COLORECTAL CANCER

Thesis submitted for the degree of

Doctor of Philosophy

at the University of Leicester

Ricky A Sharma MA MB BChir MRCP

Oncology Department, University of Leicester

July 2001

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

COPYRIGHT

ABSTRACT

Preclinical and Clinical Development of Biomarkers of the Efficacy of Curcumin in the Prevention and Treatment of Colorectal Cancer

RA Sharma

A major shortcoming of cancer chemoprevention trials has been the absence of suitable methods of assessing the efficacy of intervention prior to the development of malignancy. Curcumin is a polyphenolic antioxidant, derived from *Curcuma* plants or the spice turmeric, which prevents colorectal cancer in chemical and genetic rodent models and which may have cancer chemotherapeutic potential.

This project explored the suitability of three biological indices as potential markers of the efficacy of curcumin in preventing or treating colorectal carcinoma: glutathione S-transferase (GST) activity; a deoxyguanosine adduct (M_1G) associated with oxidative DNA damage and malondialdehyde (MDA) production *via* lipid peroxidation (LPO); and levels of cyclooxygenase-2 (COX-2) protein and its product prostaglandin E_2 (PGE_2). The viability of these biomarkers was studied at three levels: malignant and non-malignant human colon cells *in vitro*; a rat model of LPO and the multiple intestinal neoplasia (MIN) mouse model; and a pilot study of a standardised oral preparation of *Curcuma* extract in patients with advanced colorectal cancer.

The results *in vitro* demonstrated an association between COX-2 activity and intracellular MDA levels, and a correlation in malignant cells between MDA concentration and M_1G adduct levels. A diet containing 2% curcumin decreased M_1G levels in rat colon mucosa and increased GST liver activity, compared to controls; the same diet completely prevented chemically induced rises in M_1G levels in colon mucosa. Elevated levels of M_1G were discovered in intestinal adenomas in MIN mice, relative to normal mucosa, and 0.1% dietary curcumin significantly decreased those levels. Although addition of curcumin to human blood *in vitro* was shown to inhibit leukocyte COX-2 activity, dose-dependent effects definitely attributable to treatment were not observed in the three biomarkers measured in the clinical trial of *Curcuma* extract. Compatible with the tissue levels described in the preclinical studies, curcumin's systemic bioavailability following oral dosing appeared to be low in humans.

Future development of curcumin as a cancer chemopreventive agent should study its effects on M_1G levels, COX-2 activity and GST isoforms in colorectal adenomas.

ACKNOWLEDGEMENTS

I would like to thank Maggie Manson, Chiara Leuratti, Simon Plummer, Larry Marnett, David Shuker, Munir Pirmohamed, Michael Festing and Chang-Kee Lim for expert advice; Steph Euden, Kirsti Hill, Sarah Perkins, Ann Hudson, Rebecca Jukes, Elke Griech, John Plastaras, Richard Verschoyle, Paul Shepherd, Chris Ireson and Raj Singh for technical assistance and team spirit; Eliot Deag for his tolerance at the outset of this project; Heather McLelland, Caroline Sanganee, Lynn Furber, and Nathan Rush for their help in the clinic; David Barnett and Penella Woll for reviewing the adverse events forms; and of course all the patients who took part in the clinical trial.

I feel deeply indebted to Andy Gescher and Will Steward for their patience, enthusiasm and wisdom as my supervisors during this project.

Gratitude to my parents for their unfaltering support and to my wife Sara for forcing me to smile through the tribulations of research.

LIST OF FIGURES

LIST OF TABLES

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 PRINCIPLES AND ETHICAL CONSIDERATIONS OF COLORECTAL CANCER CHEMOPREVENTION

1.1.1 DEFINITIONS

Cancer chemoprevention can be defined as the inhibition, retardation or reversal of carcinogenic processes by chemical means[1]. Although the history of cancer chemoprevention covers less than three decades, hundreds of studies have been reported and agents such as tamoxifen and retinoids have been shown to prevent or delay the onset of human cancer (see Appendices 2A and 2B).

Unfortunately the reputation of cancer chemoprevention has been damaged by adverse results. A notable example is the α-tocopherol β-carotene prevention study, in which lung cancer incidence was actually *increased* in individuals who continued to smoke at least twenty cigarettes per day and among those in the highest quartile of alcohol consumption[2]. Subsequent research has suggested mechanisms that may underlie this unexpected effect[3], thus highlighting the need for detailed mechanistic information on putative chemopreventive agents *before* they reach large-scale clinical trials (see Appendix 2C).

One of the shortcomings of many clinical chemoprevention studies is the absence of suitable biomarkers of efficacy. These biomarkers can be defined as biological events which take place between exposure to external or endogenous carcinogens and the subsequent development of cancer[4]. It is important to differentiate these 'preneoplastic' biomarkers from tumour markers that are used to diagnose or monitor

malignancy (see Appendix 2A). 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[4]. Such biomarkers should be based on mechanistic hypotheses, then developed and validated in preclinical models and small pilot clinical studies (see below). Study of biomarkers may not only facilitate the formulation of further mechanistic hypotheses regarding the actions of chemopreventive agents, but may also allow researchers to monitor progress in future clinical trials of such agents. Criteria used to identify and judge the potential efficacy of biomarkers of colon carcinogenesis, as defined by Eugene Gerner and colleagues[5], 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

Generally speaking the epidemiology and molecular biology underlying colorectal cancer are better understood than for most other neoplasias, and their interrelationship is now becoming clearer[6]. This knowledge offers increased potential for intervention at varying stages in the carcinogenic process (see Appendix

2D). Increasing numbers of pilot screening programmes for sporadic cancer in normal populations[7] are likely to identify individuals with early stages of this disease, which may augment the importance of chemoprevention. Studies of human premalignant lesions also offer opportunities to elucidate the relationship between putative preneoplastic biomarkers and the subsequent risk of cancer.

1.1.2 BIOMARKERS OF CARCINOGENESIS

Histological biomarkers alone may not offer sufficient sensitivity to detect carcinogenic processes, since it is estimated that less than 5% of adenomas become malignant and may take 5 to 10 years to transform[8]. Complementary or alternative classes of biomarkers are discussed in Appendix 2D. Study of genetic biomarkers in particular remains intense, and increasingly highlights the need to consider the entire biochemical pathway in which a gene plays its part. An example is provided by the *adenomatous polyposis coli* (*APC*)[a] gene, which is commonly mutated in polyps and early cancer[9]. Loss of wild-type *APC* leads to transcriptional activation of *c-myc* expression through the β-catenin Tcf-4 complex[10], which may affect a number of downstream targets including the initiation factor eIF-4E [11]. Elucidation of the signalling pathway downstream from the wild-type *APC* gene has pinpointed a crucial role for β-catenin and E-cadherin, two proteins whose expression is frequently abnormal in colorectal cancer[12]. Although *APC* function may be normal, mutations in β-catenin can result in the abnormalities of adhesion-migration and proliferative signalling found in neoplasia[13]. Thus the investigation of biochemical

[a] Abbreviations used are listed in Appendix A

biomarkers related to the genetic pathways implicated in colorectal carcinogenesis may provide broader mechanistic understanding of the agent under scrutiny.

Biomarkers relevant to the agent under scrutiny are increasingly incorporated in the design of contemporary chemoprevention trials. Based on mechanistic understanding of the agents' actions *in vitro* or *in vivo*, recent clinical studies of broccoli supplements and α-difluoromethylornithine in individuals at risk of developing cancer have explored biomarker levels in target tissues[14-16], and the development of aspirin as a chemopreventive agent has included measurement of colon mucosal levels of prostaglandins (PG) and cyclooxygenase (COX)-2 in conjunction with histological measures of cell proliferation[17]. These studies illustrate the utility of incorporation of biomarkers of efficacy into pilot studies of potentially chemopreventive agents, in which they may offer novel pharmacodynamic information.

Since chemoprevention trials may ultimately involve large numbers of healthy volunteers who may gain no benefit from the intervention and may even be harmed by it, cancer incidence or mortality cannot be justified as the *only* endpoint measured in long-term studies. Minimising risk to the participants of chemoprevention trials is essential, and there are a number of ways in which this goal can be achieved. One way is to perform short-term pilot studies in the early clinical development of putative agents, principally to study the pharmacokinetic parameters of the agent but also to investigate toxicity or potential effects on biomarkers relevant to carcinogenesis. The need for such pilot trials, usually involving ten to twenty

individuals, is discussed more fully in Appendices 2C and 2D. Another way of minimising risk to the participants of chemoprevention trials is to bear in mind the risks associated with the measurement of the efficacy of intervention. When biomarkers are being selected, it is important to consider whether they can be measured in peripheral blood or urine rather than necessitating invasive biopsies. Indeed, choice of less invasive measurements will also facilitate more frequent biomarker assessment. An example of this concept was provided in a recent pilot study[18] followed by a chemoprevention study[14], in which a correlation was demonstrated between total glutathione S-transferase (GST) activity of colon mucosa and that of blood lymphocytes in volunteers considered 'high-risk' for developing colorectal cancer. Similarly, development of blood and urinary biomarkers of potential inhibition of polyamine synthesis is in progress for other cancers[19,20].

