have therefore highlighted possible markers of carcinogenesis that may be utilised in clinical trials. Levels of COX-2 protein and M_1G adducts are significantly higher in adenomas of Min/+ mice. Curcumin modified levels of both M_1G and 8-oxo-dG adducts in adenomas of Min/+ mice and also altered the expression of COX-2 protein in these adenomas. Futhermore, a strong negative correlation exists between haematocrit and tumour burden in Min/+ mice and dietary administration of curcumin can modify the haematocrit of these mice. To conclude, the effect of curcumin on these markers has been determined and may offer starting points for the determination of the mechanism of action of curcumin in reducing tumour burden in the Min/+ mouse.

CHAPTER 5
THE EFFECT OF CURCUMIN ON GENE TRANSCRIPTION IN MIN/+ AND WILDTYPE MICE USING cDNA MICROARRAYS

5.1 Introduction

Since the introduction of DNA microarray in the mid 1990's (Schena *et al.*, 1995) the applications of this relatively nascent technique have been shown to be immense. Microarray enables the total gene compliment of a cell to be analysed in a single experiment. This is particularly important in cancer, a disease that is caused by an accumulation of abnormalities in the sequence and expression of a number of critical genes. With respect to carcinogenesis, this technique maybe employed to include:

1) Development of a more global understanding of the gene expression abnormalities that contribute to carcinogenesis;
2) Discovery of new diagnostic and prognostic indicators and biomarkers of therapeutic response;
3) Identification and validation of new molecular targets for drug development;
4) Provision of an improved understanding of molecular mode of action during compound identification and optimisation, including structure activity relationships;
5) Prediction of potential side effects during preclinical development and toxicology studies;
6) Confirmation of a molecular mode of action during hypothesis-testing clinical trials;
7) Identification of genes involved in conferring drug sensitivity and resistance;
8) Characterisation of patients most likely to benefit from the drug and use in general pharmacogenomic studies (reviewed in Clarke *et al.*, 2001).

Gene expression microarray technology is based on hybridisation of labelled RNA or DNA in solution to DNA molecules attached at specific locations on a surface. It is founded on a number of important methodological developments including blotting techniques, key recombinant DNA technologies such as PCR, availability of gridded libraries and the accumulation of tens of thousands of sequenced cDNA and expressed sequence tags (ESTs) clones from the various genome-sequencing initiatives.

The array may take one of two forms: a) those where oligonucleotides are synthesised on the array *in situ* using photolithographic or other techniques (Lipshutz *et al.*, 1999) and b) arrays where nucleic acids (PCR products, plasmids or oligonucleotides) are robotically deposited onto the solid support. Technological advances over the last 4

years now allow in the order of 100,000 oligonucleotides or 10,000 PCR products to be arrayed per cm^3. The arrays are probed with labelled single-strand cDNA representations of the cellular RNA pool. In the case of arrays printed on glass slides, as in the experiments described in this chapter, RNA from test and reference samples can be compared simultaneously on a single array by using fluorescent labels that emit light at different excitation wavelengths (Figure 5.1.1). This approach yields data on relative RNA abundance based on direct comparison between test and reference samples.

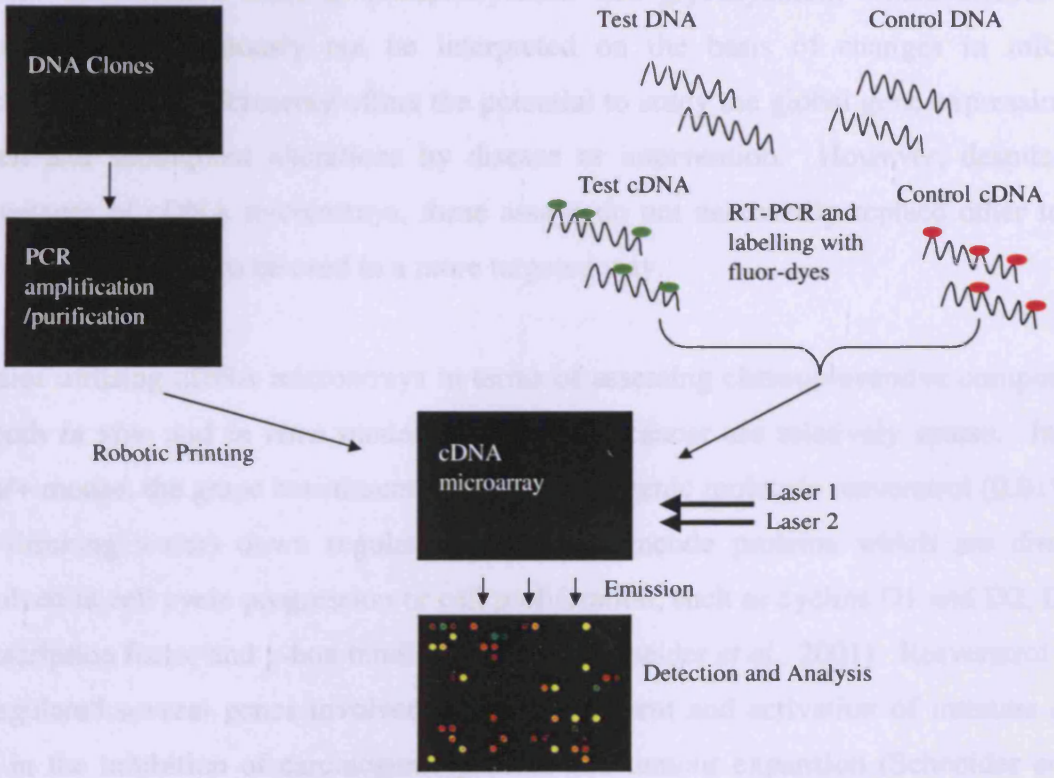


Figure 5.1.1 Scheme for the detection of altered gene expression using cDNA microarrays.

Microarray offers many advantages over traditional methods for measuring mRNA abundance and gene expression, for example, Northern blotting, polymerase chain reaction following reverse transcription (RT-PCR) cDNA sequencing, in which only a limited number of genes can be studied in any one experiment. The use of mRNA is advantageous over protein-based assays as these are generally less sensitive and are not suitable for high throughput analyses. However, mRNA is an "intermediate" molecule and it is essential that differentially regulated genes and the final expression of the protein product should be validated by alternative methodologies. Posttranslational protein modification such as phosphorylation and glycosylation, which affects cell phenotype, can obviously not be interpreted on the basis of changes in mRNA. Therefore, cDNA microarray offers the potential to study the global gene expression of a cell and subsequent alterations by disease or intervention. However, despite the advantages of cDNA microarrays, these assays do not necessarily replace other tools, but may allow them to be used in a more targeted way.

Studies utilising cDNA microarrays in terms of assessing chemopreventive compounds in both *in vivo* and *in vitro* models of colorectal cancer are relatively sparse. In the Min/+ mouse, the grape constituent and anticarcinogenic molecule resveratrol (0.01% in the drinking water) down regulated genes that encode proteins which are directly involved in cell cycle progression or cell proliferation, such as cyclins D1 and D2, DP-1 transcription factor and y-box binding proteins (Schneider *et al.*, 2001). Resveratrol also upregulated several genes involved in the recruitment and activation of immune cells and in the inhibition of carcinogenic process and tumour expansion (Schneider *et al.*, 2001). Gene expression analysis of curcumin modifying colonic cell maturation shows that this agent induces G_2-M arrest and limited transient changes (Mariadason *et al.*, 2000). Park *et al.*, (2002) showed that the upregulation of cyclin dependant kinase inhibitors by curcumin in ECV304 cells plays a critical role in the regulation of the cell cycle. Studies describing the effect of curcumin on gene expression in the Min/+ mouse by cDNA microarray have not as yet been performed. Most importantly, the effect of the Min/+ genotype on gene expression on a C57BL/6J background is also unknown.

Microarray experiments facilitate the comparison of overall changes in gene expression in response to different compounds, treatment intervals, as well as differences between normal and disease states. The purpose of this study was to elucidate gene expression

changes in Min/+ mice, compared to wild type mice, and to determine target genes for curcumin in both the normal and the mutant mouse. These studies focus on the early changes in carcinogenesis as the mice were culled prior to the development of visible adenomas. Reference and test mice were bred from identical parents and exposed to appropriate identical conditions to limit exogenous factors affecting mRNA expression. Each study arm was performed four times, using separate mice under controlled conditions. During the isolation of the RNA appropriate sterile conditions were implemented to limit contamination. All samples were of acceptable purity (OD 260nm/ 280nm = 1.6-1.9) and run on agarose gel to check RNA integrity prior to microarray (section 2.1.13).

To analyse the gene changes from the replicates of each of the three experimental settings, the absorbance data for both the red and green channels were normalised. The mean and standard deviation of the four replicates for both channels including any replicate clones was calculated and a t-test applied. The ratios were calculated when the significance was reached. The data obtained was analysed using the Treeview programme (Mike Eisen, Stanford, USA). The Treeview programme clusters genes in groups according to their differential expression over the three treatment arms. This sensitive method assigns a coloured block for each gene depending on the degree of upregulation (red) or downregulation (green). Unchanged gene expression is shown in grey. There are several gradations of each colour to depict the extent of the change. All significant changes for each experiment can be viewed in appendix 2. The abbreviations for the genes are taken from Weizmann Institute of science (http://bioinfo.weizmann.ac.il, the National Cancer Institute (NCI) Genecards website (http://nciarray.nci.nih.gov/cards and from Jackson Laboratories. (http://www.informatics.jax.org). As large numbers of genes were differentially expressed in each experiment, genes germane to carcinogenesis were identified and arranged into clusters for further discussion.

5.2 Results

The global effect of curcumin on gene expression in wildtype and Min/+ mice was investigated using cDNA microarray. The mouse microarrays used in these studies contained 4246 ESTs. Figure 5.2.1 shows a typical array from these experiments and the clearly visible red and green "spots" depicting upregulated and downregulated genes respectively.

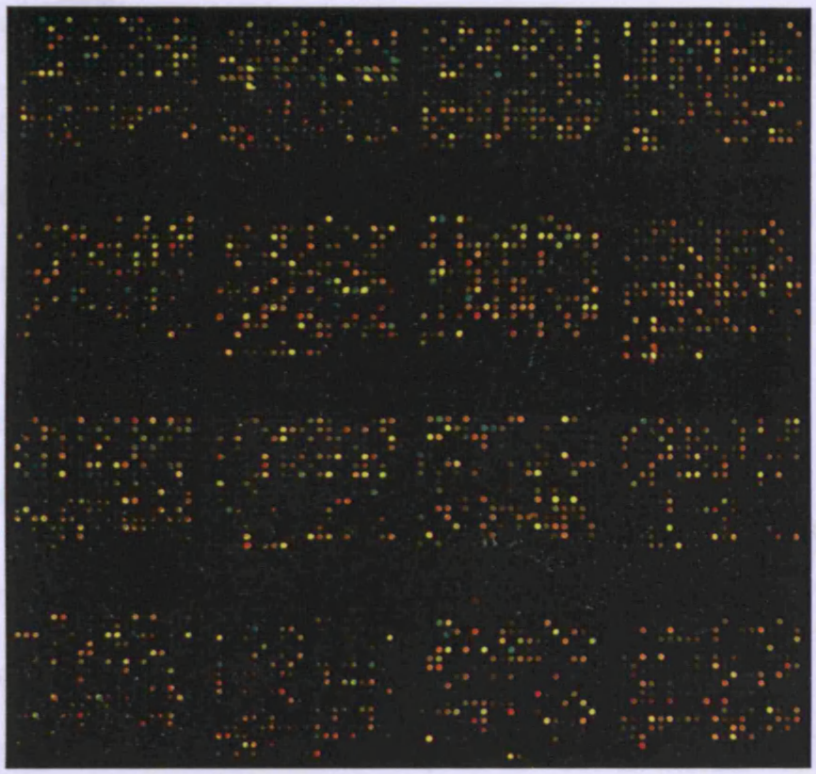


Figure 5.2.1 Representative microarray slide

From the scatter plots shown below (Figure 5.2.2), it is possible to observe a lot of low level hybridisation, depicted by the amount of absorbance observed in each channel, and small changes either up or down regulation depicted by overall red or green fluorescence detected at 635 and 532nm respectively.

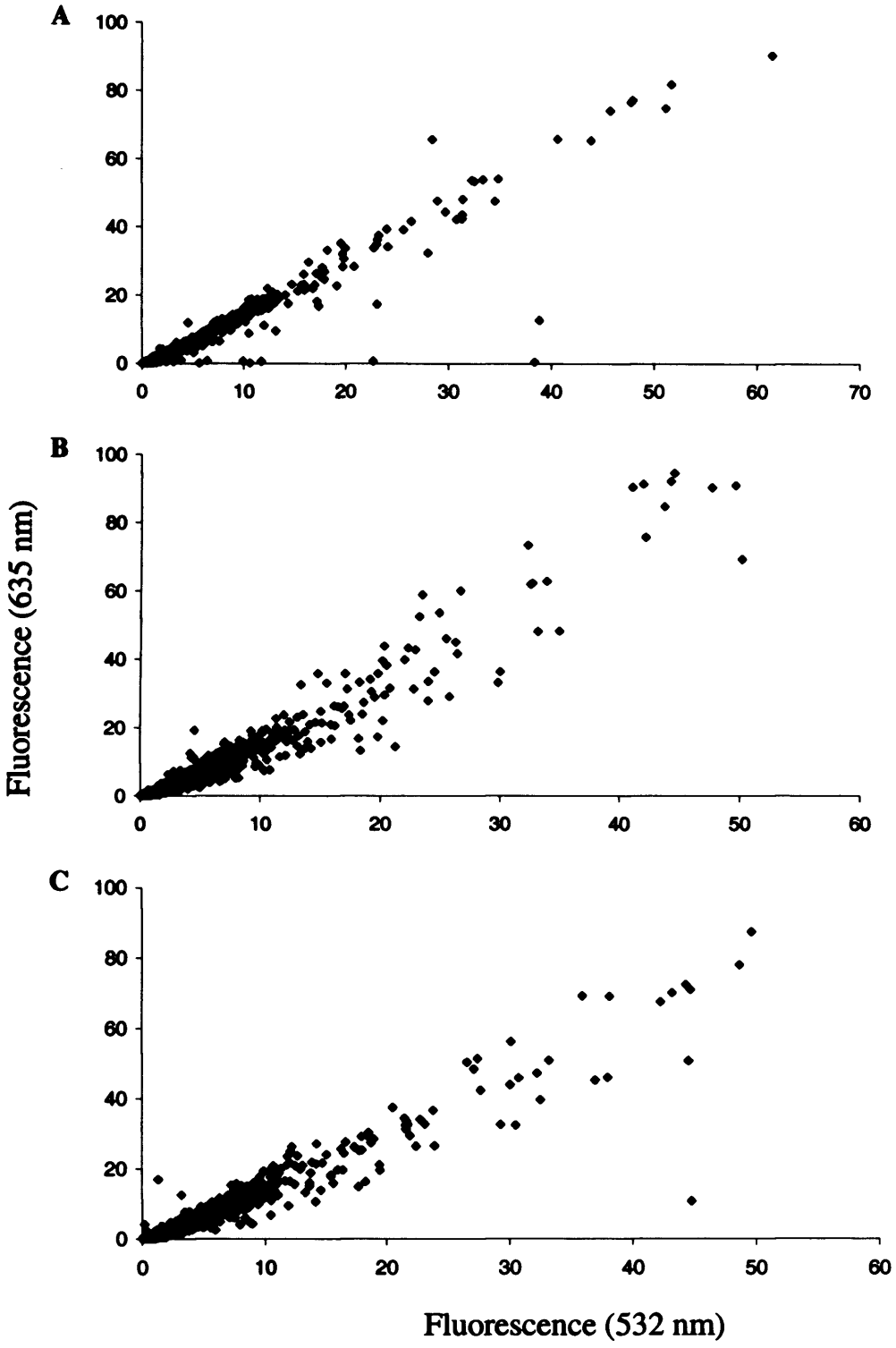


Figure 5.2.2 Scatter plot including signals from all the genes in the microarray. The plots are derived from analysis of RNA from A) wildtype and Min/+ mice B) wildtype and wildtype mice which received curcumin (0.2%) for 5 weeks and C) Min/+ and Min/+ mice which received curcumin (0.2%) for 5 weeks. (Details of methodology are described in section 2.1.13)

5.3 Analysis of changes in gene expression

Microarray is an important technique for the identification of genome wide gene changes, which can then be further investigated using reverse-transcription polymerase chain reaction (RT-PCR) or Northern blotting. Microarray was utilised in these experiments to study early gene changes in the development of adenomas in the intestine of Min/+ mice and gene changes induced by curcumin in both the normal and Min/+ mice. In these experiments a plethora of significant gene changes were detected, many of which were not germane to carcinogenesis or chemoprevention and have therefore been omitted from further discussion. This analysis, therefore, cannot provide conclusive mechanisms of action pertinent to curcumin, or definitive insights into changes associated with loss of APC function, but it highlights possible genes that may warrant further investigation as markers of carcinogenesis. These changes were chosen on the basis of the gene clusters obtained from the experiments and an understanding of the carcinogenic process and known alterations induced by curcumin (see section 1.9).

Clusters of significant differential gene expression associated with the Min/+ genotype and the effect of curcumin on both normal and Min/+ mice are shown in appendix 2. In total 420 genes were significantly altered over the three treatment arms. Comprehensive literature searches were performed for each gene and interesting genes with respect to colon carcinogenesis and the chemopreventive efficacy of curcumin are discussed below. The full dataset is available at http://www.le.ac.uk/cmht/microarray_lab/collaborations/ (Sarah Perkins) and are summarised in appendix A. Gene changes associated with the Min/+ genotype are listed in Table 5.3.1 and 5.3.2.

Table 5.3.1 Upregulated genes associated with the Min/+ genotype. The description of genes was taken from http://www.informatics.jax.org and http://nciarray.nci.nih.gov/cards. The log ratio indicates the relative significance of the gene changes between the Min/+ and wildtype genotypes (n = 4).

Gene	Description	Log Ratio
Ccng	Cyclin G	0.4898
Cct6b	Chaperonin subunit 6b	0.5916
Ctsl	Tumour protease	0.9586
D3Wsu133e	G-elongation factor	0.2879
Daf1	Decay accelerating factor 1	0.9127
Daf2	Decay accelerating factor 2	0.7834
E2a	Tcfe2a – transcription factor	0.4617
Eif4g2	Eukaryotic translation initiation factor (4γ2)	0.7764
Expi	Extracellular proteinase inhibitor	2.765
Foxa1	Forkhead box A1	0.518
Gclm	Glutamate cysteine ligase	0.5839
Glns	Glutamine synthetase	0.587
Idb3	Inhibitor of DNA binding	0.7523
Pcna	Proliferating cell nuclear antigen	0.6894
Ppp1cb	Protein phosphatase 1	0.4825
Rbp2	Retinol binding protein 2	1.057
Slc2a1	Solute carrier family 2	1.198
Sparc	Secreted acidic cysteine rich glycoprotein	0.5544
Tpd52	Tumour protein d52	0.5807
Unp	Ubiquitin specific protease 4	0.4289

Table 5.3.2 Downregulated genes associated with the Min/+ genotype. The description of genes was taken from http://www.informatics.jax.org and http://nciarray.nci.nih.gov/cards. The log ratio indicates the relative significance of the gene changes between the Min/+ and wildtype genotypes. (-) Indicates downregulation (n = 4).

Gene	Description	Log Ratio
Adcy9	Adenylate cyclase 9	-0.8979
Cbx4	chromobox homolog 4	-0.7354
Cdx2	Caudal type homeobox 2	-0.9501
Cd28	Antigen	-1.279
Hmox1	Hemeoxygenase 1	-0.7446
Junb	Oncogene	-1.123
Usf1	Upstream transcription factor 1	-0.8933

Clustering together of gene changes observed in the intestinal tract of wildtype and Min/+ mice, which received curcumin, pinpoints genes upregulated by curcumin treatment that may be particularly significant. Notable changes were upregulation of *SOD2, tgfbi* and *fabpi* (or *fabp2*) and are shown in Table 5.3.3. Curcumin did not downregulate any genes in both wildtype and Min/+ mice germane to carcinogenesis or chemoprevention.

Table 5.3.3 Genes upregulated by curcumin in both Min/+ and wildtype littermates. The description of genes was taken from http://www.informatics.jax.org and http://nciarray.nci.nih.gov/cards. The log ratio indicates the relative significance of the gene changes between the Min/+ and wildtype genotypes. (-) Indicates down regulation. B) Represents the effect of 5 weeks dietary administration of curcumin in Min/+ mice and C) represents the effect of 5 weeks dietary administration of curcumin in wildtype mice (n = 4).