1.1.3 PRECLINICAL VALIDATION OF BIOMARKERS

Validation of preneoplastic biomarkers selected for the particular agent being tested is currently performed in preclinical model systems before advancement to human studies. Such validation benefits from the use of preclinical models to determine the reliability and accuracy of the method, optimise sample acquisition and storage, and collect information on the potential significance of changes observed at various dose levels. Validation of a biomarker requires description of the reproducibility and reliability of the method used to obtain tissue and the assay used to analyse it. It also requires consideration of all potential sources of variability[5], *e.g.* assay conditions, organ or tissue heterogeneity, sex, age, ethnicity, diet, lifestyle, circadian rhythm, etc.

Although a variety of cell models with reasonable predictive value are available *in vitro*, their limitations must be borne in mind. Cells grown *in vitro* are clearly different from those growing in the natural host due to environmental factors such as the matrix to which they adhere and the medium to which they are exposed. In particular, many cellular metabolising enzymes may be lost within a day of primary culture (see Chapter 5, Section 5.1.3). On the other hand, cultured cells exist under controlled conditions, free of the complex milieu of contaminating factors present *in vivo*; they usually possess sufficient characteristics of colon tumour cells to permit their use in testing particular hypotheses regarding cancer cell biology[21] and the screening of potential chemopreventive agents using biochemical markers[22].

With regard to *in vivo* preclinical models available, it is not clear which ones are the most representative of colorectal carcinogenesis, especially the sporadic form (which, despite its name, is the commonest form of colorectal cancer). Aberrant crypt foci assays have been used widely in the short-term screening of putative chemopreventive agents, based on the assumption that dysplastic areas may become adenomas[23]. The adenoma has been regarded as a premalignant lesion in the human colon for many decades[24]. These assumptions are almost certainly true for familial adenomatous polyposis (FAP) patients, but they may not hold for all cases of colorectal cancer.

In the longer term screening for potentially chemopreventive activity, the preclinical models in use can be subdivided into chemically-induced and genetic. Chemically-induced colon cancer models use large doses of certain carcinogens to induce tumours, and are therefore different from the multifactorial aetiology considered

important in humans[6]. However they may provide a suitable model of the post-initiation period of carcinogenesis and of established neoplasia, including the efficacy of intervention[25]. For example, the F344 rat treated with azoxymethane develops ten or less large intestinal polyps, which develop into adenocarcinomas at 36 weeks following treatment[22]. On the other hand, genetic models may be more relevant to certain stages of carcinogenesis and chemoprevention. An example is the multiple intestinal neoplasia (MIN) mouse. This model was developed by chemical induction of a germline mutation in the *APC* gene[26], which was first identified in its inherited mutated form in FAP patients but is now known to exist in 80% of sporadic colorectal cancers[27]. These mice develop multiple intestinal and a few colonic adenomas by 100 days of age, but die before carcinomas develop[26]. Nevertheless they have provided a genetically relevant model system for intervention studies of agents such as piroxicam[28] and curcumin[29] during the premalignant stages of intestinal carcinogenesis.

1.1.4 DESIGN OF CLINICAL TRIALS

The design of clinical chemoprevention trials continues to evolve, but a few generalities about each phase can be defined[30]. Phase I studies are short-term pilot projects that determine the pharmacokinetics and dose-related tolerability of drugs by dose escalation in volunteers or patients. These pilot studies may also be used to provide information on specific mechanism-based biomarkers of the efficacy of an agent in humans, or to monitor potentially cancer preventive activity in the short and intermediate terms. Such pilot studies have been part of the US National Cancer Institute (NCI) prevention research programme since 1992. Initial doses and schedule should be based on safety and efficacy data from preclinical models.

Although preclinical models can provide some biological information, they are not entirely predictive of an agent's pharmacology in humans. In particular, it is important to emphasise that there is currently a sparseness of knowledge regarding the pharmacokinetics in humans of putative cancer chemopreventive agents. A need therefore exists for Phase I studies of promising agents investigating relationships between pharmacokinetics and potential pharmacodynamic effects. Such pilot studies of certain promising agents may be conducted in individuals with pre-malignant lesions or patients cured of an initial cancer but at risk of developing a second primary tumour. Furthermore, since agents such as retinoids or curcumin (see Section 1.2.3) have been shown in preclinical models to affect processes considered relevant to late carcinogenesis and metastasis[31,32], and agents such as curcumin and piroxicam may have some degree of potentially chemotherapeutic benefit in humans[33,34], pilot studies of these agents may use cancer patients as a subject group. Findings regarding pharmacokinetics and toxicity are likely to equate to those in healthy volunteers, although conclusions regarding the biomarkers studied should be more cautious since many of the biochemical or molecular indices associated with carcinogenesis are dysregulated in established malignancy. The ethical justification for performing pilot studies of chemopreventive agents in patients with cancer depends on the theoretical benefits to the patients from potential modification of malignant disease behaviour, a concept incorporated in the earliest definitions of cancer chemoprevention[1].

In contrast to small Phase I pilot studies, Phase II chemoprevention trials use a randomized, blinded, placebo-controlled design to evaluate dose-response and

common toxicities likely to result from prolonged administration, preferably 3 months or longer. Multiple dosage levels are evaluated in these trials, which should incorporate measurement of preneoplastic biomarkers validated in prior preclinical and clinical projects. If safety and biomarker results are judged to be satisfactory in these trials, agents procede to randomised, prospective large-scale Phase III clinical trials. These trials test the efficacy of an agent in preventing cancer relative to dose and toxicity, ultimately measuring the incidence of primary tumours in addition to the interim assessment of preneoplastic biomarkers. The long timeframes involved necessitate assurances of the reproducibility of the formulation administered and of satisfactory patient compliance. Traditionally, at least two controlled clinical studies are considered necessary for drug approval[30].

1.2 CURCUMIN AS A CANCER CHEMOPREVENTIVE

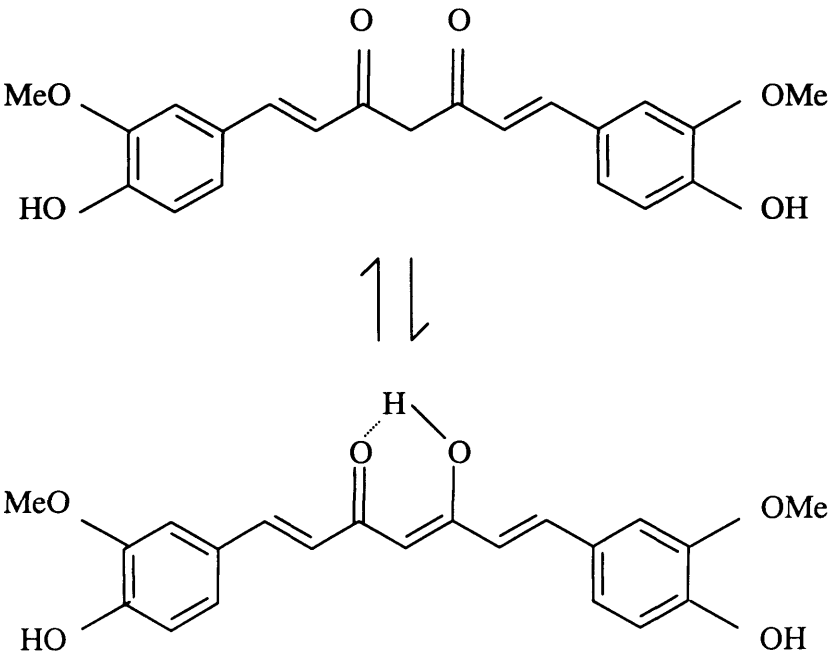
There has been considerable public and scientific interest in the use of phytochemicals derived from the diet to combat human diseases, especially the two commonest killers in the developed world, cardiovascular disease and cancer (see Appendix 2C). The dried ground rhizome of the perennial herb *Curcuma longa* Linn., called turmeric in English, haldi in Hindi and ukon in Japanese, has been used in Asian medicine since the second millenium BC[35]. In addition to its aromatic, stimulant and colouring properties in the diet, turmeric is mixed with other natural compounds such as slaked lime and has been used topically as a treatment for wounds, inflammation and tumours. In contrast to the maximum dietary consumption of 1.5 g per person per day, smaller quantities of turmeric tend to be used for medicinal purposes[36].

Curcuma spp. contain turmerin (a water-soluble peptide), essential oils (such as turmerones, atlantones and zingiberene), and curcuminoids including curcumin. Curcumin (diferuloylmethane) is a low molecular weight polyphenol first chemically characterised[37] in 1910, that is generally regarded as the most active constituent and comprises 2 - 8% of most turmeric preparations[38]. Curcumin has been the subject of hundreds of published papers over the past three decades, studying its antioxidant, anti-inflammatory, cancer chemopreventive and potentially chemotherapeutic properties. Relevant findings are reviewed below.