Gene	Description	Log Ratio	
		B	C
Fabpil Fapp2	intestinal fatty acid binding protein	0.9256	0.7024
		0.8532	0.702
SOD2	superoxide dismutase 2	0.8073	0.6153
Tgfbi	transforming growth factor, beta induced	1.18	0.6442

Tables 5.3.4 and 5.3.5 show changes in gene expression induced by curcumin in either the Min/+ mouse or its wildtype counterpart.

Table 5.3.4 Genes affected by curcumin in Min/+ mice. The description of genes was taken from http://www.informatics.jax.org and http://nciarray.nci.nih.gov/cards. The log ratio indicates the relative significance of the gene changes between the Min/+ and wildtype genotypes. (-) Indicates down regulation (n = 4).

Gene	Description	Log Ratio
Aop1	peroxidoxin	0.6301
Gstpi	glutathione s-transferase π	0.6419
Txn2	thioredoxin	0.6319
Akt	thymoma viral proto-oncogene	-0.6977
Ddx21	Atp-dependant helicase	-0.4674
Dnmt	DNA methyl transferase	-0.6958
Gtmbp	mutS homolog 6	-0.5946
Msk2	ribosomal protein S6 kinase, 90 kD, polypetide 4	-0.9448
Pex7	Peroxisome biogenesis factor 7	-0.8701
Pola2	Polymerase α 2	-0.9563
Rad51	RAD51 homolog	-0.3013
Tra1	tumour rejection antigen gp 96	-0.5716
Trp53	transformation related protein 53	-0.7402

Table 5.3.5 Genes affected by curcumin in wildtype mice. The description of genes was taken from http://www.informatics.jax.org and http://nciarray.nci.nih.gov/cards. The log ratio indicates the relative significance of the gene changes between the Min/+ and wildtype genotypes. (-) Indicates down regulation (n = 4).

Gene	Description	Log Ratio
Cdkn2b	Cyclin dependant kinase inhibitor 2b	0.4056
Tff3	Trefoil factor 3, intestinal	0.65
Tsg101	Tumour susceptibility gene	0.3134
Txn	thioredoxin	0.7542
Fasn	fatty acid synthase	-0.6414
iNos	inducible nitrogen oxide synthase	-0.1627
Pparg	peroxisome proliferator activated receptor γ	-1.139
Vegfc	vascular endothelial growth factor c	-1.973

Table 5.3.6 shows changes in gene expression induced by Min/+ genotype and curcumin in its wildtype counterparts. Tables 5.3.7 show changes in gene expression induced by the Min/+ genotype and curcumin in either the Min/+ mouse or its wildtype counterpart.

Table 5.3.6 Genes affected by both the Min/+ genotype and curcumin in wildtype mice. The description of genes was taken from http://www.informatics.jax.org and http://nciarray.nci.nih.gov/cards. The log ratio indicates the relative significance of the gene changes between the Min/+ and wildtype genotypes. (-) Indicates down regulation. A) Represents the effect of the Min/+ genotype on gene expression in C57BL/6J mice C) represents the effect of short-term administration curcumin on wildtype mice (n = 4).

Gene	Description	Log Ratio	
		A	C
Tnf	tumour necrosis factor	-0.9932	-1.289
Trp63	transformation related protein 63	-3.42	-2.386

Table 5.3.7 Genes affected by the Min/+ genotype and curcumin in both wildtype and Min/+ mice. The description of genes was taken from http://www.informatics.jax.org and http://nciarray.nci.nih.gov/cards. The log ratio indicates the relative significance of the gene changes between the Min/+ and wildtype genotypes. (-) Indicates down regulation. A) Represents the effect of the Min/+ genotype on gene expression in C57BL/6J mice B) represents the effect of short-term administration of curcumin in Min/+ mice and C) represents the effect of short-term administration of curcumin on wildtype mice (n = 4).

Gene	Description	Log Ratio		
		A	B	C
Abcb7	ATP-binding cassette, sub-family B (MDR/TAP), member 7	-1.213	-1.61	-1.348

5.4. Discussion

In general, these experiments highlighted a large number of genes that were modified by both the Min/+ genotype and the effect of curcumin on gene expression profiles in both wildtype and Min/+ mice. To reiterate, the changes in gene expression highlighted in Tables 5.3.1- 5.3.6 are those that are germane to carcinogenesis and/ or genes of interest relevant to potential mechanisms of action of curcumin, or potential markers of carcinogenesis.

In total the Min/+ genotype caused significant upregulation in 124 genes and down regulation in 122 genes compared to wildtype mice. Many of these gene changes were associated with carcinogenesis as highlighted in Tables 5.3.1 and 5.3.2. The work presented here is the first study of the effect of *Apc* gene mutation on C57BL/6J mice. It is noteworthy that the *Apc* gene was not altered in this experiment. The mutated *Apc* RNA may still be able to bind to the EST, as the EST may not be a gene sequence found after codon 850 or the deletion may not be large enough to prevent hybridisation onto the microarray slide.

5.4.1 Genes altered in the Min/+ genotype

The Min/+ genotype caused the altered expression of a number of genes that are modulated by carcinogenesis. Many genes over expressed in the Min/+ mouse are known to be upregulated genes in carcinogenesis, particularly in gastrointestinal neoplasms. These include *ppp1cb*, *glns* and *ccng*, which are genes known to be upregulated in tumours. Ppp1cb is the catalytic subunit of protein phosphatase, which is required for the growth of cancer cells (Takakura *et al.*, 2001). These authors showed that *Ppp1cb* was ubiquitously expressed in 55 cancer cell lines, including 11 colorectal cancers. *Glns*, glutamine synthetase, was also upregulated in the Min/+ intestine. Neoplastic cells require increased glutamine for increased protein synthesis and oxidation (Shapot, 1979), and increased mRNA levels of glutamine synthetase have been demonstrated *in vitro* (Collins *et al.*, 1997). Cyclin G (*ccng*) is an essential regulatory factor required for cell cycle progression from G0 through G1 and S phase (Wang *et al.*, 1998). Facilitated glucose transporter, *Slc2a1*, was upregulated in Min/+ mouse intestine. This upregulation correlates with reports of increase glucose metabolism in the progression of normal to neoplastic cells (Lambert *et al.*, 2002). *Tpd52*, tumour protein D52, is also upregulated in neoplastic tissue (Byrne *et al.*, 1995).

Sparc, which encodes an extracellular matrix protein that plays a role in cell migration, survival, or angiogenesis was also upregulated (Bradshaw and Sage, 2001). *Unp* encodes a nuclear ubiquitin protease whose over expression leads to oncogenic transformation of cell lines (Gray *et al.*, 1995).

Gclm, *daf 1* and *daf 2*, *cct6b*, *D3Wsu133e* and *ctsl* have been shown to be upregulated in gastrointestinal tumours, and may therefore warrant further investigation as potential markers in the Min/+ mouse. *Gclm*, the modifier subunit of glutamate cysteine ligase is upregulated in colon tumours in response to oxidative stress (Ozdemirler *et al.*, 1998). Daf1 and Daf2 are decay accelerating factors that prevent complement-mediated cytotoxicity of tumour cells and encoded by genes which are frequently upregulated in neoplastic colon cells both *in vitro* (Juhl *et al.*, 1997) and *in vivo* (Koretz *et al.*, 1992). *Cct6b*, chaperonin subunit 6β, and *D3Wsu133e* (G elongation factor) are frequently over expressed in colon cancer (Yokota *et al.*, 2001; Frazier *et al.*, 1998). In particular, *ctsl* encodes cathepsin L, a tumour protease upregulated in colon tumours (Adenis *et al.*, 1995). Cathepsin L catalyses the degradation of the interstitial matrix and basement membranes, allowing cancer cells to invade locally and metastasise to distant sites. *Ctsl* was underexpressed in wildtype mice that received curcumin and was therefore highlighted as a gene that may warrant further investigation.

A number of genes encoding proteins required for transcription were upregulated in Min/+ mice and have previously been shown to be upregulated in neoplastic tissue. These include *Rbp2* (retinol binding protein) (Levin, 1993), *foxa1* (forkhead box A1) (Lin *et al.*, 2002) and *e2a* (transcription factor e2a) (Dang *et al.*, 2001). *Eif4g2* encodes eukaryotic translation initiation factor 4, gamma 2 which is required for translation initiation (Gingras *et al.*, 1999). A number of genes required for cell proliferation were upregulated. These include *Idb3* (inhibitor of DNA binding 3) and *pcna* (proliferating cell nuclear antigen). Inhibitors of DNA binding proteins negatively regulate cellular differentiation and they induce proliferation by modulating different cell cycle regulators both by direct and indirect mechanisms (Hasskarl and Munger, 2002). Curcumin has been shown to decrease elevated pcna levels in murine hepatocarcinogenesis (Chuang *et al.*, 2000) therefore highlighting another gene that may be investigated as a potential marker of chemoprevention.

Genes characteristically downregulated in tumour tissue were also underexpressed in intestinal tissue of Min/+ mice compared to wildtype littermates. *Cdx2* (caudal type homeobox 2) and *adcy9* (adenylate cyclase 9) are often decreased in malignant tissue. Cdx2 is an intestine-specific transcription factor required for differentiation and maintenance of intestinal epithelial cells. Cdx2 expression has been shown to decrease with tumour grade in human colorectal cancers and also in rat and mouse models of the disease (Ee *et al.*, 1995; Chawengsaksophak *et al.*, 1997). Adenylate cyclase, an integral component of many signalling cascades, is frequently underexpressed in tumour tissue. In particular, in cells mutant in *Apc* gene, down regulation of adenylate cyclase leads to decreased cell-cell communication and adhesion with other epithelial cells without corrective capability (Waddell and Miesfeld, 1995). Junb is an AP-1 protein that negatively regulates cell proliferation through upregulation of tumour suppressor genes and repression of Cyclin D1 (Shaulian and Karin, 2001). Junb is underexpressed in neoplastic gastrointestinal tissue in accordance with activation of the Wnt signalling pathway (Tice *et al.*, 2002). *Usf1* encodes upstream stimulating factor 1, which is critical for *Apc* gene expression and is an integral part of the Wnt signalling pathway (Jaiswal and Narayan, 2001). Underexpression of *Usf1* leads to decreased activation of APC leading to increased cell proliferation. *Cbx4*, chromobox homolog 4, is required for stable gene expression and this is under expressed in the Min/+ mice intestine, which may lead to uncontrolled transcription (Alkema *et al.*, 1997). A decreased resistance to tumour progression is correlated with a decreased expression of *Cd28*, therefore, favouring tumour growth in the min/+ mouse (Chaux *et al.*, 1996). Cross-linking of the CD3 and CD28 molecules on T lymphocytes represents one of the most effective signals for T lymphocyte activation and triggering of their cytotoxic effector function. Decreased expression of the gene encoding the CD28 antigen therefore reduces the rejection of neoplastic cells. Decreased expression of hemeoxygenase, *HMOX1*, was observed in Min/+ mice intestine. Decreased resistance to oxidative stress may occur due to decreased expression of the microsomal enzyme, hemeoxygenase 1. Oxidative stress has been shown previously to play a role in Min/+ mouse carcinogenesis (Chapter 4).

5.4.2 Genes altered by curcumin in both wildtype and Min/+ mice

The broad spectrum of activities of curcumin, germane to carcinogenesis, were summarised in chapter 1, section 1.9. The aim of the experiments performed in this

chapter was to study the effect of curcumin on these genes and identify potentially new genes that were the target of curcumin. Gene changes elicited by curcumin in wildtype and Min/+ mice pinpoints genes, the upregulation of which by curcumin may be particularly significant. Notably three genes were identified which may play a role in carcinogenesis, *SOD2* (superoxide dismutase), *Fabpi / Fapp2* (intestinal fatty acid binding protein), and *Tgfbi* (transforming growth factor beta) (Table 5.3.3). Superoxide dismutase is an enzyme, which scavenges superoxide radicals, in response to oxidative stress (McCord, 2000), thereby reducing oxidative DNA damage. Dietary turmeric and an analogue of curcumin have been shown to increase superoxide dismutase activity in rat liver (Devasena *et al.*, 2002; Reddy and Lokesh, 1994). Intestinal fatty acid binding (*Fabpi*) expression was also increased after administration of curcumin. Fabpi protein binds free fatty acids, which may otherwise undergo lipid peroxidation leading to DNA damage. As shown in this thesis (Figure 4.3.2), curcumin inhibits M_1G, an indirect product of lipid peroxidation. A decrease in intestinal fatty acid binding protein is also documented in rat small intestine adenocarcinomas (Davidson *et al.*, 1993). As discussed in chapter 1, there is substantial evidence to support the contention that the Smad portion of the TGF-beta signal transduction pathway provides an important tumor-suppressor function in the development of colorectal cancer. In the intestine, TGF-beta signal transduction is thought to possess a potent inhibitory effect on cell growth, therefore the over expression of *tgfbi* by curcumin treatment may be a potential beneficial action of the phytochemical (Attisano and Wrana, 2002).

5.4.3 Genes altered by curcumin in Min/+ mice

More specifically, in the Min/+ mouse, curcumin induced the overexpression of 129 and underexpression of 46 genes. Genes of note include *Txn2* (thioredoxin) and *Aop1* (peroxidoxin), enzymes considered to act as an endogenous antioxidant system. Thioredoxin exerts multiple mechanisms of action in redox regulation of signal transduction and in cytoprotection against oxidative stress (Powis and Monfort, 2001). Peroxidoxin, like thioredoxin, scavenges radicals. *Gstpi* (Glutathione s-transferase π) was also increased by the presence of curcumin in the Min/+ mouse diet. The gene encodes GSTπ, a phase II detoxifying enzyme which is one of four glutathiones s-transferase subtypes that are involved in the activation and detoxification of many potential carcinogens and may therefore be important in susceptibility to cancer

induction. Curcumin and an analogue of curcumin have been shown to upregulate glutathione s-transferase in rat intestinal mucosa (Sharma *et al.*, 2001b) and rat *in vivo* model of colon carcinogenesis (Devasena *et al.*, 2002)

Table 5.3.4 highlights some of the genes under expressed in the Min/+ mouse after administration of curcumin (0.2%). *Akt* is a proto-oncogene, overexpression of which is reported in early stages of colon carcinogenesis leading to inhibition of apoptosis (Roy *et al.*, 2002). Akt plays an integral role in the phosphatidylinositol-3-OH kinase (PI (3) K) signalling cascade. Msk2, ribosomal protein S6 kinase, 90kD, functions in a protein kinase C- (PKC) dependent pathway to promote cell survival via phosphorylation and inactivation of Bad-mediated cell death (Tan *et al.*, 1999). Curcumin therefore may induce apoptosis by interfering in both PKC and PI (3) K signalling pathways.

Dnmt, *ddx21* and *tst* are overexpressed in tumour tissue and therefore their modification by curcumin may warrant further investigation. Elevated *Dnmt*, which encodes DNA methyl transferase, is a prevalent feature of neoplasias. DNA methylation can act alone or in concert with deletion or mutation to disrupt tumour suppressor gene function in human tumours *in vivo* (Jones and Laird, 1999). Ddx21 is a DEAD box helicase, one of a group of enzymes implicated in cancer development, many of which are upregulated in colon cancer (Nakagawa *et al.*, 1999; Causevic *et al.*, 2001). The tumour specific antigen gene, *Rad51*, was down regulated, suggesting the propensity for curcumin treated animals to develop fewer tumours. Rad51 is involved in DNA repair and recombination and is overexpressed in cancer cell lines (Raderschall *et al.*, 2002). *Pola2*, polymerase (DNA directed, α2), a gene encoding a protein required for DNA replication was downregulated suggesting decreased cell growth. *Pex7* was also downregulated. *Pex7* encodes a protein required for peroxisome development. Peroxisome activity is a source of oxidative damage and as previously shown, oxidative damage may be reduced by curcumin (Chapter 4 and Chapter 1, section 9).

Trp53, a tumour suppressor gene, *tral* and *Gtmbp* were genes downregulated by curcumin administration. These are potential negative effects of curcumin. *Trp53* (*p53*), a tumour suppressor gene implicated in colon carcinogenesis (section 1.4). *P53* is a tumor-suppressor gene that codes for a multifunctional DNA-binding protein involved in cell cycle arrest, DNA repair, differentiation, and apoptosis. *Gtmbp* is a gene

encoding the G/T mismatch binding protein and member of DNA mismatch repair genes (MSH6). *Apc*^{1638N} mice defective in *Msh6* show reduced survival and a 6-7-fold increase in intestinal tumour multiplicity (Kuraguchi *et al.*, 2001). Tra 1, tumour rejection antigen gp96, is a stress protein that elicits protective immune response against neoplastic tissue (Panjwani *et al.*, 2002).

5.4.4 Gene altered by curcumin in wildtype mice

In wildtype mice, curcumin induced changes in the expression of 144 genes. Pertinent gene changes with respect to mechanisms of action of curcumin and carcinogenesis are tabulated in Table 5.3.5. *Cdkn2b* (or p15) encodes a cyclin dependant kinase inhibitor previously shown to be upregulated by curcumin *in vitro* in colon cell lines (Park *et al.*, 2002). Cdkn2b is a down stream element in the TGF-β signalling pathway, alterations of which is implicated in the pathogenesis of colorectal cancer. Down regulation of Cdkn2b is decreased in azoxymethane colon mouse tumours suggesting a role of abberant TGF-β signalling in colorectal carcinogensis (Guda *et al.*, 2001). Aspirin has been shown to promote gene activation of *Tff* (trefoil factor family) genes in gastric cell lines (Azarschab *et al.*, 2001). Trefoil factor family peptides promote cell migration, heal the mucosa and may inhibit tumour growth. Curcumin possesses a similar mechanism of action to aspirin and upregulated *Tff3* in the intestine of wild type mice. *Txn2*, (thiorexodin) was again upregulated by curcumin in wildtype mice. Curcumin upregulated *Tsg 101*, a tumour susceptibility gene, inactivation of which in mice results in genome instability, cell transformation and the ability to form metastatic tumours in nude mice (Xie *et al.*, 1998). Interestingly, administration of resveratrol to Min/+ mice upregulated the tumour susceptibility gene *Tsg 101* (Schneider *et al.*, 2000).

Curcumin induced the underexpression of four genes germane to carcinogenesis in wildtype mice, *Fasn* (fatty acid synthase), *iNos*, *PPARγ* and *Vegfc*. Fatty acid synthase is elevated in tumours and has been shown to be decreased by curcuminoids in rat liver (Visca *et al.*, 1999: Yasni *et al.*, 1993). PPARγ, peroxisome proliferator-activated receptor gamma, is also elevated in colon tumours both in mice and humans (Lefebvre *et al.*, 1998; Saez *et al.*, 1998; Dubois *et al.*, 1998). The PPARs are ligand-activated transcription factors that bind as heterodimers with members of the retinoid X receptor subfamily to PPAR response elements in the promoters/enhancers of responsive genes.

PPARγ may modulate colon carcinogenesis, as prostaglandins may bind to the PPARγ receptor leading to the upregulation of vascular endothelial growth factor (vegf), a tumour angiogenic factor. Curcumin decreased the expression of both PPARγ and vegf suggesting a possible pathway of carcinogenesis inhibited by curcumin. Inducible nitric oxide synthase (iNOS) is a source of nitric oxide (NO) and other reactive nitrogen species, which may lead to oxidative DNA damage, oncogene expression, angiogenesis, inhibition of DNA repair enzymes, deregulation of apoptosis, and modulation of transcription factors (Jaiswal *et al.*, 2001).

5.4.5 Genes altered in the Min/+ genotype and by curcumin in wildtype littermates

A study of the gene changes associated with both the Min/+ genotype and administration of curcumin to wildtype mice revealed downregulation of two interesting genes, *tnf* (tumour necrosis factor) and *trp63* (transformation related protein 63; a homolog of p53) (Table 5.3.6). Tnf is a proinflammatory cytokine, which increases gene transcription, notably of NF-κB and c-Jun and induces apoptosis. Therefore downregulation of this gene in Min/+ mice is not unexpected, leading to increased survival of neoplastic cells. However, curcumin inhibits Tnf activity *in vitro* (Chan 1995), possibly mediating an anti-inflammatory response in both wildtype and Min/+ mice. Trp63 is known to be downregulated in many tumours (e.g. human squamous cell carcinomas; Senoo *et al.*, 2001). It mediates tumour suppressor and oncogenic properties, possibly explaining its underexpression in both Min/+ mice and curcumin treated animals (Irwin and Kaelin, 2001).

5.4.6 Genes altered in the Min/+ genotype and by curcumin in Min/+ and wildtype mice

A study of the genes affected by the Min/+ genotype and curcumin in both wildtype and Min/+ mice highlighted an interesting gene change *Abcb7* (ATP-binding cassette, sub-family B) (Table 5.3.7). Although not directly relevant to carcinogenesis, all three experiments showed an underexpression of the gene encoding ATP-binding cassette protein, Abcb7. ATP-binding cassette proteins play important role in multi-drug resistance, hydrolysing ATP to translocate specific substrates across membranes. This is an important mechanism of resistance to certain chemotherapies, such as doxorubicin. It is worth noting that ABC transporters are also important in the development the host-

pathogen interactions in Leishmaniasis. Curcumin is under investigation as a treatment of this parasitic disease, although its antiprotozoal mechanism of action is unclear (Saleheen *et al.*, 2002).

5.4.7 Summary of gene changes

The presence of the *Apc* gene mutation, or the administration of curcumin diet induced a plethora of gene changes. This discussion aims to highlight possible alterations in the expression of genes relevant to carcinogenesis or chemoprevention rather than discuss the genome wide changes induced by dietary administration of curcumin and/or Min/+ genotype. In both Min/+ and wildtype mice, dietary administration of curcumin induced a number of gene changes associated with antioxidation such as the gene encoding superoxide dismutase. Therefore further studies should involve an investigation of the gene changes at a protein level. As shown in the previous chapter, the scavenging of radicals by curcumin is an important mechanism of action in reducing tumour burden in Min/+ mice. This action confirms the importance of studying biomarkers of carcinogenesis germane to oxidative damage, such as 8-OH dG and M_1G, carried out in the work described here (Chapter 4). Other genes involved in decreasing oxidative stress were altered in Min/+ mice that received curcumin, again emphasising that the antioxidant properties of this phytochemical might be the key to reducing tumour burden. In wildtype mice curcumin altered the expression of a number of genes relevant to chemoprevention. These may be of particular importance if curcumin is administered prior to the development of neoplasia in healthy individuals. However, the extent of gene changes induced by dietary administration of curcumin, shows that curcumin possesses a broad spectrum of activity.

The experiments described in this chapter, include the first assessment of genome wide gene changes that occur in the early stage of carcinogenesis in the intestine of Min/+ mice. It is clear that the *Apc* gene mutation confers a number of genomic alterations including the upregulation of numerous genes relating to transcription and signalling pathways integral to carcinogenesis.

To confirm the gene changes observed in these studies, validations experiments would need to be performed. These would include an assessment of RNA levels by Northern

blotting or RT-PCR or at the protein level using Western blotting. A further study is planned to study the effect of curcumin administration and the presence of the *Apc* gene mutation at a later stage of carcinogenesis in Min/+ mice. These experiments may elucidate changes in gene expression relating to the development of established neoplasm. Further analysis of the change in expression of genes emphasised in this discussion may confirm their importance in the development of neoplasia in the Min/+ mouse.

CHAPTER 6
PHARMACOKINETICS OF CURCUMIN IN C57BL/6J MICE

6.1 Introduction

The aim of this project was to relate the chemopreventive efficacy of curcumin in Min/+ mice with concentration of the agent or its metabolites. The work presented in chapters 3 and 4 has demonstrated the chemopreventive efficacy and potential mechanisms of action of curcumin in Min/+ mice. In order to permit interpretation of these findings, they need to be related to drug levels at target tissues. This study therefore was designed to determine the levels of curcumin and its metabolites in the target organs, which elicited the reduction in tumour burden. Studies, thus far, have not been performed on the pharmacokinetics of dietary administration of curcumin in mice. Studying the subsequent disappearance of curcumin after cessation of dietary curcumin would then aid the identification of a suitable dosing regime.

Curcumin is poorly bioavailable when given orally (as described in section 1.9). The aim of this work was to explore the bioavailability of dietary curcumin in mice, assessing levels of curcumin in the systemic circulation and excreta. Curcumin undergoes phase I and II metabolism with the formation of reduced species and less pharmacologically active conjugated metabolites (Figure 1.9.5). A further aim of this work was to identify possible metabolites in target tissue and faeces after dietary administration of curcumin and draw comparisons with previous reports of the metabolism of curcumin after dietary administration both in humans (Sharma *et al.*, 2001c) and rodents (Ireson *et al.*, 2001a).

To define the disposition of curcumin, [^{14}C]-labelled curcumin was synthesised, administered *via* the intraperitoneal (*ip*) route and the disappearance of radioisotope from tissues and plasma was determined. This sensitive method of identifying the radiolabelled species enables the detection of all of the [^{14}C]-labelled curcumin and its derivatives, which other methods of analysis (for example, HPLC) may overlook. All of the [^{14}C] detected will be derived from the radiolabelled curcumin. These studies were designed to aid the pharmacokinetic evaluation of curcumin.

6.2 Determination of steady-state levels of curcumin and metabolites in target tissues and excreta after dietary administration

Steady-state levels were determined in the plasma, excreta and intestinal mucosa, the target tissue, and, for comparative purposes, also in liver tissue of C57Bl/6J mice, which had received dietary curcumin at 0.1, 0.2 or 0.5% for eight days. The concentration of curcumin in the intestinal mucosa and excreta are shown in Table 6.1. Representative chromatograms of curcumin treated mice, and mice that received RM3 diet for 8 days are shown in Figure 6.2.2 to 6.2.6.

Large amounts of curcumin were found in the faeces (3.2-3.8 μmols/g, Table 6.1). In the mucosa of the small intestine its concentration varied between 39 and 240 nmoles per g tissue and in the colonic mucosa between 15 and 715 nmoles per g tissue depending on the dietary level of curcumin (Table 6.1). Curcumin levels in the small intestine reflect differences in dose ($r^2 = 0.986$), whereas levels in the colon and faeces do not mirror dose levels (Figure 6.2.1).

Table 6.1 Curcumin levels in tissue and excreta of C57BL/6J mice after 8 days dietary administration of 0.1%, 0.2% or 0.5% curcumin in RM3 diet.

Curcumin content of diet (%)	Curcumin levels (nmoles/ g)		
	Small intestinal Mucosa	Colon Mucosa	Faeces
0.1	39 ± 9[A]	15 ± 9	3770 ± 1246
0.2	111 ± 40	508 ± 149	3590 ± 231
0.5	240 ± 69	715 ± 448	3186 ± 2411

[A] Values are the mean ± SD of 4 animals. Extraction and HPLC analysis were performed as described in section 2.2.17.2.

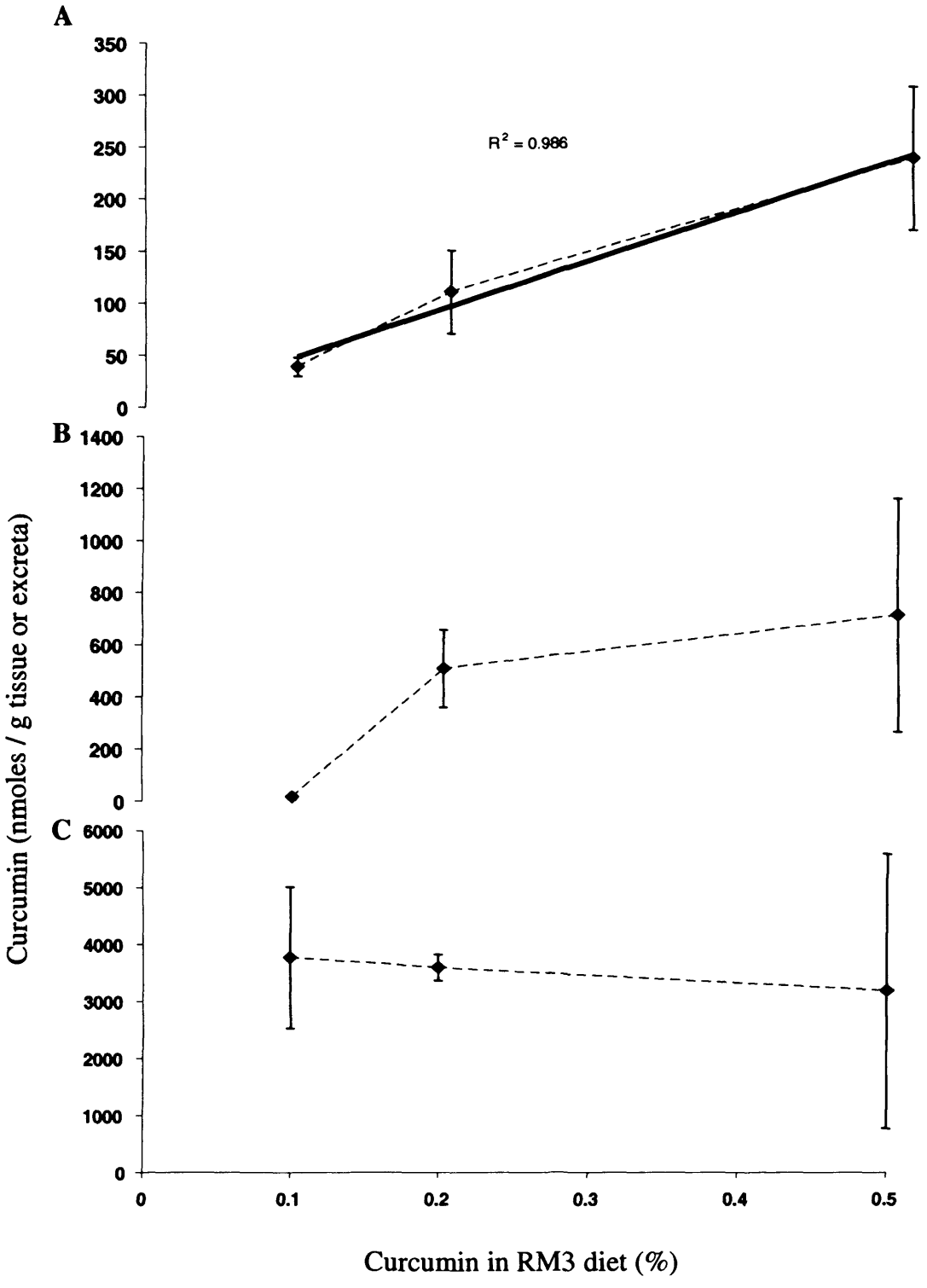


Figure 6.2.1 Mean curcumin concentrations in A) small intestine mucosa, B) colonic mucosa, C) faeces at each dose level (n = 3, ± SD). The solid black line represents the line of best fit correlating dose and curcumin concentration in the small intestine mucosa. Dose dependency was observed in small intestine mucosa (A) as shown by the high correlation coefficient (r^2). Extraction and HPLC analysis was performed as described in section 2.2.17.2.

Irrespective of the dose, curcumin was present in the plasma at levels near the limit of detection (10 pmol/ ml). This is represented chromatographically in Figure 6.2.5. The concentration of curcumin in liver tissue of mice, which were fed 0.2 % curcumin in the diet, was 119 ± 31 pmoles/ g tissue (n = 3), a value which constitutes approximately a thousandth of that observed in the intestinal mucosa. A representative chromatograph of an HPLC analysis of an extracted liver sample from a C57BL/6J mouse fed 0.2% curcumin for eight days is shown in Figure 6.2.6.

Mice were housed in metabolism cages for 24 h prior to study termination, during which time faecal and urine samples were collected. Analysis of the urine samples revealed high levels of curcumin that were considered to be nonrepresentative of the elimination of the compound (and suggested poor separation of urine and faeces with faecal contamination of urine). Therefore urine samples were collected directly from the bladder post cardiac exsanguination and extracted as for plasma (as described in section 2.2.18). Curcumin levels were below the limit of detection for this assay (10 pmol/ ml, Sharma *et al.*, 2001b).

To ensure that curcumin was present only in the plasma of the whole blood and not in the blood cells, the cellular fraction was extracted as for plasma (as described in section 2.2.18) and analysed by HPLC (chromatogram not shown). Due to the presence of a large asymmetric peak at 37 - 40 min, this fraction was collected, reanalysed by mass spectrometry (as described in section 2.2.19) and curcumin was not detected. In a confirmatory experiment, plasma levels of curcumin were measured in Min/+ mice at the end of the lifetime feeding study (as described in section 2.2.3.1) and levels were found to be very similar to those seen in wild type C57BL/6J mice kept on curcumin containing diet for 8 days.

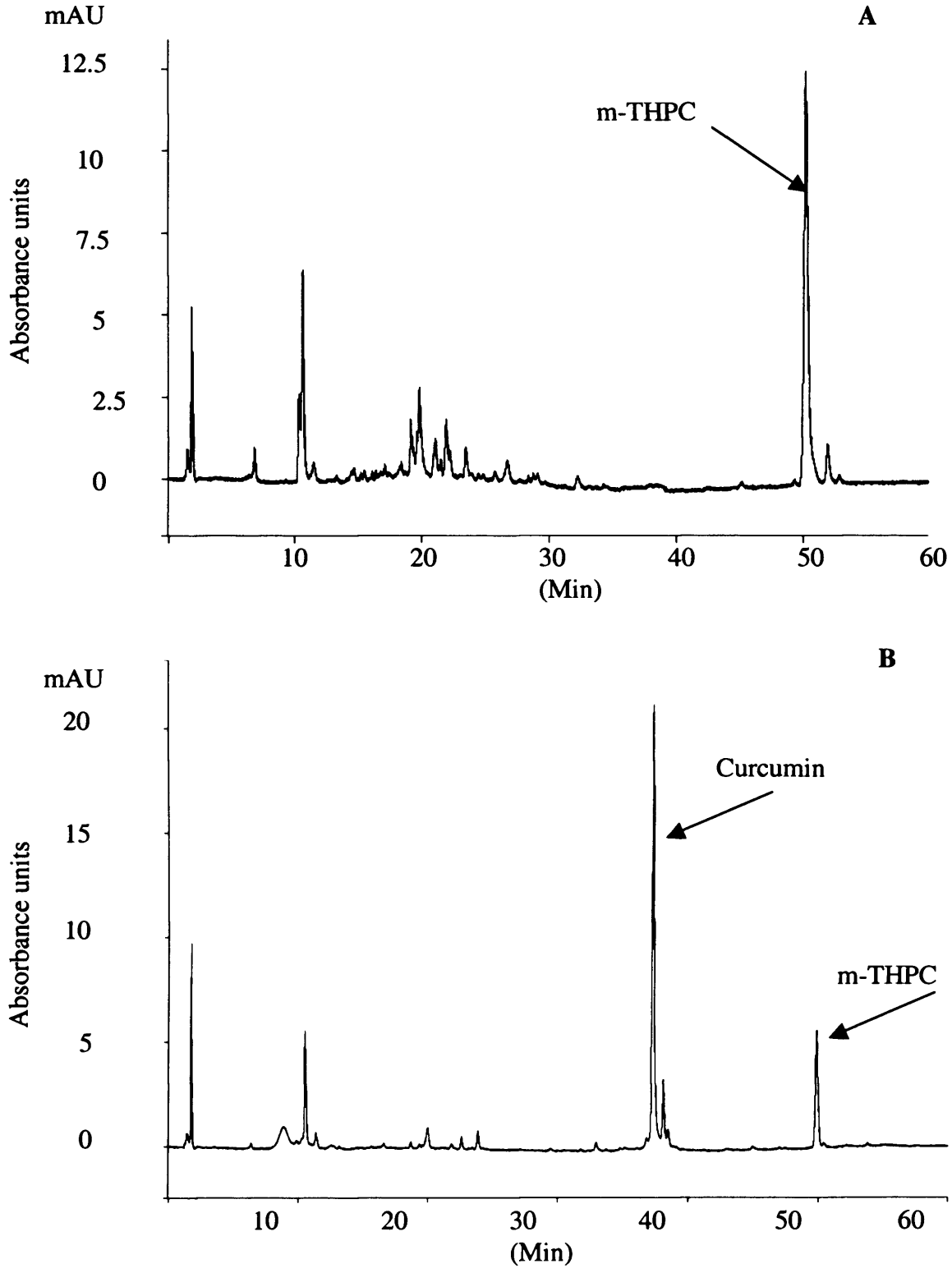


Figure 6.2.2 HPLC chromatogram of extract of small intestinal mucosa from C57BL/6J mice which received A) control diet (RM3) or B) curcumin (0.2% in RM3 diet) for 8 days. The internal standard (m-THPC) and curcumin retention times are 50 and 37 minutes respectively. Extraction and HPLC analysis was performed as described in section 2.2.18.

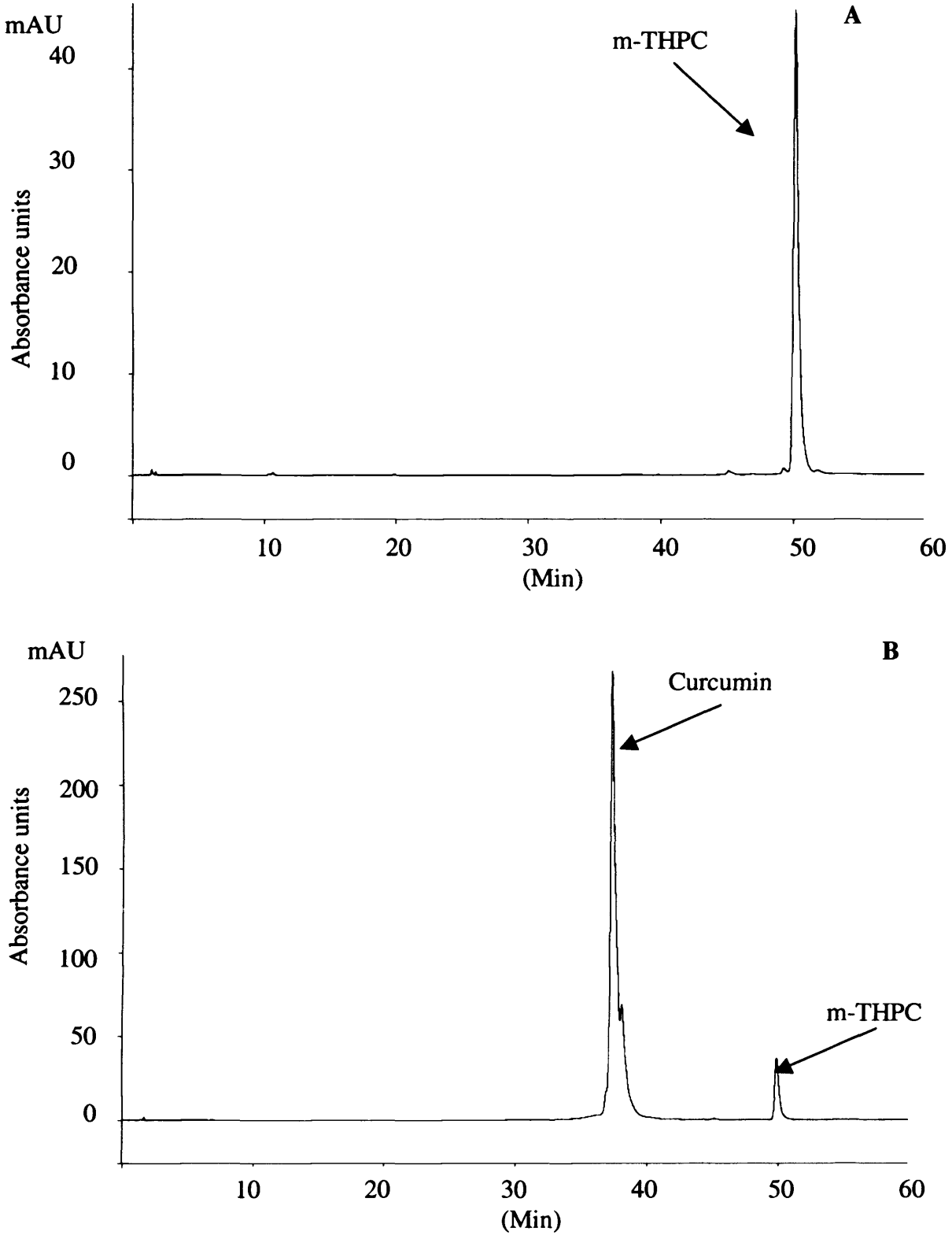


Figure 6.2.3 HPLC chromatogram of extract of colonic mucosa from C57BL/6J mice which received A) control diet (RM3) or B) curcumin (0.2% in RM3 diet) for 8 days. The internal standard (m-THPC) and curcumin retention times are 50 and 37 minutes respectively. Extraction and HPLC analysis was performed as described in section 2.2.18.