1.2.1 CHEMISTRY

Curcumin, 1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, is a bis-α,β-unsaturated β-diketone. As such, it exists in equilibrium with its enol tautomer. The bis-keto form predominates in acidic and neutral aqueous solutions and in the cell membrane[39]. At pH 3 to 7, curcumin acts as an extraordinarily potent H-atom donor[40]. This is because, in the keto form of curcumin, the heptadienone linkage between the two methoxyphenol rings contains a highly activated carbon atom, and the C-H carbon bonds on this carbon are very weak due to delocalisation of the unpaired electron on the adjacent oxygens (see Figure 1.1). In contrast, above pH 8, the enolate form of the heptadienone chain predominates, and curcumin acts mainly as an electron donor, a mechanism more typical for the scavenging activity of phenolic antioxidants[40]. Curcumin appears unstable at basic pH, and is degraded within 30 minutes (mins) in buffer systems to trans-6-(4'-hydroxy-3'-methoxyphenyl)-2,4-dioxo-5-hexanal, ferulic acid, feruloylmethane and vanillin[41]. 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 pH 7 [39]. Under acidic conditions, the degradation of curcumin is much slower, with less than 20% of total curcumin decomposed at 1 hour (h)[39].

Figure 1.1: Tautomerism of curcumin under physiological conditions. Under acidic and neutral conditions, the bis-keto form (top) predominates, whereas the enolate form is found above pH 8.



1.2.2 PHARMACOKINETICS AND SAFETY

The absorption, metabolism and tissue distribution of curcumin has been studied in rats. At a dose of 1 g/kg in the diet, about 75% of the dose was excreted in the faeces and negligible amounts appeared in the urine[42]. Intravenous and intraperitoneal administration resulted in large quantities of curcumin and metabolites in the bile, mainly tetrahydrocurcumin and hexahydrocurcumin (see Figure 1.2)[43]. Results obtained using suspensions of isolated hepatocytes or liver microsomes suggest that metabolic reduction occurs very rapidly, in a matter of minutes[42]. Use of radiolabelled curcumin has confirmed that the vast majority of the oral dose is excreted in the faeces, but only one third is excreted unchanged[44]. Intestinal metabolism may account for this discrepancy[42,45]. After intravenous dosing, more than 50% of the dose was excreted in the bile within 5 h and curcumin may have undergone enterohepatic recirculation[43]. A recent study of intraperitoneal curcumin in the mouse suggested that curcumin was first biotransformed to dihydrocurcumin and tetrahydrocurcumin, and that these compounds were subsequently converted to monoglucuronide conjugates[46]. After oral dosing in rats, small amounts of curcumin were detected in plasma with higher levels of curcumin glucuronide and curcumin sulphate, and small quantities of hexahydrocurcumin, hexahydrocurcuminol, and hexahydrocurcumin glucuronide (Figure 1.3)[45]. In summary, curcumin exhibits low oral bioavailability in rodents and may undergo intestinal metabolism; absorbed curcumin undergoes rapid first-pass metabolism and excretion in the bile.

Figure 1.2: Chemical structures of major metabolites of curcumin in rodents and humans

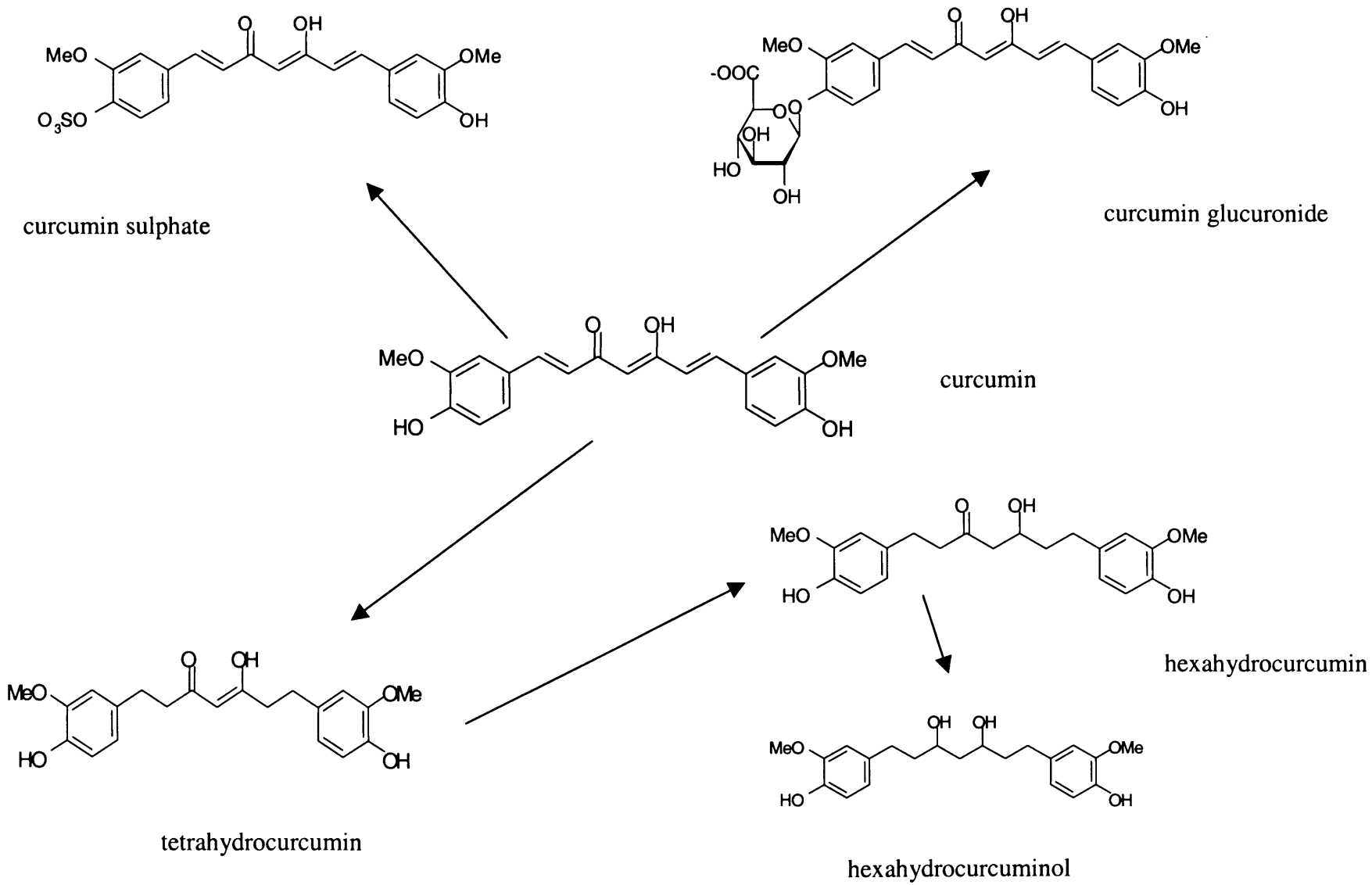
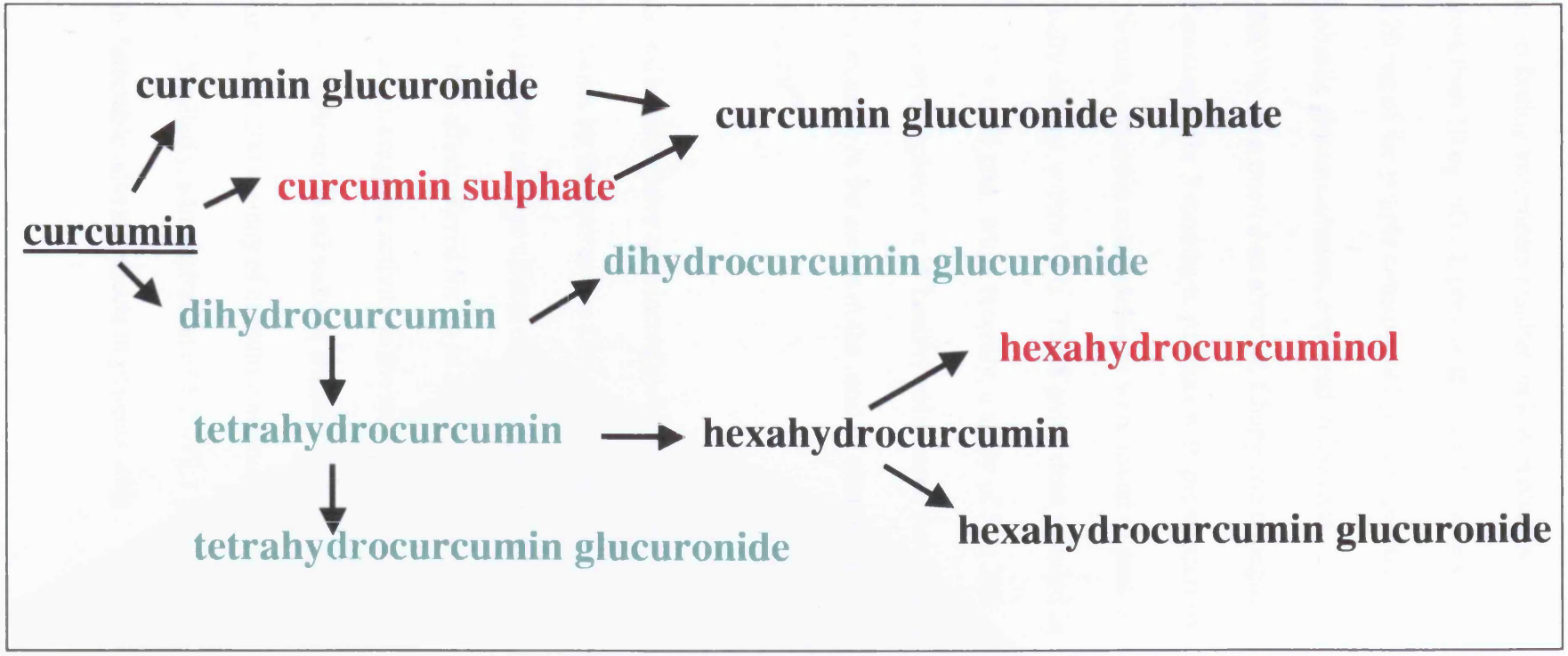