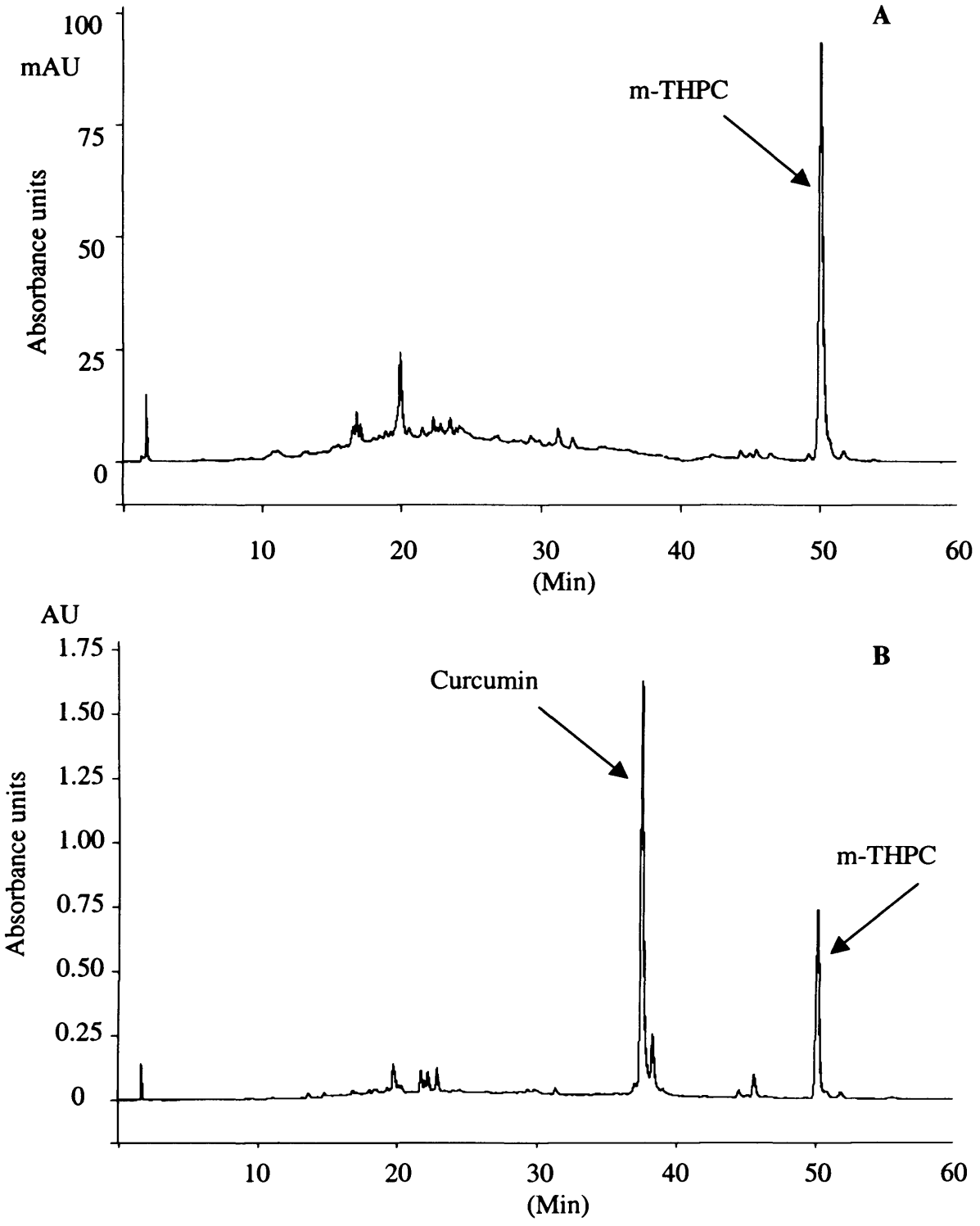


Figure 6.2.4 HPLC chromatogram of extract of faeces from C57BL/6J mice which received A) control diet (RM3) or B) curcumin (0.2% in RM3 diet) for 8 days. The internal standard (m-THPC) and curcumin retention times are 50 and 37 minutes respectively. Extraction and HPLC analysis was performed as described in section 2.2.18.

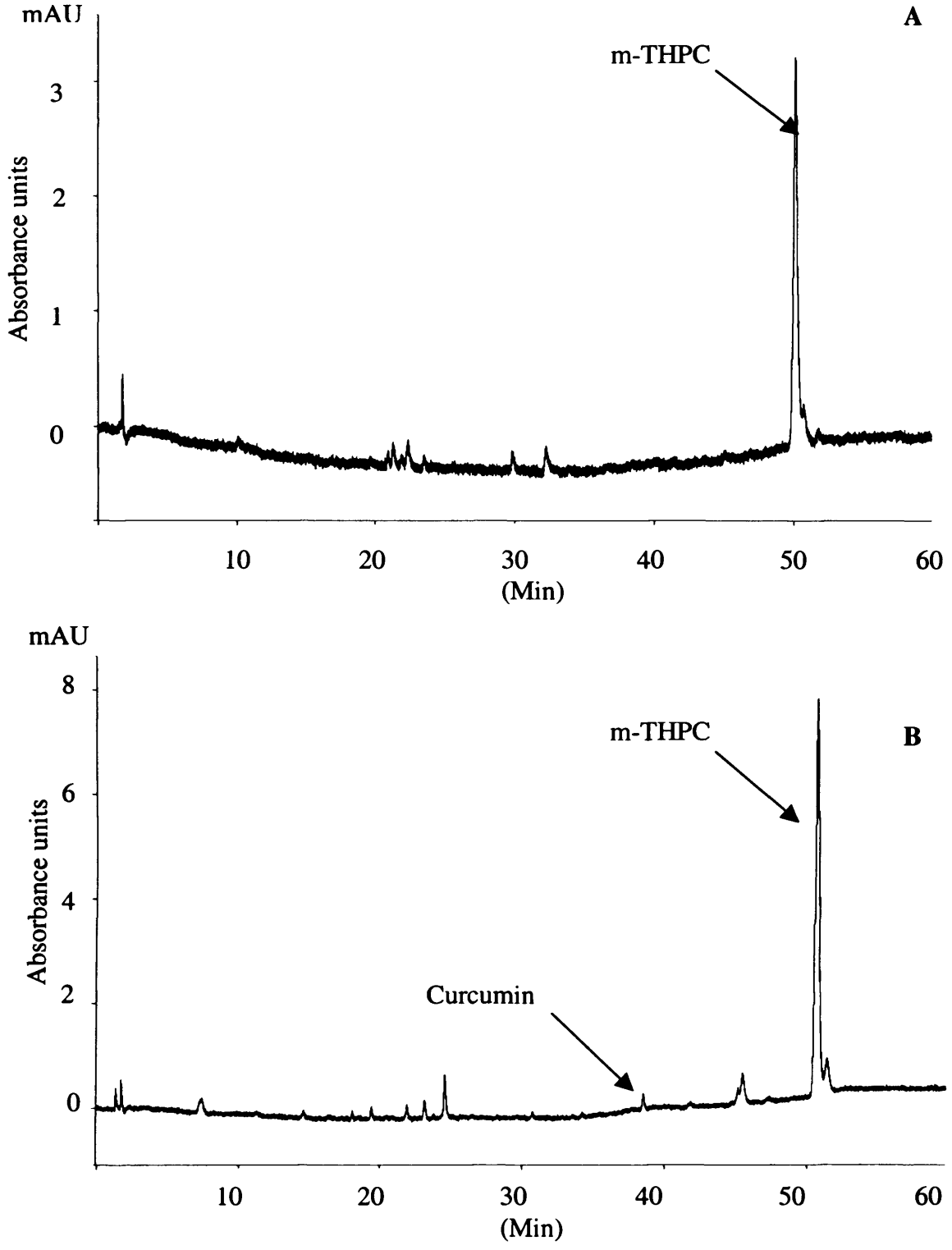


Figure 6.2.5 HPLC chromatogram of extract of plasma from C57BL/6J mice which received A) control diet (RM3) or B) curcumin (0.2% in RM3 diet) for 8 days. The internal standard (m-THPC) and curcumin retention times are 50 and 37 minutes respectively. Extraction and HPLC analysis was performed as described in section 2.2.18.

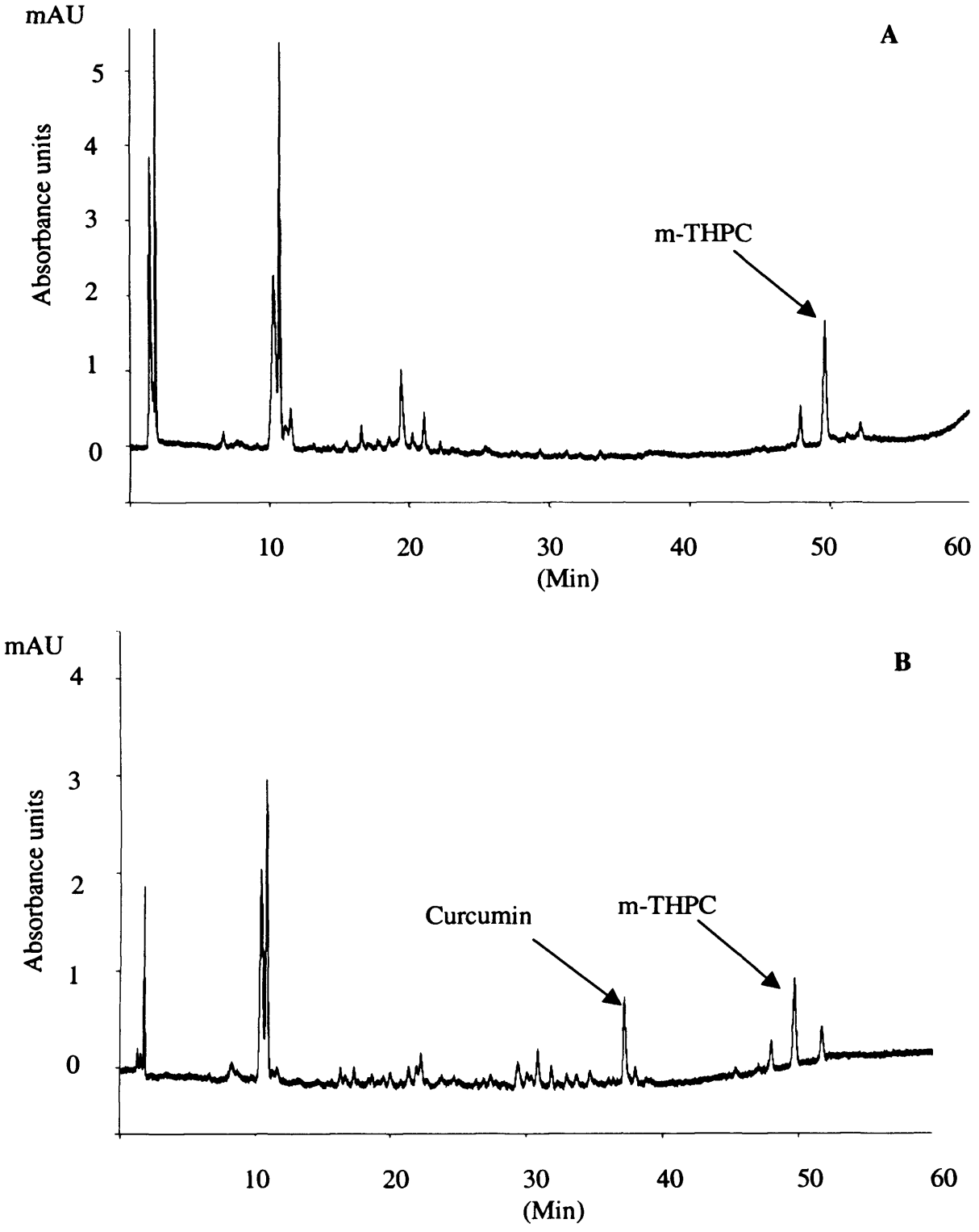


Figure 6.2.6 HPLC analysis of extract of liver of mice which received A) control diet (RM3) and B) curcumin (0.2% in RM3 diet) for 8 days. The internal standard (m-THPC) and curcumin retention times are 50 and 37 minutes respectively. Extraction and HPLC analysis was performed as described in section 2.2.18.

In the intestinal mucosa and faeces, analysis of the HPLC chromatograms showed the presence of traces of species, which co-eluted with authentic curcumin sulphate. An example of a representative chromatogram showing the presence of curcumin sulphate from the analysis of extracted small intestine mucosa is shown in Figure 6.2.7.

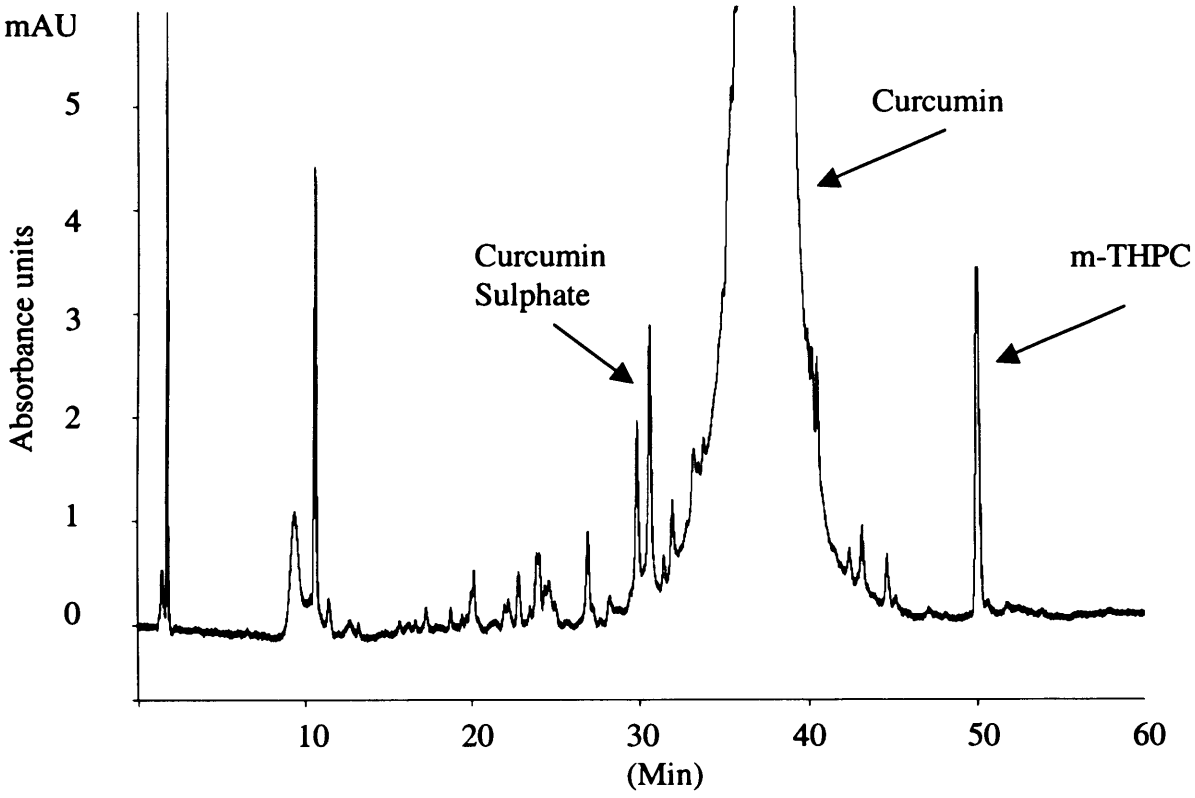


Figure 6.2.7 HPLC analysis of extract of small intestinal mucosa from a mouse that received curcumin (0.2% in RM3 diet) for 8 days. The internal standard (m-THPC) and curcumin sulphate retention times are 50 and 31 minutes respectively. Extraction and HPLC analysis was performed as described in section 2.2.18

Mass spectral investigation of the HPLC peak in the intestinal mucosa, by selected ion monitoring, afforded the molecular ion of *m/z* =447, corroborating the identity of the peak as curcumin sulphate (Figure 6.2.8).

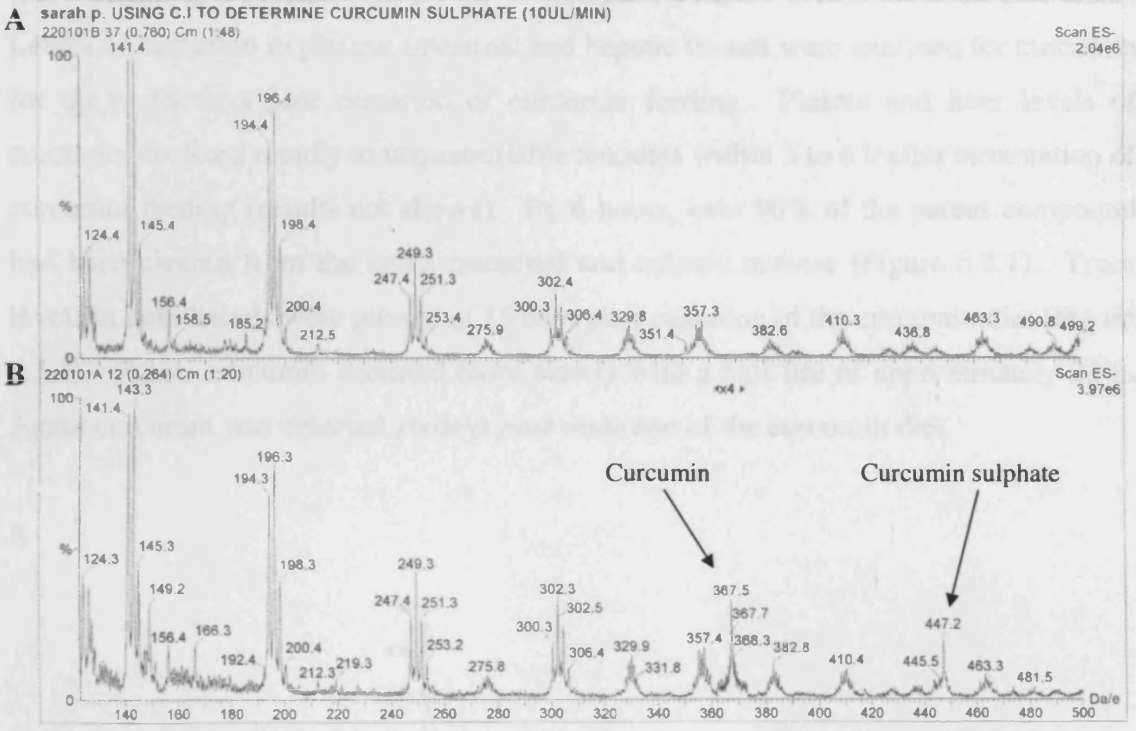
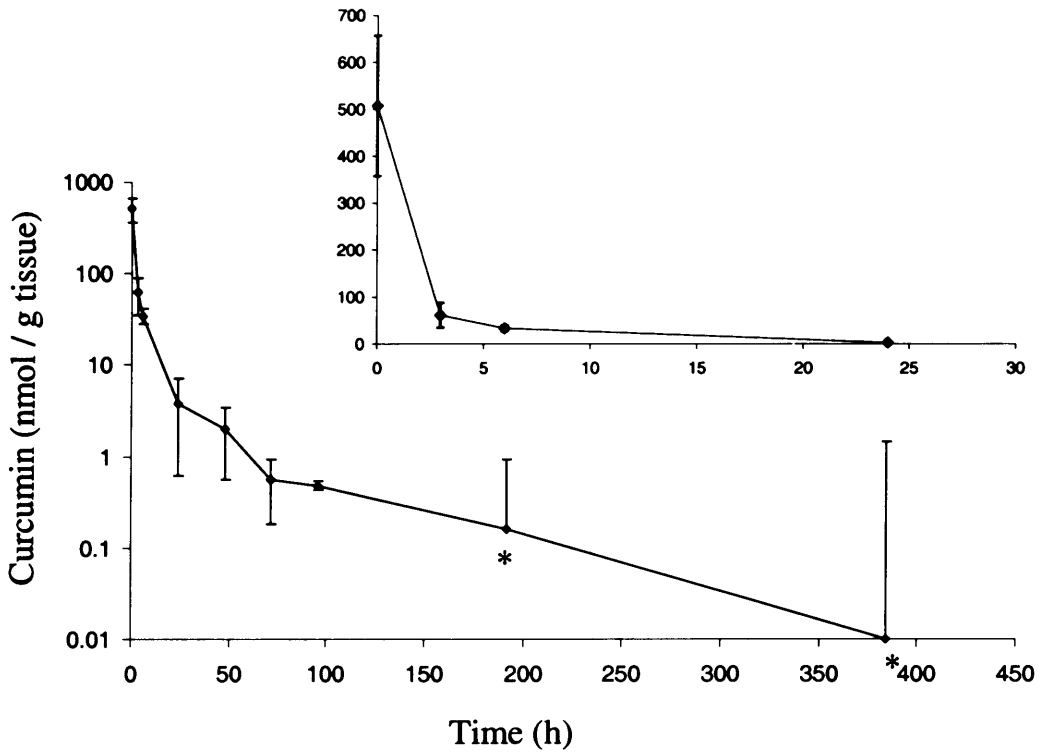


Figure 6.2.8 Mass spectra analysing extract of A) control small intestine mucosa B) collected HPLC fraction from small intestine mucosa containing a putative peak for curcumin sulphate (*m/z* =447) (Mass spectral analysis was performed as described in section 2.2.19)

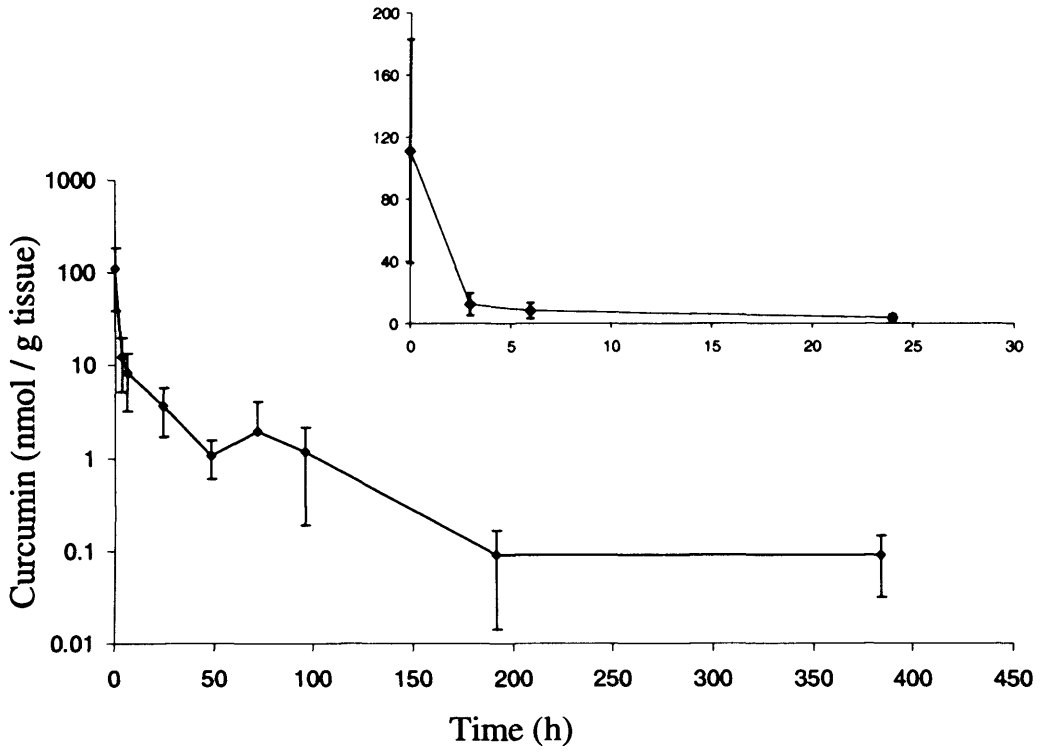
6.3 Determination of the clearance of curcumin from target tissues and excreta of C57BL/6J mice after termination of 8-day curcumin diet.