Figure 1.3: Metabolic pathways of curcumin in rodents and *ex vivo* culture of rat and human hepatocytes (as described in reference 45). Metabolites shown in green have been identified in mice; those shown in **red** have been observed in human hepatocyte culture. Metabolites shown in **black** and **red** have been demonstrated in rat hepatocyte culture or in rat plasma *in vivo*.



Thus far, comprehensive pharmacokinetic data in humans is lacking. 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[47]. In the same study, co-ingestion of curcumin with 20 mg of the pepper constituent l-piperoylpiperidine, which is thought to inhibit xenobiotic glucuronidation, appeared to increase curcumin's bioavailability by 2000%. In a published abstract, Cheng and collegues administered 0.5 - 8 g daily of curcumin for 3 months to patients with pre-cancerous lesions or resected cancers[48]. Serum curcumin concentrations were found to peak 1 - 2 h after oral intake, and gradually decline within 12 h. The 8 g/day dose resulted in a peak serum concentration of 1.75 ± 0.80 μM. More recently, a study of 50 - 200 mg of micronised curcumin has been completed in 18 healthy volunteers, resulting in no evidence for the presence of curcumin in the serum at the limit of quantitation which was approximately 0.63 ng/ml[49].

Studies of curcumin in animals and humans have convincingly shown lack of significant toxicity. Preclinical studies by the Prevention Division of the US National Cancer Institute did not discover adverse effects in rats, dogs or monkeys at doses up to 3.5 g/kg-body weight (bw) administered for up to 3 months[50]. One early report of dietary curcumin suggested ulcerogenic activity in the stomach of the albino rat[51], but this finding has not been confirmed in subsequent studies[50]. Dietary doses of turmeric, equating to a maximum of 200 mg/day of curcumin are not associated with adverse effects in humans[36]. Similarly, administration of 1.2 to 2.1 g curcumin for 2 - 6 weeks did not result in detectable adverse effects in patients with rheumatoid arthritis[52].

1.2.3 PHARMACOLOGICAL ACTIVITY

(a) Inhibition of Cyclooxygenase

It has been known for a decade that curcumin can inhibit cyclooxygenase (COX) activity in rat peritoneal neutrophils and human platelets[53]. 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 an 'housekeeping' enzyme; 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[54].

Curcumin's ability to inhibit induction of COX-2 gene expression has been demonstrated in oral and colon epithelial cells *in vitro*[55,56]. 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[45]. One of the mechanisms for this effect is inhibition of the activity of the IKK signalling complex responsible for phosphorylation of IκB [57]. The IKK signalling complex is integral to activation of the transcription factor, NF-κB, and is also inhibited by aspirin[58], a drug whose regular use may decrease the incidence of colorectal cancer by as much as

50% (discussed in Appendix 2D). Unlike selective COX-2 inhibitors, which inhibit the catalytic activity of the COX enzyme, curcumin appears to decrease COX-2 expression at the transcriptional level[57]. The ability of many natural agents derived from the diet to act at more than one level in a cellular pathway (reviewed in Appendix 2C) may be important in the prevention or treatment of diseases with multifactorial aetiologies such as cancer. Curcumin's ability to inhibit activation of pathways that interact with the NF-κB pathway, notably those involving activator protein-1 (AP-1) and c-Jun NH_2-terminal kinases (JNK)[59,60], 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.

(b) Phase I and II Metabolising Enzymes

The cytochromes P450 (CYP) enzyme system is important in the metabolic conversion and activation of many compounds, such as tetrachloromethane and aflatoxin B_1, to toxic reactive metabolites. Inhibition of CYP isoenzymes by curcumin has been demonstrated in cells cultured *in vitro*[61] 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, curcumin's inhibition of CYP1A1-mediated activation of dimethylbenzanthracene resulted in less DNA adduct formation[62]. Adducts are covalent interactions formed between toxic chemicals and constituents of DNA, and have been associated with mutagenesis and carcinogenesis (see section 1.3.4 below). Understanding of

curcumin's effects 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 GSTs are regarded as detoxifiers; induction rather than inhibition is therefore generally regarded as favourable in early carcinogenesis, potentially conferring a protective effect. Epoxide hydrolase and various hepatic GST isoenzymes were significantly increased upon curcumin feeding in mice[63], and total GST activity has been induced by dietary curcumin in both mice and rats in other studies[64-66]. 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[67].

(c) Reactive Oxygen and Nitrogen Species

Reactive oxygen species, such as superoxide anions and hydroxyl radicals, play a pivotal role in atherosclerosis and are also thought to be involved in carcinogenesis[68]. Consequently mopping up activated oxygen species is an important mechanism invoked in the prevention of cardiovascular disease and cancer. Impairment of reactive oxygen species generation in rat peritoneal macrophages by 10 μM curcumin has been shown[69], and similar effects have been observed in red blood cells at similar concentrations[70]. More specifically, curcumin has been shown to scavenge superoxide anion radicals[71] and hydroxyl radicals[72]. However, in keeping with other dietary phytochemicals, 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[73].

Nitric oxide (NO) is a short-lived, lipophilic molecule generated from L-arginine by various NADPH-dependent enzymes called NO synthases (NOS)[74]. NO is involved physiologically in vasorelaxation, neurotransmission, inhibition of platelet aggregation, immune defence and intracellular signalling[74]. NO has an unpaired electron, and is therefore a free radical species; its bioactivity is related to production of many reactive intermediates, but many of these reactive nitrogen species are capable of damaging DNA or hindering DNA repair[75-77]. Peak inducible NOS (iNOS) activity may relate to the transition of colonic adenomas to carcinomas[78]. 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*[79,80]. *Ex vivo* studies have suggested that the inducibility of macrophage NOS activity is inhibited by 1 to 20 μM concentrations of curcumin[81]. Subsequently, it has been shown that nanomoles of curcumin per g bw can significantly inhibit murine hepatic *iNOS* gene induction by lipopolysaccharide as an aqueous alkaline solution mixed into the drinking water (consumption equated to a mean of 92 ng curcumin per g bw)[82]. Since inhibition of iNOS activity may represent a mechanism of intervention during carcinogenesis, curcumin's activity at such low concentrations offers potential for cancer chemoprevention.

(d) Apoptosis and Angiogenesis

Curcumin inhibits cell proliferation and induces apoptosis in fungi[83], viruses[84] and cancer cells[85]. Its effects on the cell cycle appear manifold, and may be tissue-specific. In mouse embryo fibroblast, mouse sarcoma, HT29 human colon carcinoma, human kidney carcinoma, and human hepatocellular carcinoma cell lines grown *in vitro*, Jiang and colleagues observed cell shrinkage, chromatin

condensation and DNA fragmentation secondary to 9 μM curcumin treatment[85]. In other colon carcinoma cells cultured *in vitro*, curcumin induced apoptotic cell death by cell cycle arrest in the S and G2/M phases[86], and in the MCF-7 human breast tumour cell line the same was observed at G2 or M phases[87]. The presence of the diketone moiety may be essential for such antiproliferative activity[87]. 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*[88], 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[89].