To determine a suitable dosing regime, studies were performed to explore how rapidly curcumin levels declined on termination of treatment. C57Bl/6J mice received RM3 diet containing curcumin (0.2 %) for 8 days prior to continuance on RM3 diet alone. Levels of curcumin in plasma, intestinal and hepatic tissues were analysed for curcumin for up to 16 days *post* cessation of curcumin feeding. Plasma and liver levels of curcumin declined rapidly to unquantifiable amounts within 3 to 6 h after termination of curcumin feeding (results not shown). By 6 hours, over 90% of the parent compound had been cleared from the small intestinal and colonic mucosa (Figure 6.3.1). Trace levels in both tissues were present at 16 days *post* cessation of the curcumin diet (Figure 6.3.1). Faecal curcumin declined more slowly with a half life of approximately 23 h. Again curcumin was detected 16 days *post* cessation of the curcumin diet.

A



B



C

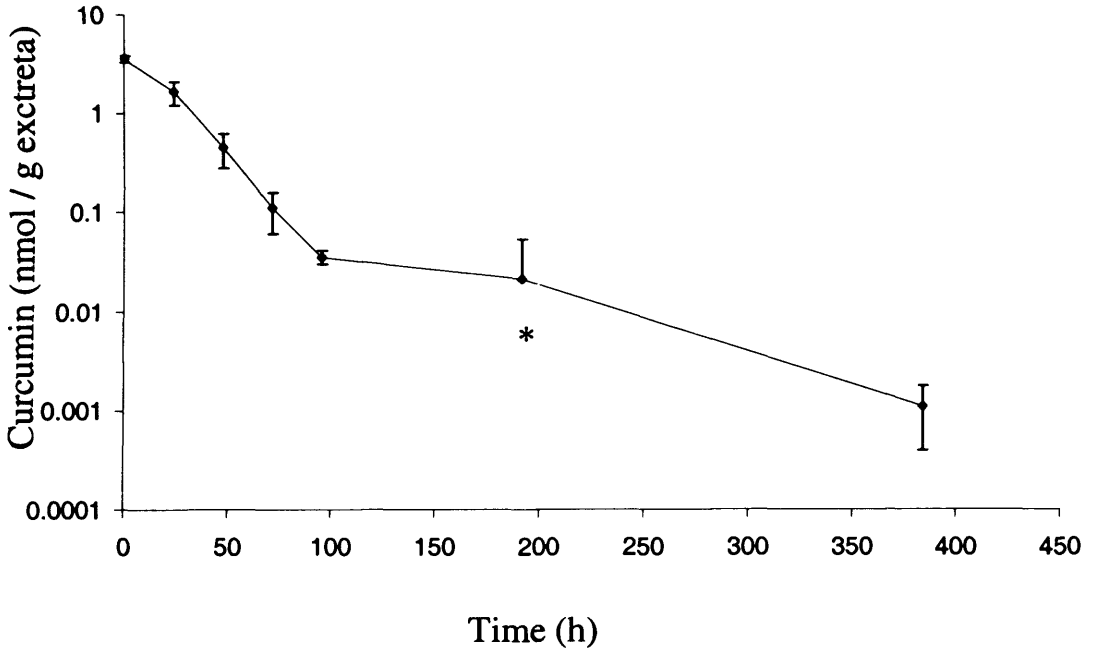


Figure 6.3.1 Disappearance of curcumin from A) colon B) small intestine and C) excreta of C57BL/6J mice. Inset shows the disappearance of curcumin up to 24 h (n=3, mean ± SD). Asterisks denote incomplete standard deviation Y- error bars due to limitations of logarithmic scale. Extraction and HPLC analysis were performed as described in section 2.2.18

6.4 Disposition of radioisotope after *ip* administration of [^{14}C] curcumin (100mg / kg)

Mice received [^{14}C] curcumin (vehicle: DMSO, dose: 100 mg/ kg) *via* the *ip* route, and the disappearance of radioactivity associated with the curcumin molecule was studied in plasma, intestinal tract, liver, heart, lung, kidney, brain and muscle tissues. Plasma and other tissues were collected 0.25, 0.5, 1, 2, 4, 8, and 24 h after *ip* administration. (Justification of the chosen route of administration is discussed in section 6.5.) Peak levels of radioactivity measured in the plasma and tissues after *ip* injection of [^{14}C], expressed as nmol curcumin equivalents per ml plasma or per g tissue are shown in Table 6.4.1. Beyond the peak, radioactivity declined swiftly to reach levels of between 20 and 33 % of peak values at 4 h - or in the case of the small intestine 8 h - *post* dosing (Figure 6.4.1). From this time point onwards radioactivity levels hardly decreased up to 24 h. A similar pattern of disposition was observed in heart, lung, kidneys, brain and muscle tissues, in that levels decreased within 2-4 h *post* dosing to 10-20 % of peak levels, and radioactivity levels then remained at this residual level for up to 24 h (Figure 6.4.1 and Figure 6.4.2)

Table 6.4.1 Peak levels of radioisotope and time of peak levels in tissues after *ip* administration of [^{14}C] curcumin (n = 3, mean ± SD). (Details of methodology of radioisotope analysis are described in section 2.2.20)

Tissue	Time (h)	Radioisotope peak level (nmol / g tissue or ml plasma)
Intestinal Mucosa	1	200 ± 23
Kidney	0.25	78 ± 3.0
Liver	0.25	73 ± 20
Plasma	0.5	25 ± 2.0
Lungs	0.25	16 ± 3.0
Heart	0.25	9.1 ± 1.1
Muscle	0.25	8.4 ± 6.0
Brain	0.25	2.9 ± 0.4

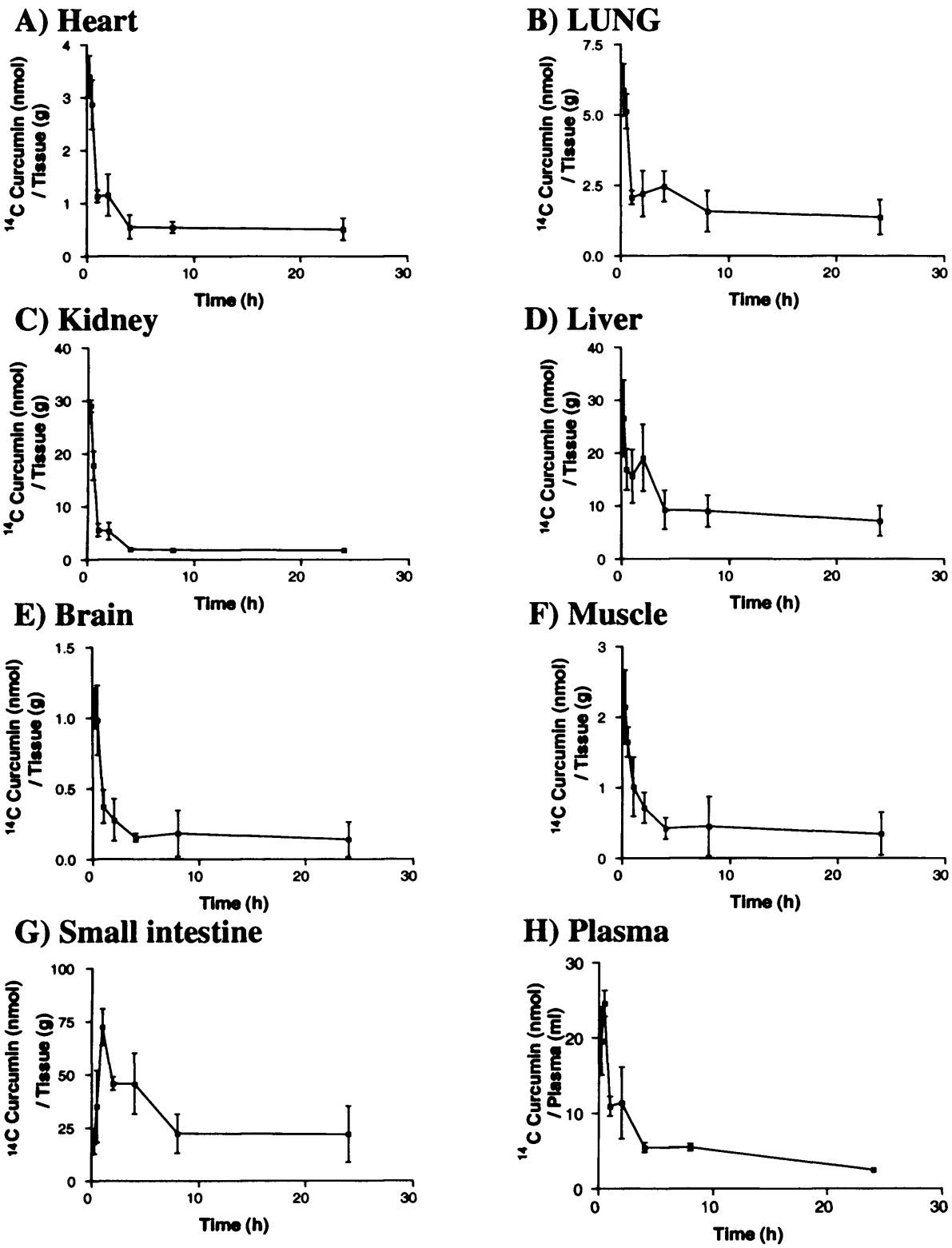


Figure 6.4.1. The distribution of radioisotope over time for A) heart, B) lung, C) kidney, D) liver, E) brain, F) muscle, G) small intestine and H) plasma (n = 3, mean ±SD). (Details of methodology of radioisotope analysis are described in section 2.2.20)

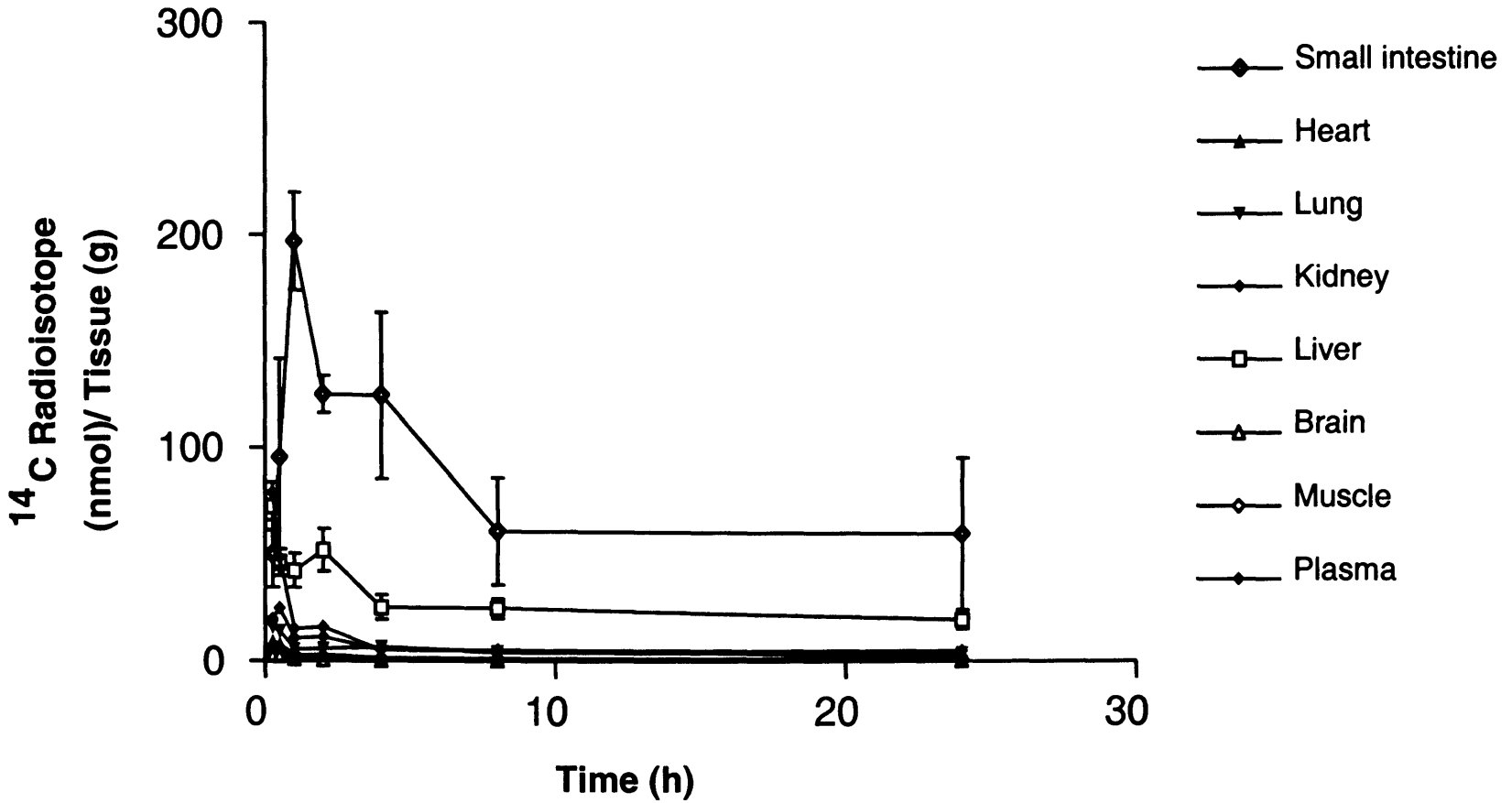


Figure 6.4.2 Disposition of [^{14}C]- radioisotope in C57BL/6J mice after *i.p* administration of 100mg/kg [^{14}C] curcumin (n= 3 ± SD)(Details of methodology of radioisotope analysis are described in section 2.2.20)

6.5 Discussion

From the work described in this thesis, novel conclusions can be drawn with regard to the pharmacokinetics of curcumin in mice. These conclusions may facilitate the rational development of suitable dosing regimes in humans with colorectal cancer. Moreover, this work is the first assessment of levels of curcumin in target tissues after dietary administration at several dose levels and is the first assessment of the distribution of radiolabelled curcumin in a broad range of tissues after *ip* administration of [^{14}C] curcumin.

The low systemic bioavailability of orally administered curcumin is well documented in both rodents (Wahlstrom and Blennow, 1978; Holder *et al.*, 1978; Ravindranath and Chandrasekhara, 1980; Ireson *et al.*, 2001a; Pan *et al.*, 1999) and humans (Shoba *et al.*, 1998; Cheng *et al.*, 1998; Sharma *et al.*, 2001c;). The work reported here is in accordance with these findings. The majority of the dietary curcumin was excreted unchanged in the faeces (Table 6.2.1) and the systemic availability of curcumin after oral intake was poor; the substantial dietary doses resulted in only ρM plasma levels (Figure 6.2.5). Curcumin levels in urine were undetectable suggesting that curcumin undergoes biliary excretion. Consideration of the tissue and plasma concentration of curcumin after dietary intake is pertinent for two separate pharmacological reasons. Curcumin levels in the gastro-intestinal mucosa, for which systemic absorption is not a prerequisite, determine its gastro-intestinal cancer preventive efficacy. In this respect curcumin offers great potential as a chemopreventive agent for the treatment of preneoplasias of the gastro-intestinal tract. *Post*-absorption levels of curcumin in the systemic circulation reflect the ability of curcumin to access tissue sites distant from the region of absorption. However, levels of curcumin in the systemic circulation were found to be poor. This finding mitigates against the usefulness of dietary curcumin in the prevention of malignancies remote from the gastro-intestinal tract.

Concentrations of curcumin observed in the small intestine were correlated with dose, although this correlation was not observed in the faeces or colon. The lack of dose dependency in the colon may well be a reflection of the small amounts of mucosal tissue that can be collected in this region. Increasing the dose level from 0.1% to 0.5% did not offer a significant increase in curcumin concentration in plasma and liver. This

may be a consequence of saturation of absorption or induction of metabolism at higher doses.

The presence of curcumin sulphate in the intestinal mucosa and faeces described here is consistent with the suggestion that curcumin undergoes metabolic conjugation in the gut (Holder *et al.*, 1978; Ireson *et al.*, 2001b, Ireson *et al.*, 2002). Identification of this metabolite is consistent with the report of Sharma and colleagues (2001c) who showed that curcumin sulphate was the only metabolite to be identified in faeces of one of 5 patients on *Curcumin* extract at a dose equivalent to 180 mg of curcumin *pd.* Previous reports suggest that following oral dosing, curcumin undergoes metabolic conjugation to curcumin glucuronide and curcumin sulphate and reduction to tetrahydrocurcumin, hexahydrocurcumin and hexahydrocurcuminol in rats and mice (Ireson *et al.*, 2001a; Pan *et al.*, 1999; Asai and Miyazawa, 2000) and in suspensions of human and rat hepatocytes (Ireson *et al.*, 2002). In the study reported here, intestinal faecal samples were analysed for the presence of curcumin glucuronide as HPLC fraction collection was performed at 25 minutes (retention time for curcumin glucuronide). However, curcumin glucuronide was undetected in these samples as confirmed by mass spectrometry (data not shown). HPLC analysis at a wavelength of 280 nm did not reveal any major peaks at retention times that would co-elute with the reduced metabolites of curcumin. The retention time of hexahydrocurcuminol is 22 minutes and the retention time of tetrahydrocurcumin is 24 minutes. The lack of metabolites is somewhat surprising in light of the fact that curcumin undergoes avid metabolic conjugation and reduction in hepatic cells (Ireson *et al.*, 2001a) and in hepatic and gut fractions *in vitro* (Ireson *et al.*, 2002). The lack of metabolites is inconsistent with the work by Pan and colleagues (1999). Discrepancies may be due to the dietary administration of curcumin rather than gavage administration, the formulation of curcumin (Pan and colleagues emulsified the curcumin) or extraction methods.

The lack of metabolites observed in this study suggest that the chemopreventive efficacy of curcumin in the Min/+ mouse is highly likely to be due to the parent compound rather than a metabolite. Previous work has shown that the metabolites such as tetrahydrocurcumin possess anti-inflammatory (Mukhopadhyay *et al.*, 1982) and antioxidant activities (Sugiyama *et al.*, 1996; Osawa *et al.*, 1995) similar to those of their metabolic progenitor. However the curcumin conjugates are probably devoid of

biological activity (Ireson *et al.*, 2001a). Therefore the presence of curcumin sulphate in the intestine mucosa may offer little chemopreventive efficacy in the reduction in tumour burden after administration of curcumin. Future work may involve the study of the chemopreventive efficacy of the major metabolites of curcumin in the Min/+ mouse model of colorectal cancer. However this is to finalise the assessment of the chemopreventive efficacy of curcumin and its metabolites in the Min/+ mouse rather than investigating the possibility that these agents may be more efficacious, as this is unlikely.