The mechanisms of apoptosis induced by curcumin may be varied. Inhibition of cell signalling pathways involving NF-κB, AP-1 or JNK have been implicated, as has downregulation of the expression of survival genes *egr-1*, *c-myc*, and *bcl-X(L)* or abnormal tumour suppressor genes such as *p53* [57,59,60,90]. Curcumin has also been shown to have immunomodulatory effects, involving activation of host macrophages and natural killer cells[91] and modulation of lymphocyte-mediated function[92]. In addition to the colon, breast, kidney, fibroblast, liver, and sarcoma cell lines mentioned above, curcumin has shown growth inhibitory effects *in vitro* in cancer cell lines derived from human prostate, skin, bone, leukaemia and lymphoma[93-97].

Preclinical studies *in vivo* have also suggested that induction of apoptosis is a chemopreventive mechanism displayed by curcumin. Oral 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[98]. Indeed, curcumin's cancer chemopreventive properties in rodents are extremely diverse and it has been shown to inhibit tumour formation in the skin, forestomach, duodenum, liver and colon of mice and in the tongue, colon, mammary glands and sebaceous glands of rats[99,100]. Such chemopreventive properties have been demonstrated in both chemical and genetic models of colorectal carcinogenesis[29,99].

Curcumin also affects carcinogenic processes associated with the growth and dissemination of established malignancy. Angiogenesis, the formation of new blood vessels from existing vasculature, appears necessary for tumours to grow beyond 1 - 2 mm in diameter and metastasise[101]. Inhibition of angiogenesis by curcumin (10 μM and above) has been demonstrated *in vivo* using a mouse corneal model[31]. Inhibition of angiogenic growth factor production, integral to the formation of new vessels, has also been effected by curcumin in non-malignant and malignant cells[102,103]. Similarly, curcumin has been shown to inhibit the production of cytokines relevant to tumour growth, *e.g.* tumour necrosis factor-α and interleukin-1, and to reduce the expression of membrane surface molecules that play a role in cellular adhesion[104-106]. It is thus conceivable that curcumin may affect the behaviour of established malignancy *in vivo*.

(e) Effects in Humans

Several observations in human volunteers and patients suggest that curcumin may possess systemic biological activity at low oral doses. Two teams have studied inflammatory disease. Satoskar and colleagues found a significant anti-inflammatory effect from 400 mg thrice daily for 5 days in postoperative patients[107]. In a double-blind study, Deodhar and team administered 1200 mg curcumin four times daily to 18 patients with rheumatoid arthritis for 2 weeks, and they reported a significant improvement in the patients' inflammatory symptomology without apparent toxicity[52]. Other research has focussed on curcumin's effect on lipid metabolism. In accordance with the suggestion that 500 mg curcumin daily for 7 days can lower cholesterol and lipid peroxide levels in healthy human volunteers[108], it has been reported that a dose as low as 20 mg curcumin daily for 45 days may significantly decrease levels of serum lipid peroxides[109].

Two small studies have suggested that curcumin may possess other effects in humans. A single oral dose of 20 mg curcumin appeared to induce contraction of the gall bladder, assessed by ultrasound scan in human volunteers, compared to amylum placebo[110]. There also exist published anecdotes of curcumin as a topical treatment for cancer, most notably Kuttan's report of turmeric as a topical treatment for oral cancers and leukoplakia[33]. This research group reported a reduction in the size of the lesions in 10% of the 62 patients treated, but there was no control group, no assessment of anti-inflammatory activity and no chemical analysis of the preparation applied.

1.2.4 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 inhibition of cell signalling pathways at multiple levels, its diverse effects on cellular enzymes, and its scavenging of reactive oxygen and nitrogen species. In particular, curcumin's ability to inhibit malignant tumour growth and induce apoptosis in preclinical models offers potential for cancer chemoprevention and chemotherapy, although its low systemic bioavailability may limit the tissues in which the oral route of administration proves beneficial.

1.3 BIOLOGICAL MARKERS OF CURCUMIN'S ACTIVITY

The assumption made from existing knowledge, upon which this research has been based, is that the biochemical and molecular events associated with colon carcinogenesis are suitable indicators of the efficacy of cancer chemopreventive agents such as curcumin. Should this subsequently prove not to be the case for any of the biomarkers chosen, the findings from this research should still provide pharmacokinetic and pharmacodynamic insights regarding curcumin, valuable if this agent is to advance into clinical practice. It should be noted that curcumin resembles some new synthetic cancer chemotherapeutic drugs that fit into the current trend towards agents which target signalling molecules and the cancer cell environment[111]. Since these drugs appear less toxic at biologically effective doses than conventional cytotoxic agents in clinical use, and may result in cancer cytostasis rather than tumour volume reduction, the study of mechanism-based endpoints that may undergo subtle modulation is more important than for chemotherapeutic drugs used traditionally[111]. The biomarkers outlined below were selected on the basis of their potential for modification by curcumin; their relevance to colorectal carcinogenesis is also discussed. The principles and criteria used for biomarker selection have been summarised in sections 1.1.1 and 1.1.2.

1.3.1 GLUTATHIONE S-TRANSFERASE ACTIVITY

The GSTs are phase II detoxification enzymes that catalyse the conjugation of glutathione to a variety of electrophilic compounds. In general, induced GST enzyme activity results in more efficient elimination of carcinogens and may lead to cancer prevention[112]. Much research has been directed at investigating the cellular

protective role of this multigene family, and preclinical and clinical studies have established an association between decreased GST activity and increased susceptibility to developing colorectal cancer[112,113]. Paradoxically, in human colorectal adenocarcinoma and its liver metastases, *increased* levels of the GST-π isoenzyme have been found[114,115], and may have prognostic value for the overall survival of these patients[114]. In contrast, α- and μ-isoenzyme levels do not appear to be elevated. It thus appears that GST isoenzyme expression may be aberrant as a consequence of malignant transformation. Such expression may also contribute to drug resistance in established tumours[112].

Preclinical models have shown that putative chemopreventive agents such as oltipraz, NSAIDs, curcumin and other polyphenols can induce GST expression in the colon and liver[64-67,116-118]. Curcumin's effects on GST activity in rodents have been discussed in section 1.2.3(b). Phase I clinical evaluation of oltipraz has demonstrated its ability to induce GST activity as well as the level of transcripts encoding other detoxification enzymes in the colon mucosa of individuals at increased risk of colorectal cancer[119]. Furthermore, in individuals at risk of developing colorectal cancer, the total GST activity of blood lymphocytes has been shown to correlate with that of colon tissue[18]. In the same study, no relationship between lymphocyte and colon levels was demonstrated for the GST-μ and GST-π isoenzymes. Knowledge of an agent's effects on GST activity and isoenzyme levels, particularly in the liver, is also a predictor of potential drug interactions[112]. Modification of total GST in lymphocytes, liver or colon mucosa by oral curcumin is therefore a biological effect of potential relevance to the chemoprevention of colorectal cancer.

1.3.2 POLYAMINE CONCENTRATIONS

Polyamines are short-chain aliphatic amines required for normal cellular growth, and their concentrations have been shown to correlate with cellular proliferation[120]. Rapidly proliferating tissues such as tumours not only produce increased amounts of polyamines, but also have concentrations of individual polyamines that differ from those of normal tissues[120]. In particular, N1-acetylspermidine which is generally undetectable in normal mammalian tissues, is present at high levels in colorectal cancer[121,122]. Polyamines can also induce the expression of oncogenes[120].

The rate-limiting step in polyamine synthesis is the level of the enzyme ornithine decarboxylase (ODC), and this enzyme is constitutively overexpressed in colorectal dysplasia and neoplasia[123,124]. ODC and polyamine (especially putrescine) levels are significantly elevated in the colorectal mucosa of individuals with the *APC* germ-line mutation before the development of polyposis[125], suggesting a potential role for these assays in risk assessment for colorectal cancer. ODC has been postulated as critical for cell transformation[126] and is a possible preneoplastic biomarker or potential target for chemopreventive agents in preclinical and clinical trials[15,16,127].

A number of naturally occurring polyphenols, including curcumin and indole-3-carbinol, have been shown to inhibit ODC activity in preclinical models[128-130]. Clinical trials of the ODC inhibitor, difluoromethylornithine, have shown changes in colon mucosal polyamine levels, but dose escalation has been limited by toxicity[15]. Polyamine metabolites can be measured in 24-h urine samples, and appear to be abnormally elevated in rats with chemically induced colon cancer[131] and in patients with colorectal cancer[132]. This urinary biomarker may therefore offer a biochemical

index of malignant disease activity and potential inhibition of ODC *in vivo* by oral curcumin.

1.3.2 CYCLOOXYGENASE ACTIVITY

The COX (or prostaglandin H synthase) enzyme occurs as two isozymes, COX-1 and COX-2. There are several reasons to support the hypothesis that COX-2 is important in the development of colorectal cancer. Firstly a marked increase in COX-2 mRNA has been observed in a majority of colon carcinomas compared to normal surrounding mucosa, and in many adenomas[133,134]. Higher levels of eicosanoids, including PGs, have also been found in tumour tissue[135,136]. Secondly, inhibition of COX-2 expression or activity suppresses polyp formation in the MIN mouse[137] model of inherited polyposis. Thirdly, 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[138]. These findings suggest that COX-2 is important in the promotion/progression stages of CRC carcinogenesis, and is therefore a potential target for chemopreventive and chemotherapeutic agents.

Inhibition of COX-2 by curcumin in preclinical models[55-57] has been discussed in section 1.2.3(a). An indirect assay for measuring COX-2 inducibility in whole blood has been developed[139,140] and is recognised as "an established technique" for studying the potency and selectivity of COX inhibition by various agents *ex vivo*[141]. It involves inhibiting platelet COX-1 activity by the addition of aspirin, and then inducing COX-2 *in vitro* by the addition of the pro-inflammatory stimulus, bacterial

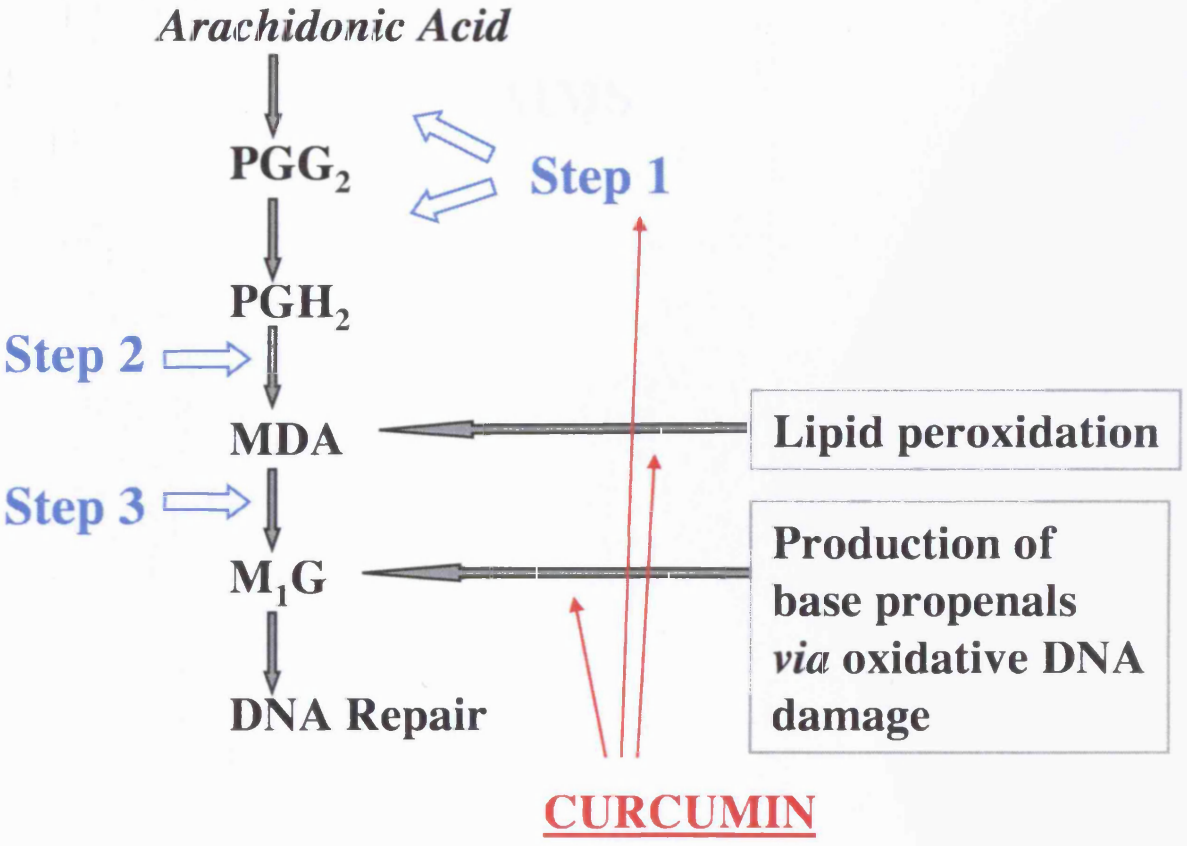
lipopolysaccharide (LPS). The degree of COX-2 activity is measured *via* plasma concentration of its product, PGE_2. This PG is principally formed by the COX-2 isozyme[142].

1.3.4 MALONDIALDEHYDE AND M_1G ADDUCTS

Malondialdehyde (MDA) is a mutagenic carbonyl compound generated in tissues by lipid peroxidation (LPO) and during PG biosynthesis[143]. Both enzymatic processes appear relevant to colorectal carcinogenesis. High levels of MDA and lipid peroxide have been found in colorectal cancer tissue[144,145] and MDA concentration may correlate with levels of PGE_2 [145]. Under physiological conditions, MDA reacts avidly with deoxyguanosine in DNA to form M_1G adducts, which have been associated with mutagenesis in bacteria and carcinogenesis in rodents[146]. DNA adducts arising directly or indirectly (*e.g. via* MDA arising from oxidation of lipid molecules in the cell membrane) from oxygen radicals may act as biomarkers of mutagenesis and carcinogenesis in humans[147]. M_1G adducts have been detected in liver, leukocyte, pancreas and breast from healthy human beings at levels ranging from 1 to 120 per 10^8 nucleotides[146]. Levels of M_1G and other base adducts formed by MDA were found to be higher in normal breast tissue from patients with breast cancer than from matched controls without the disease[148]. Adduct levels in colon mucosa and CRC tissue have not been described in any species. Diet appears particularly important in modifying the background levels of this biomarker, and significant potential appears to exist for modification of adduct levels by dietary intervention[146]. M_1G can be removed by mammalian nucleotide excision repair and mismatch repair pathways[146], and the existence of repair systems that appear to have

evolved to remove oxidised DNA bases is strong supporting evidence for their involvement in spontaneous DNA damage in humans[147]. Curcumin is a potent inhibitor of LPO *in vitro*[53,149] and in humans at oral doses as low as 20 mg/day[109], and it also inhibits PG production (see previous section). Furthermore, curcumin is a potent antioxidant under physiological conditions[40]. Even in the absence of LPO or MDA, oxidative DNA damage can contribute to formation of M_1G adducts *in vitro*, *via* base propenals[150]. In theory, there are thus at least 3 mechanisms by which curcumin may affect the formation of M_1G adducts (see Figure 1.4). Curcumin's effects on MDA and M_1G levels have not been described previously.

Figure 1.4: Conversion of arachidonic acid to PG endoperoxides resulting in formation of MDA and potentially M_1G adducts. Step 1 incorporates both oxygenase and peroxidase activities of the COX enzyme. Step 2 represents breakdown of PGH_2 to MDA and hydroxyheptadecatrieonic acid, which can occur spontaneously or *via* catalysis by thromboxane synthases or other cytochromes p450 (reference 144). Step 3 is one possible mechanism of formation of the M_1G adduct. Other routes of malondialdehyde production and generation of M_1G adducts are also shown, with potential mechanisms of inhibition by curcumin shown in red.



CHAPTER 2

AIMS

(1) The initial aim of this project was to develop assays for biomarkers relevant to the prevention or treatment of colorectal cancer by curcumin, and apply them in preclinical studies.

These experiments were based on the hypothesis that the biochemical and molecular events associated with carcinogenesis act as suitable biomarkers of chemopreventive efficacy in preclinical models. Curcumin was chosen as the agent to be scrutinised on account of its pleiotropic biological activity in preclinical models, with apparent lack of toxicity in animals and humans. The biomarkers were selected on the basis of their potential for modification by curcumin and their relevance to colorectal carcinogenesis, as outlined in Chapter 1. The normal F344 rat was chosen as a suitable model to study pharmacokinetic and pharmacodynamic effects of curcumin *in vivo*, partly because of the similarities of its biliary and gastrointestinal systems to those of humans. The MIN mouse model of an *APC* gene defect was used specifically to look at curcumin's effects on intestinal adenoma development.

Experiments were designed to provide novel mechanistic information on COX-2 activity and M_1G adduct levels *in vitro* and *in vivo*. In particular, the hypothesis was tested in human colon cells cultured *in vitro* that COX-2 activity results in formation of MDA and generation of M_1G adducts. Apart from the stipulation that they be of human origin, the main prerequisite for the five cell lines to be used was that they should be relevant to colorectal carcinogenesis, with varying constitutive levels of COX-2 activity.

(2) The principal aim of this project was to design and implement a pilot clinical trial of a standardised turmeric preparation in patients with colorectal cancer. Its main goal was the evaluation of the pharmacokinetics and safety profile of oral curcumin in humans. Measurements of potential changes in biomarkers relevant to cancer chemotherapy and chemoprevention constituted secondary objectives.