Curcumin disappears relatively rapidly from murine tissues, including the target tissue, once treatment is discontinued (Figure 6.3.1). However, trace levels could be detected in the faeces and intestine up to 16 days after termination of the curcumin (0.2%) diet. This result is important given the observation (discussed in chapter 3) that curcumin needs to be present consistently in the gastro-intestinal mucosa of Min/+ mice for several months in order to achieve chemopreventive activity. The data suggests that daily administration of curcumin is required to achieve an efficacious concentration. Potentially rapid dispositional removal of curcumin from the target tissue needs to be taken into account, if sustained levels are to be achieved in humans. To delay clearance of curcumin from the organism might well pose a formidable pharmaceutical technological challenge. Further work may include the analysis of target tissues and excreta in Min/+ mice that receive curcumin (0.2%) from 4 – 18 weeks of age. Drug levels could be determined in intestinal adenomas and surrounding normal mucosa to provide an assessment of levels required to hinder the growth of the adenomas.

Interestingly, in contrast to its low systemic availability when consumed as a constituent of the diet, [^{14}C]-curcumin formulated in DMSO, after administration *via* the *ip* route, achieved substantial levels of curcumin in the plasma and a variety of tissues remote from the gastro-intestinal tract. This finding hints at the possibility that development of an imaginative curcumin formulation for humans may help considerably to improve its absorption and bioavailability. It needs to be stressed that the curcumin pharmacokinetics observed in plasma and tissues after *ip* administration cannot be compared directly with those observed after dietary intake, not least because the agent was formulated for *ip* administration in DMSO, an amphiphilic solvent which seemed to enhance curcumin absorption considerably. After achieving peak levels,

[^{14}C] curcumin, or species derived from it, fell swiftly to low, but detectable, residual levels of radioactivity, which remained almost stationary for at least 24 h. Whilst the exact chemical nature of this radioactive residue was not explored further, the results illustrated in Figure 6.4.1 show that curcumin and/ or its metabolite(s) can be released from a deep compartment giving rise to low levels of drug-derived species for a considerable period of time. Further work would require *ip* administration of cold curcumin (100 mg/ kg) and HPLC analysis of curcumin and its major metabolites. The inclusion of a radiolabelled curcumin study enabled a sensitive method of tracing all the radioactive species in the mice. However, to follow this with a "cold experiment" would then facilitate an assessment of the formation of metabolites in the tissues studied.

After evaluation of the current information on curcumin and its pharmacokinetic and pharmacodynamic properties in animal models of colorectal cancer, no studies had aimed to assess the relationship between these pharmacological parameters in mice. The work presented in this chapter, in conjunction with the previous chapters, has aimed to explain this discrepancy, providing a pharmacokinetic assessment of dietary administration of curcumin in the mouse. This assessment may in turn aid the optimisation of a suitable dosing regime in patients with colorectal cancer.

CHAPTER 7
FINAL DISCUSSION

7.1 Final discussion

The rationale for preclinical chemoprevention research using rodent models of carcinogenesis is to provide mechanistic, pharmacokinetic and safety data on the compound under investigation. This information is then utilised to optimise the design of clinical trials. The main aim of this project was to provide, at least in part, this type of information. The objective was to elucidate the relationship between pharmacokinetics and efficacy of curcumin as reflected by its effect on tumour number and biochemical events associated with tumour development in the Min/+ mouse. Addressing this objective was thought to assist in the optimisation of clinical trials of curcumin in individuals at risk of developing colorectal cancer.

These studies have demonstrated the chemopreventive efficacy of curcumin in the Min/+ mouse model of colorectal cancer. Both the effect of dose and duration of dietary administration of curcumin on tumour development have been studied and results provide a comprehensive assessment of the effect of curcumin on the size and location of intestinal adenomas in Min/+ mice. At a level of 0.2% in RM3 diet, curcumin reduces tumour number in Min/+ mice by 39%. These results (discussed in detail in chapter 3) are in agreement with the studies of Mahmoud *et al.*, (2000) and Collett *et al.*, (2001) who report chemopreventive efficacy of curcumin at similar dose levels, 0.1% and 0.2% respectively. However, the results reported here show that curcumin has a rather narrow therapeutic window, as increasing the dietary level of curcumin from 0.2% to 0.5% failed to further reduce tumour burden in Min/+ mice. The failure to yield any additional gain in efficacy was irrespective of the fact that curcumin concentrations in the small intestine adequately mimicked the stepwise increase in the dose consumed as described in chapter 6. Although such studies have not been performed previously, the lack of dose dependant reduction in tumour burden is surprising. The reason for the discrepancy may be revealed by studies of the dose dependant biochemical mechanisms of action of curcumin, rather than changes in the gross pathology.

Curcumin appears to have to be present throughout the carcinogenic process to reduce tumour burden in Min/+ mice. Unlike other, more potent agents, such as piroxicam, curcumin does not reduce tumour burden when administered either *in utero* or for short time periods (Jacoby *et al.*, 2000). Targeting either the initiation or promotion stages of

carcinogenesis, rather than both, failed to affect tumour burden. The poor chemopreventive efficacy of curcumin administered pre-weaning may be a consequence of the poor bioavailability of curcumin and consequently the low concentrations that reached the foetuses or passed to the offspring through the mother's milk. Curcumin disappears quickly from target tissues after cessation of dietary administration as described in chapter 6, necessitating continual ingestion of this chemopreventive agent. Curcumin is without embryotoxicity towards Min/+ offspring, unlike the NSAID piroxicam (Jacoby *et al.*, 2000). The main advantage of curcumin over these traditional prescription NSAIDs is arguably the lack of toxicity even at dose levels of approximately 750mg/ kg body weight per day in Min/+ mice. No abnormal curcumin associated pathology was observed. The dietary levels of curcumin required to reduce tumour number are around 10-fold higher than efficacious levels of synthetic compounds such as sulindac and celoxocib, which possess biological properties not unrelated to those of curcumin. However, curcumin is a safe, inexpensive and efficacious agent for the treatment of intestinal adenomas in the Min/+ mouse.

It is difficult to directly compare results from different research projects on the same agent as chemopreventive efficacy is affected by factors such as the basal diet utilised, the time scale of the study in terms of the commencement and termination of the test diets and the genetic background of the animal model. This fact is highlighted by the preliminary study (described in chapter 3) in which the effect of changing basal diet from AIN76A to RM3, increased adenoma number by 45%. Food restriction has recently been shown not to affect tumour multiplicity in Min/+ mouse (Kakuni *et al.*, 2002), although the growth of the polyps was reduced and this correlated with increased apoptosis (Kakuni *et al.*, 2002). However, modification of the carbohydrate and fat intake could possibly be an area of further investigation in the Min/+ mouse considering the breadth of data concerning Western style high calorific diets and disposition to colorectal cancer (Le Marchand *et al.*, 1997).

Work reported in this thesis shows that, in contrast to curcumin, aspirin on its own has to be present pre-weaning, during the initiation and early promotion stages of adenoma formation in the Min/+ mice to reduce tumour burden. The experiments utilising aspirin, described in chapter 3, are novel and may partially explain the discrepancy concerning reports of chemopreventive efficacy of aspirin in the Min/+ mouse. The

work reported here concurs with the hypothesis of Sansom *et al.*, (2001) which suggests that the earlier the aspirin diet is started, the more likely it is to reduce tumour burden in the Min/+ mouse. The combination of curcumin and aspirin offered no additive effect in reducing tumour burden compared to sole administration of curcumin (as described in chapter 3). The lack of synergism may have been a consequence of both agents acting via similar mechanisms of action, thus not providing any additive effect. Other studies, which have utilised a combination strategy, have used agents with differing mechanisms of action, for example sulindac and EKI-569 (a novel irreversible inhibitor of the epidermal growth factor receptor kinase) (Torrance *et al.*, 2000), or nonselective COX inhibitor piroxicam and the ornithine decarboxylase (ODC) inhibitor difluoromethylornithine (DFMO) (Jacoby *et al.*, 2000). Such a strategy might be usefully employed in further investigations with curcumin. Possible synergistic agents for curcumin, include the insulin-like growth factor (IGF) inhibitor silibinin, derived from milk thistle or the bile salt ursodeoxycholate. Increased IGF-II supply in the Min/+ mouse results in increased growth of polyps and an increased progression from adenoma to carcinoma (Hassan and Howell, 2000). Furthermore, reduced IGF-II supply in Min/+ crosses with *Igf2* paternal allele knockout mice resulted in reduced adenoma size and frequency (Hassan and Howell, 2000). Ursodeoxycholate when given with low dose sulindac is an effective chemopreventive agent combination that is well tolerated and decreases adenoma multiplicity in Min/+ mice (Jacoby *et al.* 2002). Ursodeoxycholate has anti-inflammatory and chemoprotective effects in animal models of inflammatory bowel disease and colon cancer through a variety of mechanisms including inhibition of iNOS (Invernizzi *et al.*, 1997) and induction of apoptosis (Schlottman *et al.*, 2000).

Analysis of the pharmacokinetics of the dietary administration of curcumin is an intrinsic factor in the optimisation of clinical trials and one frequently overlooked in preclinical studies of chemopreventive agents. The work described here (chapter 6) suggests that curcumin, when administered to mice, is poorly bioavailable, disappears rapidly from tissues, and is metabolised in the gut to curcumin sulphate. Therefore, a frequent dosing regime in humans should be implemented. These studies also highlight the small relative contribution of metabolites to the chemopreventive efficacy of curcumin, unlike the prodrug sulindac, in which the anti-tumour effect is mediated by the sulphide metabolite (Mahmoud *et al.*, 1998).

The picomolar plasma levels of curcumin after dietary administration, described in chapter 6, mitigates against the usefulness of dietary curcumin in the prevention of malignancies remote from the gastrointestinal tract. However, curcumin levels in the gastro-intestinal mucosa, for which systemic absorption is not a prerequisite, determine its gastro-intestinal cancer protective potency. High levels of unaltered curcumin were measured in the intestinal mucosa following dietary administration, and if the same holds true for humans, biological effects should be observed in this target organ. In this regard, human studies in which intestinal tissue levels are measured will provide valuable pharmacokinetic information. The work described in this thesis in which an efficacious dietary level of curcumin was determined might prove important in the design of future clinical trials.

For curcumin to be effective in the Min/+ mouse model, the gastrointestinal mucosa, the target tissue, needs to be exposed to curcumin at concentrations near or above 100 nmoles/ g tissue. There are a host of biochemical candidate processes, which might be compromised by curcumin at 100-500 nmoles/ g mucosal tissue. This concentration range is equivalent to 0.1–0.5 mM in experiments *in vitro* using cells or cellular fractions in culture, assuming equivalent conditions. In such systems curcumin has been shown to act as a scavenger of reactive oxygen species (Sharma 1976; Subramanian *et al.*, 1994; Tonnesan and Greenhill., 1992; Kunchandy and Rao, 1990; Reddy and Lokesh, 1994) and to interfere with lipid peroxidation as well as interfering with signalling events such as prostaglandin biosynthesis (Huang *et al.*, 1992) and the activity and expression of the enzyme COX (Huang *et al.*, 1991; Plummer *et al.*, 1999). All of these inhibitory actions of curcumin require concentrations of the agent in the 10–100 μM range, which is well within the levels achieved in the target tissue of Min/+ mice at efficacious dietary doses.

Using this knowledge, a further objective of this project was to identify possible indicators of carcinogenesis and the effect of curcumin on these markers. Firstly elevated levels of COX-2 and β-catenin proteins and increased levels of two distinct oxidative DNA adducts (M_1G and 8-oxo dG) were identified in the intestinal adenomas of the Min/+ mice (as described in chapter 4). Despite the link with M_1G production, MDA levels were not significantly higher than those of the surrounding mucosa.

Therefore, the M_1G adduct formation in adenomatous tissue occurred principally through MDA-independent pathways, such as oxidative DNA damage resulting in formation of base propenals (Dedon *et al.*, 1998; Plastaras *et al.*, 2000a). The higher levels of 8-oxo dG in the adenomatous tissue may corroborate this hypothesis (as described in chapter 4).

Curcumin was shown to significantly decrease adenomatous levels of COX-2, M_1G adduct and 8-oxo dG levels (chapter 4). The effect of curcumin on these markers of carcinogenesis in the Min/+ mouse has not been reported before. These results also highlight possible mechanisms of chemopreventive action of curcumin in the Min/+ mouse. However, the link between these mechanisms is thus far unresolved. The mechanistic cascade described in Figure 7.1 depicts feasible associations and potential targets of curcumin and similar antioxidants.

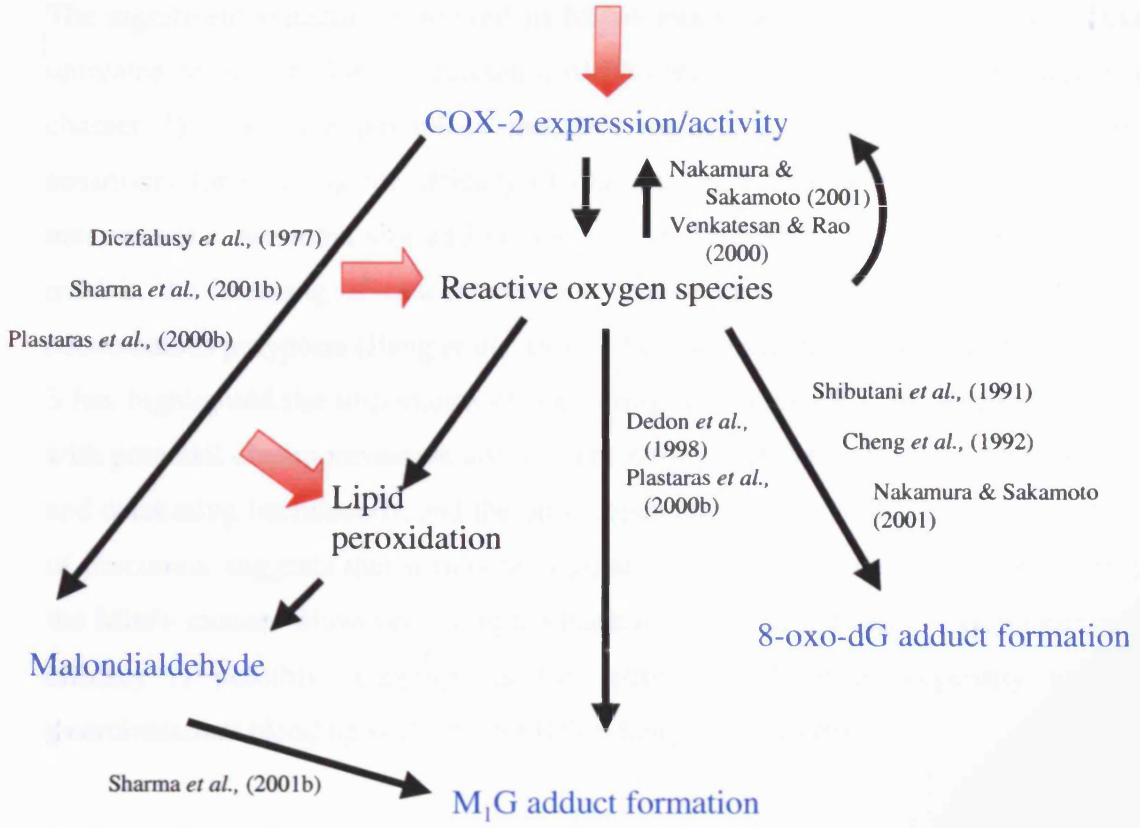


Figure 7.1 Cellular routes of oxidative DNA adduct formation involving oxidative stress and COX-2. The references refer to *in vitro* data supporting the links shown. The red arrows indicate potential points of inhibition by curcumin. Oxidative stress induced by hydrogen peroxide induced COX-2 expression in bovine luteal cells *in vitro* and this stress was associated with an increase in 8-oxo dG levels (Nakamura and Sakamoto, 2001). Incubation of salmon testis DNA with human recombinant COX-2 *in vitro* in the presence of large amounts of arachidonic acid substrate resulted in the formation of 8-oxo dG, and this reaction was attenuated by addition of antioxidants (Nikolic and van Breeman, 2001).

The significant reduction observed in Min/+ mouse adenoma M_1G levels relative to untreated mice at a dose of curcumin (0.1%) that did not reduce polyp number (see chapter 3) raises the possibility that this molecular biomarker may offer greater sensitivity for studying the efficacy of oral curcumin treatment than the macroscopic measures (*i.e.* adenoma size and number). These macroscopic measures are currently used in the licensing of cancer chemopreventive drugs for individuals with familial adenomatous polyposis (Jiang *et al.*, 1996). Furthermore, the work described in chapter 3 has highlighted the importance of measuring the haematocrit of Min/+ mice treated with potential chemopreventive agents. The strong correlation between tumour number and decreasing haematocrit, and the dose dependant elevation of haematocrit with dose of curcumin, suggests that it may be a good indicator of chemopreventive efficacy in the Min/+ mouse. However, using the haematocrit as an indicator of chemopreventive efficacy is possibly inappropriate for agents that have a propensity to induce gastrointestinal bleeding such as NSAIDS (Henry *et al.*, 1996).

As biomarkers of carcinogenesis should identify changes prior to the development of neoplasia, the potential markers studied here, COX-2, M_1G adduct and 8-oxo dG levels should also be studied at earlier time points in the Min/+ mouse. The study of the gene expression profile of both Min/+ and curcumin treated Min/+ mice aged 9 weeks attempted to identify further markers of carcinogenesis. Interesting gene changes after curcumin administration included superoxide dismutase and glutathione transferase (π). These gene changes may warrant further investigation as potential biomarkers that may be modulated by curcumin. Overall, the results presented here strongly suggest the clinical relevance of oxidative DNA adducts to intestinal carcinogenesis. Furthermore, their use as biomarkers of efficacy in clinical trials of chemopreventive antioxidants should be investigated. In conjunction, the measurement of COX-2 expression or activity should be considered for agents known to be capable of modulation of these parameters.

There are several implications of the project described here for the potential clinical evaluation of curcumin. The work corroborates the notion that curcumin possesses chemopreventive activity in a model germane to human colorectal carcinogenesis (Mahmoud *et al.*, 2000; Collett *et al.*, 2001). The studies of the chemopreventive

efficacy encourage evaluation of curcumin for the adenoma-retarding efficacy in FAP patients. The applicability to humans of the relationship between dose level, chemopreventive potency and pharmacokinetics defined in this report needs to be investigated. On the assumption that there is direct comparability, the dose of curcumin required for efficacy in humans equivalent to the 0.2% dietary concentration or 300 mg/ kg *pd* dose which was 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 if 1.8m^2 accompanying a body weight of 70 kg (Freireich *et al.,* 1966). This putative efficacious clinical dose of curcumin is well within the dose range 0.5, 1.2, 2.1 and 8g *pd* for up to 6 weeks, which according to the literature has been administered to humans without adverse effect (Soni and Kuttan, 1992; Deodhar *et al.,* 1980; Satoskar *et al.,* 1986; Cheng *et al.,* 1998). Therefore curcumin is a safe, inexpensive and potentially efficacious agent, which warrants further investigation for the treatment of familial adenomatous polyposis.

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APPENDICES

Appendix 1:Diets

RM3 diet is a standard laboratory rodent diet used for conventionally for breeding, lactation and growth of young stock due to the high nutrient content. It was therefore deemed appropriate for use in the aforementioned studies of tumour burden in Min/+ mice as it adequately mimics a "Western" style diet. However, RM3 diets are not standardised for nutrient source and composition and variations may exist between batches. In contrast the AIN 76A diet is a maintenance diet that is lower in nutrients. AIN 76A diet is also a standardised synthetic diet whereby the composition is guaranteed as shown in the table below. Initial studies (eluded to in section 3.8) used the AIN 76A diet, as this matrix provides a consistent nutritional intake. However, due to the high number of unexplained premature deaths of both wildtype and Min/+ mice this diet was abandoned in favour of the RM3 diet.