The rationale for using patients with colorectal cancer as the subject group was based on the need for early pharmacokinetic and pharmacodynamic information on the oral administration of curcumin to humans, the need for new treatments for colorectal cancer refractory to existing chemotherapy, turmeric's potential chemotherapeutic activity based on preclinical data, and contemporary Phase I trial design theory for chemopreventive agents, discussed in Chapter 6, section (3). Despite the likelihood of low systemic bioavailability following oral dosing in humans, dose escalation was performed from the lowest practical dose which appeared safe using this formulation in other subject groups, for reasons of safety. This approach also allowed the investigation of the systemic bioavailability of low dose curcumin using the most sensitive assays currently available. Even if curcumin or its metabolites were not detected in blood, analysis of the selected biomarkers would provide useful information regarding the measurement of these indices in patients with cancer, not previously reported for leukocyte M_1G adduct levels or *ex vivo* induction of COX-2 activity. The design of the measurement of chemotherapeutic parameters was based on contemporary Phase I oncology trials, and the measurement of biomarkers of curcumin's systemic activity was planned according to published data on these

indices in animals and humans and available pharmacokinetic information on curcumin, all reviewed in Chapter 1. The design of this study could possibly act as a paradigm for future Phase I trials of putative chemopreventive agents that possess potential chemotherapeutic activity.

(3) The overall aim of this research was to optimise the future development of curcumin as a chemopreventive agent by provision of preclinical and clinical information on the relationship between pharmacodynamic and pharmacokinetic endpoints.

Although it was not considered reasonable to obtain colorectal tissue from the patients in this pilot study, the investigation of the selected biomarkers in cultured cells and intact organisms in parallel with the clinical trial was intended to help bridge the gap between preclinical knowledge and lack of information resulting from pragmatic constraints in humans. Conversely, investigation of the viability of measuring M_1G adducts, GST levels and COX-2 activity in blood cells as biomarkers of curcumin's potential effects in humans was intended to reveal the clinical relevance of findings obtained in preclinical model systems, whose limitations have been discussed in Chapter 1, section 1.1.3.

CHAPTER 3

MATERIALS

Cell Culture

T75 and T150 flasks were purchased from Becton Dickinson, NJ, USA. The non-malignant HCEC cell line[57] was obtained from Dr. A. Pfeifer of Nestlé Research Institute, Lausanne, Switzerland. Cells were grown in a defined medium B50 containing bovine pituitary extracts, retinoic acid, vitamin C and dexamethasone (Biofluids Inc., Rockville, MD, USA). Malignant colorectal carcinoma cell lines SW48, SW480, HT29 and HCA-7, which have been described previously,[151,152] were obtained from Prof. C. Paraskeva of Bristol University and Dr. S. Kirkland of Imperial College, London. They were grown in Dulbecco's Modified Medium (DMEM) with Glutamax-1 (Life Technologies, Paisley), containing batch selected 10% foetal calf serum (FCS).

Rodent Experiments

Curcumin was purchased from Apin Chemicals Ltd. (Abingdon). Carbon tetrachloride (CCl_4), corn oil, fish oil, lithium-heparin, glycerol formal (consisting of 5-hydroxy-1,3-dioxane 60% and 4-hydroxymethyl-1,3-dioxalone 40%) and chremophore were purchased from Sigma Chemical Co. (Poole). Female F344 rats were obtained from Charles River UK Ltd. (Margate) and standard RM1 (for rats) and RM3 (for mice) diets from Special Diet Services (Witham). C57BL/6J MIN/+ mice were obtained from Jackson Laboratory, Bar Harbor, USA. Terminal anaesthesia was induced *via* inhalation of halothane (Astra Zeneca, Macclesfield).

Clinical Trial

A standardised proprietary turmeric preparation, P54FP, as previously described[153], was obtained as a liquid and in capsule form, from Phytopharm plc., Godmanchester, Cambs. Patient blood was collected in standardised pre-treated lithium-heparin tubes, except for samples for *GST* genotype, which were collected in tubes pre-treated with ethylenediaminetetra-acetic acid (EDTA) purchased from Sarstedt, Loughborough. Lymphocyte isolation was performed using Ficoll-paque plus©(Amersham Pharmacia, Uppsala, Sweden).

GST Activity

1-chloro-2,4-dinitrobenzene assay (CDNB), glutathione (GSH), potassium chloride and ammonium chloride were obtained from Sigma Co. (Poole). Total protein concentration of each sample collected for GST activity, and all other relevant samples in experiments (as stated), were determined using the Bradford Assay (Biorad, Hercules, CA, USA).

Polyamine Concentration

Acetyl putrescine, N8-acetyl spermidine, N1-acetyl spermidine, putrescine, 1,8-hexane diamine, acetyl spermine, spermidine, spermine and dansyl chloride were obtained from Sigma Co. (Poole). 24-h urine collection bottles containing 100 ml 1 M hydrochloric acid were purchased from Sarstedt (Loughborough). C-18 Sep-pak© cartridges for solid-phase extraction of urine were purchased from Waters Co. (Milford, MA, USA). High pressure liquid chromatography (HPLC) was performed through a LC-18 column 150 x 4.6 mm, particle size 3 μm (Supelco, Bellefont, PA, USA), wrapped in foam to maintain room temperature. Scanning fluorescence

detection was by Waters 470 machine, set at excitation wavelength 342 nm and emission wavelength 520 nm.

COX-2 Activity

COX-1 and COX-2 polyclonal antibodies and COX-2 protein standard were purchased from Oxford Biomedical Corp. (Oxford). Anti-α-tubulin and anti-actin antibodies were obtained from Amersham Pharmacia Biotech Ltd. (Little Chalfont, Bucks). Anti-rabbit and anti-murine horseradish peroxidase antibodies were purchased from Dako (Ely). Phorbol 12-myristate 13-acetate (PMA), arachidonate, ethanol, acetylsalicylic acid, and bacterial LPS were purchased from Sigma Co. (Poole) and the selective COX-2 inhibitor, NS-398, from Cayman Chemical Co. (Ann Arbor, MI, USA). Both agents were dissolved in dimethlysulphoxide (DMSO) from Sigma Co., Poole, before dilution in cell culture medium. PGE_2 levels were determined by competitive enzyme immunoassay purchased from Cayman Chemical Co. (MI, USA).

MDA and M_1G Levels

MDA levels were assessed by the colorimetric Lipid Peroxidation Assay Kit (Calbiochem Corp., San Diego, CA, USA)[154,155]. Murine M_1G monoclonal antibody D10A1 was prepared as previously described[156] and obtained from Prof. L.J. Marnett, Vanderbilt University, USA. Anti-murine horseradish peroxidase antibody was purchased from Dako (Ely). 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[157,158]. Genomic DNA extraction was performed using reagents and columns obtained from Qiagen (Hilden, Germany).

Chemiluminscence (Supersignal Ultra, Pierce, Rockford, IL, USA) and densitometry were analysed by Biorad Multimager (Biorad Labs., Hercules, CA, USA).

Propidium iodide was purchased from Sigma Co. (Poole).

Pharmacokinetic Analyses

The reversed-phase HPLC method for detection and quantitation of curcumin and its conjugates was developed by Mr C. Ireson, PhD student, University of Leicester. A Symmetry Shield RP 18 column (150 × 3.9 mm, particle size: 5 μ, Waters) was used, with a Varian Prostar (230 model) solvent delivery system coupled to a UV-visible detector (310 model) and autosampler (model 410). 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. A linear gradient of 5 – 45 % acetonitrile in 0.01% ammonium acetate (pH 4.5) was used for 30 min, followed by an increase over 20 min to 95% acetonitrile (flow rate: 1 ml/min). All chemicals for pharmacokinetic analyses were obtained from Sigma Co. (Poole).

CHAPTER 4

METHODS

4.1 MEASUREMENT OF COX-2, PGE_2, MDA AND M_1G IN COLON CELLS GROWN IN CULTURE

The non-malignant HCEC cells were grown in defined medium B50 containing bovine pituitary extracts, retinoic acid, vitamin C and dexamethasone. Malignant colorectal carcinoma cell lines SW48, SW480, HT29 and HCA-7, which have all been described previously as having differing constitutive levels of COX-2 gene expression[57,89,151], were grown in DMEM medium containing 10% FCS. All cells were grown to approximately 70% confluency in T75 or T150 flasks at 37°C in a 5% CO_2 incubator, and new DMEM medium containing 10% FCS substituted 24 h prior to treatment of all five cell lines. All cells were treated in 10 ml of growth medium, and repeat experiments were performed in serum-free medium to demonstrate that the presence of serum did not affect the parameters measured. Incubation with 75 nM PMA was performed for 4, 24 and 72 h. In certain experiments stated specifically in the following text, 50 μM arachidonate (diluted in ethanol) was added with PMA to further enhance the production of PGs, and ethanol added to controls.