AIN 76A

Composition (taken from data sheet for AIN76A diet, PMI Nutrition, Nottingham, UK)

Ingredients:	**Percentage composition (%)**
Edible casein	20
Choline bitartrate	0.2
Cornflour snowflake	32.5
Corn oil	5
Methionine	0.3
Solkafloc	5
Sugar icing	32.5
AIN76 vitamins mix	1
AIN76 mineral mix	3.5

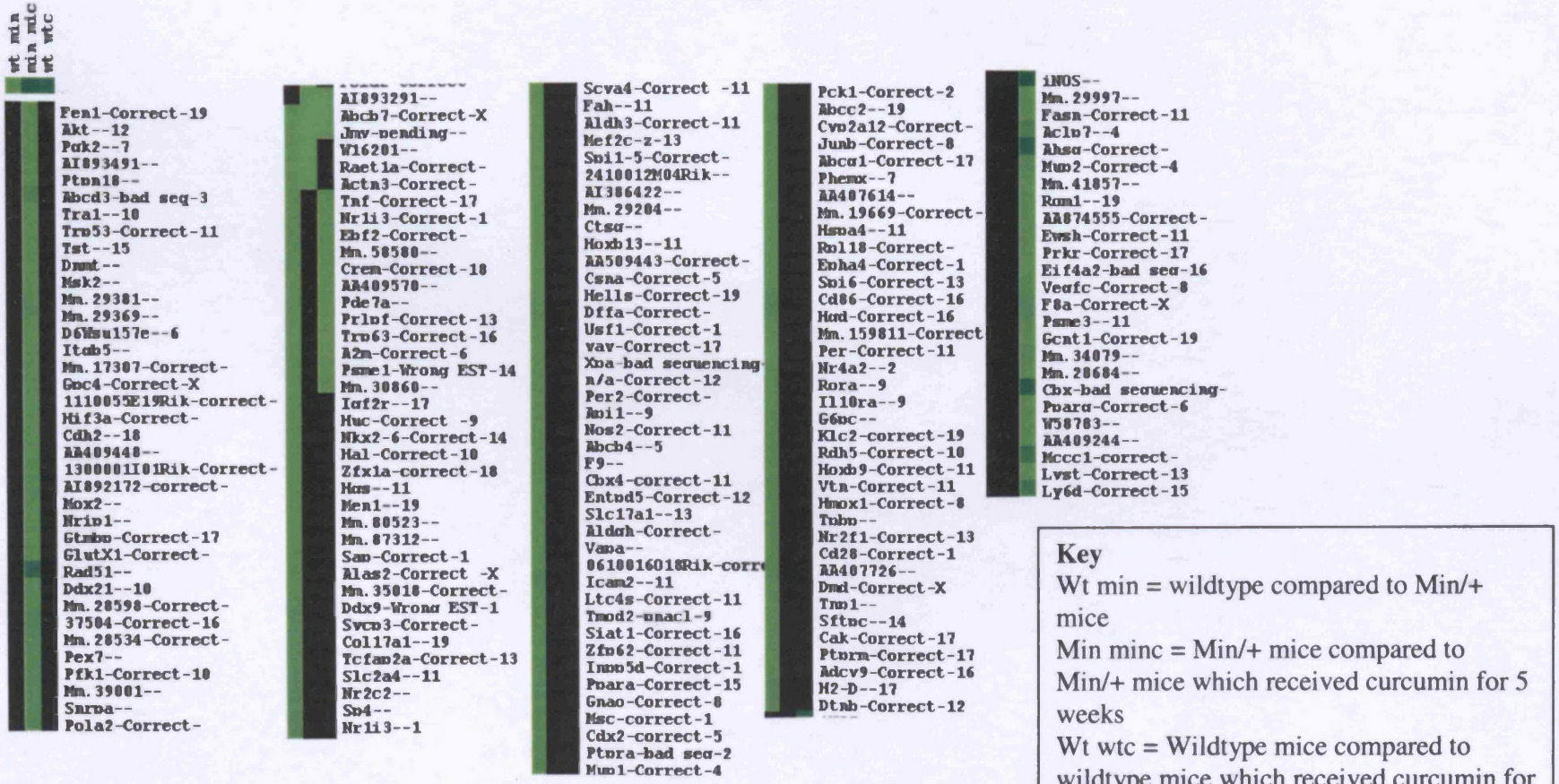
RM3 diet

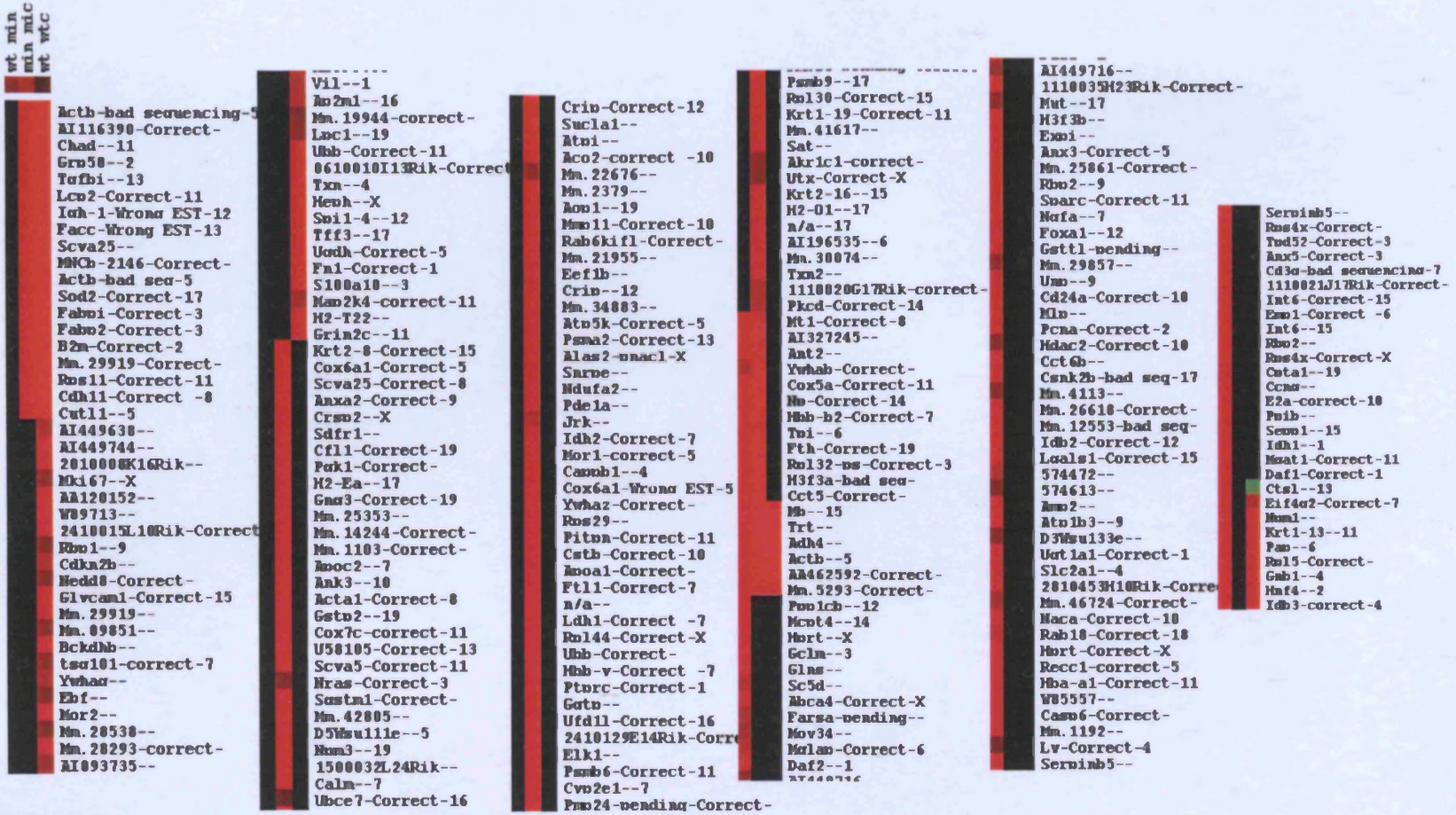
Composition (taken from data sheet for rat and mouse no.3 breeding diet, Special Diets Service, Witham, UK)

Ingredients:	**Percentage composition (%)**
Cereal products	64
Wheat	
Barley	
Wheat feed	
Vegetable proteins	16.5
Extracted Soya bean meal	
Unextracted dried yeast	
Animal proteins	15
Fish	
Whey powder	
Energy sources	2
Supplementation	2.5
Vitamins	
Minerals	
Trace minerals	
Amino acids	

Appendix 2: Microarray data

Significant gene changes detected by cDNA microarray. Downregulated genes are shown in green and upregulated genes in red (Methodology described in section 2.2.2.16; results described and discussed in chapter 5)





Key

Wt min = wildtype compared to Min/+ mice

Min minc = Min/+ mice compared to Min/+ mice which received curcumin for 5 weeks

Wt wtc = Wildtype mice compared to wildtype mice which received curcumin for 5 weeks

Appendix 3: Publications

Full papers

Perkins S, Verschoyle RD, Hill K, Parveen I, Threadgill MD, Sharma RA, Williams ML, Steward WP, Gescher AJ. Chemopreventive efficacy and pharmacokinetics of curcumin in the min/+ mouse, a model of familial adenomatous polyposis. *Cancer Epidemiol Biomarkers Prev* 2002: **11**: 535-40

Perkins S, Sharma RA, Singh R, Farmer PB, Steward WP, Gescher AJ. Involvement of cyclooxygenase-2 and oxidative DNA adducts in the chemoprevention of mouse intestinal adenoma formation by dietary curcumin. *Cancer Res,* submitted

Perkins S, Clarke AR, Steward WP, Gescher AJ. Chemopreventive efficacy in Min/+ mice of sequential intervention with dietary aspirin and curcumin. *Br J Cancer*, submitted

Abstracts

Perkins S, Versholye R, Hill K, Williams ML, Steward WP, Gescher AJ. Relationship between the chemopreventive efficacy and pharmacokinetics of curcumin in the Min/+ mouse. *Proc Amer Assoc Cancer Res* 2002: **43**: 165
(Scholar in Training Award)

Perkins S, Sharma RA, Verscholye R, Marnett LJ, Steward WP, Gescher AJ. Biomarkers of the chemopreventive efficacy of curcumin (curc) in the Min/+ mouse. *Proc Amer Assoc Cancer Res* 2002: **43**: 126

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Sharma RA, Shafayat A, Perkins S, Singh R, Steward WP, Gescher A. Translating the measurement of malondialdehyde-deoxyguanosine adducts from preclinical models to patients. *Toxicol* 2002: **178**: 32-33

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Sharma RA, Ireson CR, Perkins S, McLelland HR, Leuratti C, Singh R, Verscholye R, Marnett LJ, Gescher AJ, Steward WP. Effects of curcumin on basal and induced malondialdehyde-DNA adduct levels in F44 rats. *Proc Amer Assoc Cancer Res* 2000: **41**: 412

Chemopreventive Efficacy and Pharmacokinetics of Curcumin in the Min/+ Mouse, a Model of Familial Adenomatous Polyposis[1]

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Abstract

Curcumin, the major yellow pigment in turmeric, prevents the development of adenomas in the intestinal tract of the C57Bl/6J Min/+ mouse, a model of human familial APC. To aid the rational development of curcumin as a colorectal cancer-preventive agent, we explored the link between its chemopreventive potency in the Min/+ mouse and levels of drug and metabolites in target tissue and plasma. Mice received dietary curcumin for 15 weeks, after which adenomas were enumerated. Levels of curcumin and metabolites were determined by high-performance liquid chromatography in plasma, tissues, and feces of mice after either long-term ingestion of dietary curcumin or a single dose of [^{14}C]curcumin (100 mg/kg) via the i.p. route. Whereas curcumin at 0.1% in the diet was without effect, at 0.2 and 0.5%, it reduced adenoma multiplicity by 39 and 40%, respectively, compared with untreated mice. Hematocrit values in untreated Min/+ mice were drastically reduced compared with those in wild-type C57Bl/6J mice. Dietary curcumin partially restored the suppressed hematocrit. Traces of curcumin were detected in the plasma. Its concentration in the small intestinal mucosa, between 39 and 240 nmol/g of tissue, reflects differences in dietary concentration. [^{14}C]Curcumin disappeared rapidly from tissues and plasma within 2–8 h after dosing. Curcumin may be useful in the chemoprevention of human intestinal malignancies related to *Apc* mutations. The comparison of dose, resulting curcumin levels in the intestinal tract, and chemopreventive potency suggests tentatively that a daily dose of 1.6 g of curcumin is required for efficacy in humans. A clear advantage of curcumin over nonsteroidal anti-inflammatory drugs is its ability to decrease intestinal bleeding linked to adenoma maturation.

Introduction

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is the major yellow pigment extracted from turmeric, a commonly used spice, derived from the rhizome of the plant *Curcuma longa*. In India and Southeast Asia, turmeric has long been used as a treatment for inflammation, skin wounds, and tumors. Curcumin has broad spectrum cancer chemopreventive activity in preclinical animal models (reviewed in Ref. 1). Especially intriguing is its ability to prevent carcinogen-induced intestinal premalignancies and malignancies in rats (2, 3) and in the Min/+ mouse, a model of hereditary FAP[3] (4). The Min/+ mouse has an autosomal dominant heterozygous nonsense mutation of the mouse *Apc* gene (5); homologous to human germ-line and somatic *APC* mutations. The C57Bl/6J Min/+ inbred mouse model is particularly advantageous for investigating chemopreventive agents targeted toward early-stage lesions because, in this mutant strain, scores of adenomas grow to a detectable size within a few months on a defined genetic background (6). Because Min/+ mice develop adenomas as a result of inactivation of the same tumor suppressor gene known to underlie the pathogenesis of most colon cancers in humans, experiments using this model are likely to be germane to the design of human chemoprevention trials (7). This notion has recently been illustrated impressively by the selective COX-2 inhibitor celecoxib, the development of which was advanced substantially by evaluation of its efficacy in Min/+ mice (8). This mouse model predicted the significant reduction, or retardation, of adenoma development that was subsequently seen in FAP patients (9), a result that, in turn, led to the approval by the United States Food and Drug Administration of celecoxib for the treatment of FAP (10).

The cancer chemopreventive activity of curcumin in humans has yet to be confirmed. A recent clinical pilot study in colorectal cancer patients (11) and results of experiments in rodents (12–14) suggest that the systemic availability of curcumin is poor. This conclusion mitigates against its use for the chemoprevention of malignancies remote from the site of absorption, but it would not preclude its development for the prevention of gastrointestinal neoplasias. We wished to evaluate the link between the pharmacological activity of curcumin in the Min/+ mouse and levels of curcumin and its metabolites in target tissue and plasma. To that end, curcumin was administered in the diet at three dose levels, efficacy was assessed in terms of adenoma load, and agent and metabolites were quantitated. A major complication associated with adenoma load in

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[3] The abbreviations used are: FAP, familial adenomatous polyposis; APC, adenomatous polyposis coli; COX, cyclooxygenase; HPLC, high-performance liquid chromatography; NSAID, nonsteroidal anti-inflammatory drug; pd, per diem.

Min/+ mice is gastrointestinal bleeding, which is thought to contribute substantially to morbidity and mortality (6). To find out to what extent curcumin affects adenoma-related gastrointestinal bleeding, we measured the hematocrit in treated and control animals and compared values with adenoma numbers. Finally, to define the disposition of curcumin accurately, we synthesized ^{14}C-labeled curcumin, administered it via the i.p. route, and determined the disappearance of radioactivity from tissues and plasma. Overall, the study was designed to aid the program of clinical evaluation of curcumin in the treatment of FAP and the prevention of colorectal cancer in humans.

Materials and Methods

Chemicals. Curcumin was purchased from Apin Chemicals (Abingdon, United Kingdom) and its purity verified by HPLC analysis (see HPLC analysis below). This material contained 3% desmethoxycurcumin. Curcumin was blended into RM3 high-protein breeders diet, bought from Specialist Dietary Services (SDS, Witham, United Kingdom), using a mechanical mixer to ensure uniform distribution, confirmed by HPLC analysis. The synthesis of [^{14}C]curcumin has been described previously (15). The labeled material had a specific activity of 13.5 MBq/mmol. For the distribution study after i.p. injection, [^{14}C]curcumin was diluted with four parts unlabelled curcumin. Ethyl acetate, acetonitrile (both HPLC grade), and DMSO were obtained from Fisher Laboratory Supply Ltd. (Loughborough, United Kingdom); tissue solubilizer (Optisolve) and scintillation fluid (Optisafe High Phase) came from Wallac Scintillation Products (Milton Keynes, United Kingdom).

Animals and Treatments. To establish a breeding colony, male C57Bl/6J 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) obtained from Charles River (Margate, United Kingdom). Male Min/+ mice offspring of this cross breeding and C57Bl/6J female wild-type mice maintained the Min/+ breeding colony. Tissue samples were obtained from the weaners by ear punch and genotyped for Min/+ status by PCR and *Hin*dIII digest of the product, essentially as described previously (16). Male C57BL/6J mice (Charles River) were used in accompanying pharmacokinetic studies. All of the mice were kept in positive pressure isolators, and routine bacteriological and serological tests established that they were free of pathogens. Breeding pairs and all of the offspring were maintained throughout on a RM3 diet. This diet consists of cereal products (wheat, barley) 64%, vegetable protein (extracts of soya beans and dried yeast) 16.5%, soya oil 2%, animal protein (fish meal, whey powder) 15%, and supplements (vitamins, minerals, amino acids) 2.5%. Offspring were weaned at 3 weeks. At 4 weeks, littermates were divided following a randomized block design into control (RM3 diet only) or treatment groups, which constituted curcumin at 0.1, 0.2, or 0.5% mixed in with the RM3 diet. The choice of these curcumin concentrations was determined by the intention to span a dose range from subefficacious to markedly efficacious.

Adenoma Enumeration. Experiments in mice were conducted as stipulated by the Animals (Scientific Procedures) Act 1986 Project License 80/1250 granted to Leicester University by the United Kingdom Home Office, and the experimental design was vetted and approved by the Leicester University Ethical Committee for Animal Experimentation. Each experimental group comprised between 10 and 15 mice. At the end of the experiment when Min/+ mice reached age 18 weeks, they were killed by cardiac exsanguination under terminal anesthesia (halothane). The entire gastrointestinal tract was removed for dissection and flushed with PBS (~10 ml) to remove intestinal content. Tissue was opened longitudinally and washed extensively with PBS. Stomach and cecum were omitted from the analysis. Small intestine and colon were fixed flat in methacarn (methanol:chloroform:acetic acid, 6:3:1) for 2–4 h, after which the tissue was examined under 3-fold magnification. The small intestine was divided visually into three segments of approximately equal length (referred to in the following as proximal, middle, and distal segments). Multiplicity, location, and size of adenomas were recorded within these segments and the colon. Adenomas were differentiated by size (diameter) into <1 mm, 1–3 mm, and >3 mm.

Measurement of Hematocrit. Blood samples were collected and drawn by capillary force into heparinized microhematocrit tubes (75 mm; Richardsons, Leicester, United Kingdom). The hematocrit, which constitutes the proportion of the volume of the blood sample occupied by the erythrocytes, was determined as described previously (17).

Study of Curcumin and Metabolites in Tissues, Blood, and Excreta. Male C57BL/6J mice (8 weeks of age, ~25*g*) received the RM3 diet containing 0.1, 0.2, or 0.5% curcumin for 8 days. Animals were placed into metabolism cages for 24 h, and urine and feces were collected. After killing (exsanguination under terminal anesthesia), liver, small intestine and colon tissue were isolated, and gut epithelium was scraped off by brushing gently with a metal spatula. Plasma was separated from blood by centrifugation (10,000 × *g* for 5 min). Tissue samples were homogenized in acetate buffer [1 M (pH 4.5), 2 ml] and extracted with 10 volumes of ethyl acetate or acetonitrile. Aliquots of plasma or blood were extracted after the addition of an equal volume of acetate buffer with twice the volume of ethyl acetate. The mixtures were centrifuged (2,800 × *g* at 4°C for 15 min), the organic layer was removed, and the mixtures were evaporated under nitrogen. Fecal samples (~200 mg) were homogenized with acetate buffer (2 ml) and extracted into 10 volumes of ethyl acetate. The extraction efficiency from plasma for curcumin at 0.1 μg/ml, determined by HPLC, was 92 ± 7% (mean ± SD; n = 6); its extraction efficiency from feces was 75 ± 10% and from liver and mucosal scrapings, 50 ± 12%. Curcumin glucuronide and curcumin sulfate were extracted at 50% efficiency.

HPLC Analysis and Mass Spectrometry. Samples of blood, tissues, and excreta were analyzed for the presence of curcumin and its metabolites curcumin sulfate, curcumin glucuronide, hexahydrocurcumin, and hexahydrocurcuminol, using a reversed-phase HPLC method as described previously (18). The limits of detection for curcumin, curcumin glucuronide, and curcumin sulfate under the conditions of this assay were 5 pmol/ml of plasma or 10 pmol/g of tissue. The identity of curcumin and metabolites was established by cochromatography with authentic standard compounds and confirmed by electrospray mass spectrometry in the ion-selected mode as described previously (12).

Study of [^{14}C]Curcumin Distribution. Male C57BL/6J mice (8 weeks of age, ~25*g*) received [^{14}C]curcumin (100 mg/kg) dissolved in DMSO (injection volume, ~50 μl) via the i.p. route. Mice were killed after 0.25, 0.5, 1, 2, 4, 8, or 24 h (four mice per time point). Samples (0.2–0.3 g) of brain, heart, lung, liver, spleen, kidney, small intestine, and blood were dissolved in Optisolve solubilizer at 37°C (1.5 ml), after which, scintillation fluid was added. In the case of plasma samples, the scintillation fluid was added directly. Samples were analyzed in a Wallac 1410 liquid scintillation counter using a 10-min ^{14}C

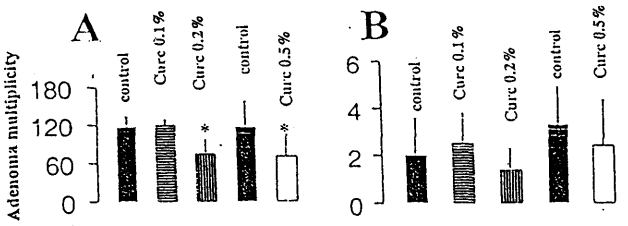

Fig. 1. Effect of dietary curcumin (*Curc*) on adenoma multiplicity in the small intestine (*A*) and colon (*B*) of Min/+ mice. Numbers of mice per group were between 10 and 12; *, number of adenomas is significantly different from that in control animals ($P < 0.05$). There were two control groups (untreated Min/+ mice), one for the study of curcumin 0.1 and 0.2% and the other for the mice on curcumin 0.5%. For details of animal husbandry and adenoma enumeration see "Materials and Methods."

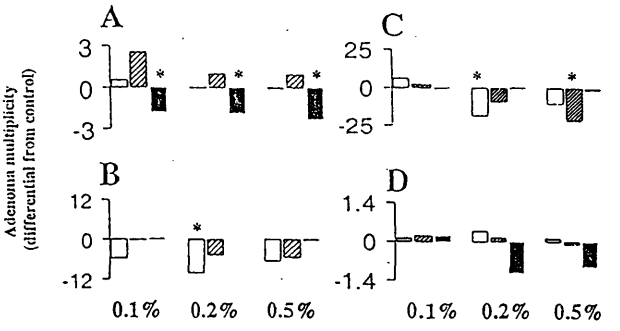

Fig. 2. Effect of dietary curcumin on multiplicity of small (<1-mm diameter, □), medium size (1–3 mm, ▨) or large (>3 mm, ■) adenomas in the proximal (*A*), middle (*B*), distal (*C*), or colonic (*D*) sections of the intestine of Min/+ mice. Results are expressed as mean number of adenomas over or below mean adenoma numbers in untreated (control) Min/+ mice. Adenoma numbers (mean ± SD) in untreated (control) Min/+ mice were as follows (in the order small, medium size, and large adenomas, respectively; because there were two control groups in separate sets of experiments, the numbers without brackets denote values for the control mice for the 0.1 and 0.2% curcumin treatment groups, and numbers in brackets refer to the control mice for the 0.5% curcumin treatment group): proximal intestine, 0.5 ± 1.0 (0.6 ± 0.8), 2.4 ± 1.4 (1.9 ± 1.7), and 2.0 ± 1.3 (3.7 ± 1.6); middle intestine, 25.6 ± 11.6 (19.5 ± 11.3), 15.2 ± 6.4 (16.7 ± 9.6), and 0 (0.4 ± 1.3); distal intestine, 43.9 ± 10.7 (34.2 ± 12.7), 26.5 ± 11.7 (37.4 ± 20.0), and 0.3 ± 0.7 (2.2 ± 3.2); colon 0 (0), 0.6 ± 0.8 (1.4 ± 1.1), and 1.4 ± 1.2 (1.9 ± 1.1). Numbers of mice per group were between 10 and 12. *, number of adenomas was significantly different from that in control animals ($P < 0.05$). The effect of 0.2 and 0.5% dietary curcumin on the numbers of small and middle-size adenomas in the middle portion of the small intestine, and of large adenomas in the colon, approached significance ($P < 0.1$ but >0.05). For details of animal husbandry and tumor enumeration see "Materials and Methods."

counting program. The measured radioactivity was converted into nmol curcumin equivalents per milliliter of plasma or gram of tissue. The plasma and tissue peak values quoted in the "Results" are the mean ± SD obtained in three animals.

Statistical Evaluation. Adenoma numbers and hematocrit values were subjected to ANOVA using Excel and Minitab software packages (Microsoft Windows 1997). Statistical significance ($P < 0.05$) was established by *post hoc* Tukey's pairwise comparison.

Results

Effect of Curcumin on Multiplicity of Intestinal and Colonic Adenomas. Min/+ mice received curcumin mixed in with their diet at three concentrations, 0.1, 0.2, and 0.5% commencing one week *post*-weaning. Animals were killed at 18 weeks of age, and tumor multiplicity and size were inspected *postmortem*. Fig. 1 shows that dietary curcumin at 0.1% was without effect on overall gastrointestinal tumor burden. However, at dietary concentrations of 0.2 and 0.5%, curcumin reduced intestinal tumor load significantly by 39 and 40%, respectively. Inspection of adenomas revealed a flattened morphology in the case of mice that had received curcumin at 0.5%, compared with untreated mice or mice on the lower doses of curcumin (result not shown). There were only a few adenomas in the colon of untreated Min/+ mice (3.5 ± 3.8, $n = 22$). Although dietary curcumin (0.2 and 0.5%) reduced their number by 30 and 27%, respectively, this decrease was not significant (Fig. 1). In a separate experiment, animals received curcumin (0.2%) for either the first (weeks 3–10.5 of age) or second (10.5–18 weeks) half of their postweaning life span. Such early or late treatment did not affect gastrointestinal adenoma multiplicity significantly (results not shown), in contrast to the effect of treatment when curcumin was applied continuously for the whole 15-week postweaning period.

Although total tumor multiplicity decreased significantly as a result of treatment with curcumin (0.2 or 0.5%), its efficacy varied depending on gastrointestinal tumor location and stage of tumor development (Fig. 2). Curcumin at 0.2% affected predominantly small adenomas (<1 mm diameter) and, to a lesser extent, middle-size tumors (1–3 mm) in both the distal and middle segments of the small intestine. Curcumin at 0.5% diminished mainly middle-size adenomas in the distal portion and reduced small and middle-size adenomas in the middle part of the small intestine. Both of the efficacious curcumin dose levels reduced numbers of large adenomas (>3 mm), rather than those of small and middle-sized ones, in the proximal small intestine and colon. Curcumin at 0.1%, a concentration that lacked effect on overall tumor number (see Fig. 1), seemed to decrease the numbers of small adenomas in the middle region and large adenomas in the proximal region of the small intestine (Fig. 2). This decrease was outweighed by increased numbers of medium-size adenomas in the proximal and small adenomas in the distal regions.

Effect of Curcumin on Hematocrit. To investigate whether dietary curcumin affects gastrointestinal bleeding associated with adenoma load in Min/+ mice, the hematocrit was studied in individual animals at the termination of the experiment. The mean hematocrit value in untreated Min/+ mice was reduced to 25% of that seen in healthy C57Bl/6J mice (Fig. 3). Dietary curcumin increased the hematocrit in Min/+ mice in a dose-dependent fashion. The highest dietary concentration (0.5%) of curcumin partially restored hematocrit values in Min/+ mice to 60% of values observed in healthy C57Bl/6J mice (Fig. 3). When individual hematocrit values were plotted against adenoma number for control C57Bl/6J mice, Min/+ mice on control diet, and those on the curcumin-containing diet, the resulting line of best fit was characterized by a correlation coefficient of −0.84 (result not shown).

Steady-State Levels of Curcumin in Plasma, Target Tissue, and Excreta. We wished to relate the chemopreventive efficacy of curcumin in Min/+ mice with the concentration of the agent or its metabolites. To that end, steady-state levels were determined in the plasma, excreta, and gastrointestinal mucosa, the target tissue, and for comparative purposes also in liver tissue of C57Bl/6J mice, which had received dietary curcumin at either 0.1, 0.2, or 0.5% for 1 week. Curcumin was not detectable in the urine. Irrespective of the dose, curcumin was present in the plasma at levels near the limit of detection (5 pmol/ml). Large amounts of curcumin were found in the feces

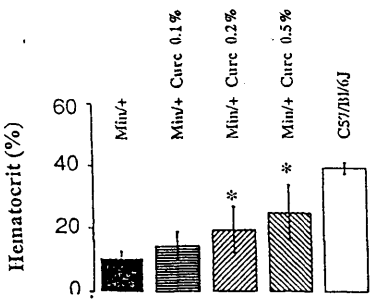

Fig. 3. Hematocrit values in C57Bl/6J mice, untreated Min/+ mice, and Min/+ mice that received curcumin (*Curc*) at 0.1, 0.2, or 0.5% in their diet. Values represent percentage proportion of the volume of the blood sample occupied by the erythrocytes and are the mean ± SD of 10–12 mice. For details of animal husbandry and determination of hematocrit see "Materials and Methods." *, values are significantly different from those in untreated Min/+ mice ($P < 0.05$).

(3.2–3.8 μmol/g; Table 1). In the mucosa of the small intestine, its concentration varied between 39 and 240 nmol/g of tissue and, in the colonic mucosa, between 15 and 715 nmol/g of tissue. Curcumin levels in the small intestine reflected satisfactorily differences in dose, whereas levels in the colon and feces did not mirror dose levels (Table 1). The concentration of curcumin in liver tissue of mice that were fed 0.2% curcumin in the diet was 119 ± 31 pmol/g of tissue ($n = 3$), a value which constitutes ~0.001 of that observed in the intestinal mucosa. Products of metabolic conjugation or reduction of curcumin were not detected, except in the colonic mucosa and feces, in which HPLC analysis revealed the presence of traces of a species coeluting with authentic curcumin sulfate. Mass spectral investigation of the HPLC peak in the colon mucosa by selected ion monitoring afforded the molecular ion of m/z = 447, corroborating the identity of the peak as curcumin sulfate. In a confirmatory experiment, plasma levels of curcumin were measured in Min/+ mice at the end of the life-time feeding study, and levels were found to be very similar to those seen in wild-type C57Bl/6J mice kept on a curcumin-containing diet for 1 week.

Disposition of Curcumin. We wished to explore how rapidly curcumin levels decline on termination of treatment. This question was addressed in two ways: firstly, C57Bl/6J mice, which had received curcumin (0.2%) in their diet for 1 week, were changed onto a curcumin-free diet, and levels of curcumin in plasma and in gastrointestinal and hepatic tissues were analyzed for curcumin for up to 16 days after the cessation of curcumin feeding. Secondly, for a more general analysis, mice received [^{14}C]curcumin (100 mg/kg) via the i.p. route, and the disappearance of radioactivity associated with the curcumin molecule was studied, not only in plasma and gastrointestinal and liver tissues, but also in heart, lung, kidney, brain, and muscle tissues. After termination of dietary curcumin intake, tissue levels of curcumin declined rapidly to unquantifiable amounts within 3 to 6 h after the termination of curcumin feeding, whereas fecal curcumin declined more slowly with a half-life of ~23 h (results not shown). Radioactivity measured in the plasma and tissues after i.p. injection of [^{14}C]curcumin achieved the following peak levels, expressed as nmol curcumin equivalents per milliliter of plasma or per gram of tissue: plasma, 25 ± 2; liver, 73 ± 20; intestinal mucosa, 200 ± 23 (see Fig. 4); brain, 2.9 ± 0.4, heart, 9.1 ± 1.1; lungs, 16 ± 3; muscle 8.4 ± 6.0; and kidney, 78 ± 3. Beyond the peak, radioactivity declined swiftly to reach levels of between 20 and 33% of peak values at 4 h, or in the case of the small intestine, 8 h, after dosing (Fig. 4). From this time point onwards, radioactivity levels decreased little, or hardly at all, up to 24 h. A similar pattern of disposition was observed in heart, lung, kidneys, brain, and muscle tissues, in that levels decreased within 2–4 h after dosing to 10–20% of peak levels, and radioactivity levels remained at this residual level for up to 24 h (results not shown). It needs to be stressed that the curcumin pharmacokinetics observed in plasma and tissues after i.p. administration cannot be compared directly with those observed after dietary intake, not least because the agent was formulated for i.p. administration in DMSO, an amphiphilic solvent that seemed to enhance curcumin absorption considerably.

Table 1 Concentration of curcumin in small intestinal and colonic mucosa and feces of mice that received curcumin at 0.1, 0.2, or 0.5% in their diet for 1 week

Curcumin content of diet (%)	Curcumin levels[a] (nmol/g)		
	Small intestinal mucosa	Colonic mucosa	Feces
0.1	39 ± 9	15 ± 9	3770 ± 1246
0.2	111 ± 40	508 ± 149	3590 ± 231
0.5	240 ± 69	715 ± 448	3186 ± 2411

[a] Values are the mean ± SD of four animals.

Discussion

This study defines for the first time the relationship between chemopreventive efficacy of curcumin and its concentration in the Min/+ mouse, a colon neoplasia model of relevance to human cancer (7). The results of this investigation allow five novel conclusions that may aid with the design, and eventual interpretation, of future clinical intervention studies with this dietary constituent. Firstly, the chemopreventive activity of curcumin in the Min/+ mouse model seems to be governed by a rather narrow therapeutic window. Intake of curcumin at a dietary level of 0.1%, approximately equal to 150 mg/kg pd, lacked overall efficacy, whereas at 0.2%, which equates to ~300 mg/kg pd, curcumin prevented, or retarded, adenoma formation. A further increase in dietary level by a factor of 2.5 to ~750 mg/kg pd failed to yield any additional gain in efficacy, irrespective of the fact that curcumin concentrations in the small intestine adequately mimicked the stepwise increase in dose consumed with the diet. The effect of curcumin on the multiplicity of tumors varied depending on their location along the intestinal tract. It was the population of small and medium-size adenomas that was most susceptible to the preventive efficacy of curcumin, and reduction in adenoma number was most prominent in the middle and distal regions of the intestinal tract, the areas in which the majority of tumors occurred. This finding is consistent with previous experience in the Min/+ mouse model using piroxicam (19) or the selective COX-2 inhibitor celecoxib (8).

The second conclusion from the work described here is that for curcumin to be effective in the Min/+ mouse model, the gastrointestinal mucosa, the target tissue, needs to be exposed to curcumin at concentrations near or above the 100-nmol/g of tissue mark. Tissue concentrations below this value are apparently without measurable chemopreventive efficacy. Thirdly, to achieve chemopreventive activity, exposure to curcumin needs to persist for the whole postweaning lifetime of Min/+ mice.

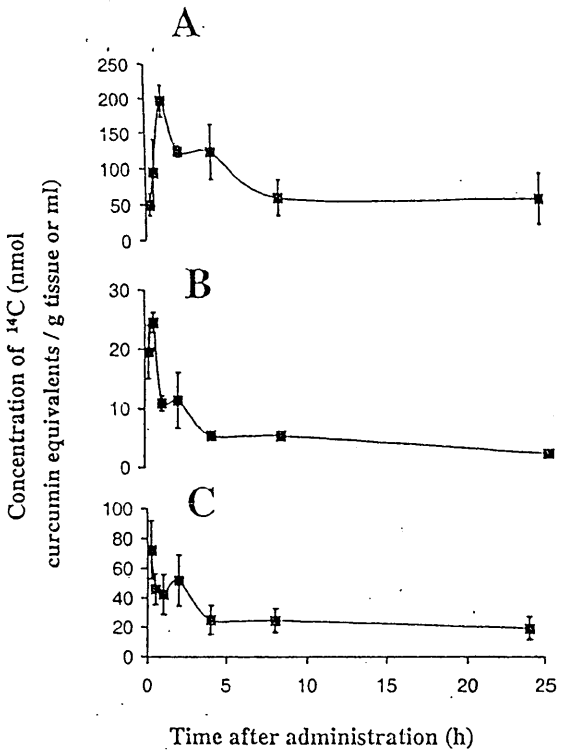

Fig. 4. Elimination of radioactivity derived from [^{14}C]curcumin from the intestinal tract mucosa (*A*), plasma (*B*), and liver (*C*) of C57Bl/6J mice, which had received a single dose of [^{14}C]curcumin (100 mg/kg) via the i.p. route. Values are expressed as nmol curcumin equivalents per gram (*g tissue*; *A*, *C*) or milliliters (*ml*) of plasma (*B*), and are the mean ± SD of four mice. For details of the analysis see "Materials and Methods."

Restriction of exposure to either the first half of this time period, the preneoplastic phase, during which adenomas are usually not yet observed, or the second half, when adenomas are established, was insufficient to decrease adenoma formation.

Fourthly, the beneficial effect of curcumin on adenoma load, as adjudged by tumor number, was accompanied by an elevation in hematocrit values, which reflect intraluminal hemorrhage consequent to adenoma maturation. Dose-dependent activity of curcumin was illustrated by the change in hematocrit. This finding is especially intriguing, when advantages and disadvantages of treatment with curcumin are juxtaposed with those reported for cancer chemopreventive NSAIDs such as aspirin, sulindac, or piroxicam. NSAIDs have the notorious drawback that they can elicit severe adverse effects in the gastrointestinal tract (20). Curcumin not only failed to exacerbate adenoma-induced intestinal bleeding, it actually ameliorated it significantly. Furthermore, there was no hint of agent-induced histopathological lesions in the gastrointestinal tract of Min/+ mice in the study described here, nor in a pilot study performed by us in which animals were fed curcumin at a dietary level as high as 2%, corresponding to ~3 g/kg pd.[4]

The fifth conclusion from the work described above is the fact that curcumin disappears relatively rapidly from rodent tissues, including the target tissue, once treatment is discontinued. This conclusion is consistent with the previous observation of rapid disappearance of curcumin and its conjugates from the plasma of rats, which received curcumin by the i.v. route (12). The conclusion is important given the observation (discussed above) that curcumin needs to be present consistently in the gastrointestinal mucosa of Min/+ mice for several months to achieve chemopreventive activity. Potentially rapid dispositional removal of curcumin from the target tissue needs to be taken into account, if sustained levels are to be achieved in humans. Some curcumin metabolites, such as tetrahydrocurcumin, may contribute to the biological potency of curcumin (21, 22), whereas, in contrast, curcumin conjugates are probably devoid of biological activity (12). Therefore, it is important to note that no significant amounts of products of the metabolic reduction or glucuronidation of curcumin were found in the plasma, gastrointestinal mucosa, or feces of mice that received a curcumin diet in the study described here. Only a trace of curcumin sulfate was detected in the intestinal mucosa of treated animals.

As to the mechanisms by which curcumin interferes with the process of carcinogenesis, there are a host of biochemical candidate processes that might be compromised by curcumin at 0.1–0.5 μmol/g of mucosal tissue, the range of levels defined here as necessary to prevent, or retard, adenoma formation in the gastrointestinal tract of Min/+ mice. This concentration range is roughly equivalent to 0.1–0.5 mM in experiments *in vitro* using cells or cellular fractions. In such systems curcumin has been shown to act as a scavenger of oxygen species, such as hydroxyl radical, superoxide anion, and singlet oxygen (23–27), and to interfere with lipid peroxidation (28–30). Curcumin also suppresses a number of key elements in cellular signal transduction pathways pertinent to growth: survival, promotion, angiogenesis, differentiation, and malignant transformation. Prominent among the signaling events inhibited by curcumin are phosporylations catalyzed by protein kinases (31), c-Jun/AP-1 activation (32), prostaglandin biosynthesis (33), and activity and expression of the enzyme COX-2 (34, 35). All of these inhibitory actions of curcumin require concentrations of the agent in the 10–100-μM range, which is well within the levels achieved in the target tissue of Min/+ mice at efficacious dietary doses.

In conclusion, the study presented here corroborates the notion that curcumin possesses chemopreventive activity in a model germane to human colorectal carcinogenesis involving *Apc* mutations. This finding encourages, in principle, the potential evaluation of curcumin for adenoma-retarding efficacy in FAP patients. The dose of curcumin required for efficacy in humans equivalent to the 0.2% dietary concentration or 300 mg/kg pd dose, which was 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 (36). This putative efficacious clinical dose of curcumin is well within the dose range, 0.5, 1.2, 2.1, and 8 g pd for up to 6 weeks, which according to the literature has been administered to humans apparently without adverse effect (37–40). The ability of curcumin to decrease intestinal blood loss linked to adenoma maturation, as adjudged by its significant effect on Min/+ mouse hematocrit, renders the clinical exploration of combinations of curcumin with NSAIDs potentially attractive.

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[4] Unpublished results.

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