In order to increase M_1G levels, HCEC cells were treated with 1 mM NaMDA for 24 h[159]. To selectively inhibit COX-2 activity, HT29 cells were incubated with 17.7 μM NS-398 (ten times the stated IC_{50} value)[160] for 24 and 72 h, with DMSO added to control flasks. At the end of each stated incubation time, 1 ml of culture medium was removed and following removal of particulates by centrifugation, stored at -80 °C for PGE_2 determination (described in section 4.6). All cells were then removed immediately using trypsin, and counted using trypan blue staining and a haemocytometer. PGE_2 levels in the medium were normalised with respect to the

number of adherent cells counted at the time of harvesting. COX-1 and COX-2 protein levels were assessed by SDS-PAGE Western blotting according to standard procedures[57], using 25 μg protein per lane; then stripping and re-probing the blot 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. Intracellular MDA and M_1G adduct levels were analysed as outlined in section 4.7. In Table 5.2, M_1G values were corrected for the cell number used for DNA extraction, in case cell viability following PMA treatment should affect total adduct levels.

4.2 STUDIES OF DIETARY CURCUMIN IN RODENTS

Water and standard RM1 or RM3 diets were available *ad libitum* to rats or mice respectively. All rodents were housed in treatment groups of four animals per cage, and animal facilities were maintained at 20°C on a 12 h light/dark cycle. For all experiments, curcumin powder was mixed into the diet to constitute the proportion of the total diet, stated as a percentage. Corn oil was added at the same time in the same proportion to enhance mixing. Control animals received a diet containing the same proportion of corn oil only. Analysis by HPLC/MS of the curcumin formulation used established that the powder contained 91% curcumin and 9% desmethoxycurcumin.

Rat experiments were designed with the help of Dr R. Verschoyle, and they were approved by the University of Leicester Ethical Committee for Animal Experimentation. Rat handling and dissection were performed by Dr R. Verschoyle, under Project Licence 80/1250 granted to the MRC Toxicology Unit by the UK Home Office. Extraction and analyses for tissue levels of curcumin and its metabolites were performed by Mr C. Ireson, PhD student, University of Leicester.

Rat Experiment 1 was designed to test the hypothesis that dietary curcumin affects the basal levels of selected biomarkers in association with detectable tissue levels of the parent compound or its metabolites. In this experiment, seven female Harlan F344 rats (6-week old, 160 - 180 g) were fed 2% curcumin for 14 days, and seven control rats were fed a 2% corn oil diet. Food intake and body weight were monitored throughout. Rats were killed under terminal anaesthesia, followed by cardiac puncture. Lymphocytes were separated from fresh blood for measurement of biomarkers, and plasma stored for pharmacokinetic analysis. Whole liver and rat colon mucosal scrapings were frozen immediately in liquid nitrogen and stored at -80°C.

Since the feeding of diets rich in polyunsaturated and diunsaturated fatty acids has been shown to increase M_1G adduct levels in rats and humans[161,162], fish oil (consisting of >26% of polyunsaturated and diunsaturated fatty acids) was used in **Rat Experiment 2**. In this experiment, the hypothesis was tested that dietary curcumin attenuates diet-related increases in M_1G adduct levels. Three female F344 rats (8-week old, 180 - 200 g) were fed a 10% fish oil diet for 26 days, and three other animals were fed the same until day 22 and then a diet containing 10% fish oil plus 4% curcumin for the final four days. Three control rats were fed a normal RM1 diet throughout the 26 days. On day 26, all rats were heart bled under terminal anaesthesia and samples of blood, liver and whole colon were frozen in liquid nitrogen and stored at -80°C.

In **Rat Experiment 3**, the hypothesis was tested that dietary curcumin prevents both endogenously and exogenously induced formation of M_1G adducts. In this experiment, consisting of eight F344 rats (6-week old, 160 - 180 g) per group (numbers based on the large standard deviation detected in Rat Experiment 2), curcumin was dissolved in corn oil and mixed with the diet to constitute an intake of 2% curcumin for 14 days. Control animals received a diet containing the same proportion of corn oil only. CCl_4 was dissolved in corn oil (2 ml/kg), and administered by intragastric tube (*ig*) at a dose of 0.5 ml/kg on day 10, to induce LPO. Control animals received the equivalent dose of corn oil (2 ml/kg) *ig* on day 10. On day 14, all rats were anaesthetised and bled by cardiac puncture. The dose regime and timing of sampling following CCl_4 treatment was based on published rodent studies of MDA and M_1G adduct levels[163,164]. Blood was collected in tubes containing approximately 10 u lithium-heparin per ml blood. The colon was flushed with PBS, and dissected out. Whole blood, liver tissue, colon mucosal scrapings and colonic contents were protected from light, frozen immediately in liquid nitrogen, and stored at –80°C. Histological specimens of colon were collected to confirm that scraping was no deeper than the epithelial layer.

MIN mice were fed dietary curcumin to investigate potential changes in biomarker levels in normal tissues and intestinal premalignant adenomas, relative to controls. A MIN/+ breeding colony was established and tissue samples were obtained from newborns by earpunching and genotyped for MIN/+ staus by polymerase chain reaction (PCR) and *HindIII* digest of the product[28]. Four-week old male MIN mice had access to water and a standard RM3 diet *ad libitum*. For dietary administration,

curcumin was mixed with the diet to constitute 0.1 and 0.2% of the total diet. The lower of these two doses of curcumin in the diet, which mice received continuously from the age of 4 weeks, has previously been shown to prevent gastrointestinal tract tumours in MIN mice[29]. Control animals received the standard RM3 diet. At the end of the 17th week, mice were subjected to terminal anaesthesia and the small and large intestines were flushed with PBS, and dissected out. All adenomas were removed using forceps under visual magnification. Normal mucosa was scraped using a blunt spatula, and histological examination ensured accurate scraping of intestinal mucosa only. Tissue samples were protected from light and analysed immediately, or frozen in liquid nitrogen and stored at –80°C until pharmacodynamic and pharmacokinetic analyses were performed.

Experiments were approved by the University of Leicester Ethical Committee for Animal Experimentation. Animal genotyping, handling and dissection were performed by Ms S. Perkins, PhD student, University of Leicester, under Project Licence 80/1250 granted to the MRC Toxicology Unit by the UK Home Office. Extraction and HPLC analysis of tissue levels of curcumin and its metabolites were performed by Ms S. Perkins using the method developed by Mr C. Ireson.

4.3 CLINICAL TRIAL OF ORAL *CURCUMA* EXTRACT

Each capsule of P54FP contained 18 mg curcumin, 2 mg desmethoxycurcumin and 200 mg essential oils derived from *Curcuma spp.* (150 mg from *Curcuma domestica* and 50 mg from *Curcuma xanthorrhiza*). Chemical analysis by HPLC/MS confirmed the content of curcuminoids. Typical constituents of *Curcuma* essential oil mixtures are tumerones, atlantone, curlone and zingiberene[165-167]. This formulation, capsules of which in the following text will be referred to as "*Curcuma* extract," was considered appropriate for the aims of this trial on account of the curcumin dose (which equates to a normal dietary intake of turmeric in certain communities, as discussed in Chapter 1, section 1.2), reproducibility of curcuminoid content and the fact that capsules contained extracts of *Curcuma* plants used in traditional Indian and Southeast Asian medicine. It is conceivable that curcumin's systemic bioavailability may be enhanced by the oral co-administration of other diet derived compounds, such as piperine or other turmeric constituents[47]. This formulation of *Curcuma* extract has been used in a previous unpublished clinical trial in patients with osteoarthritis at a dose of 2 capsules/day for one month[b]and a study of 6 capsules/day for five days in volunteers, published in abstract form[153].

[b] P54FP Investigators' Brochure, Phytopharm plc., 9 West Street, Godmanchester, Cambs PE18 8HG

A Doctors' and Dentists' Exemption was granted by the UK Medicines Control Agency for a clinical trial of P54FP in patients with colorectal cancer. A clinical trial protocol was prepared, and full local ethics committee approval was obtained for the use of blood from untreated healthy volunteers for preliminary experiments followed by a clinical trial of P54FP in patients with advanced colorectal cancer. An independent team of physicians was recruited to review the adverse events documentation before each dose escalation.

Fifteen patients, enrolled between September 1999 and September 2000 at the Leicester Royal Infirmary, met the following eligibility criteria: Histologically proven adenocarcinoma of the colon or rectum for which no further conventional therapies were available; measurable or evaluable progressive malignant disease within 4 weeks of enrolment; age >18 years; WHO performance status of 0 - 2 and life expectancy greater than 12 weeks; absolute neutrophil count $\geq 1.5 \times 10^9$/l; haemoglobin $\geq$ 10 g/dl; platelets $\geq 100 \times 10^9$/l; aspartate aminotransferase and alanine aminotransferase < 2.5 times the upper limit of normal (ULN); serum bilirubin and creatinine < 1.5 x ULN; and no previous investigational or chemotherapeutic drugs within 28 days prior to enrolment. Exclusion criteria included: Active chronic inflammatory or autoimmune disease; active infection, including viral infection; significant impairment of gastrointestinal function or absorption; active peptic ulcer disease; known biliary obstruction or biliary insufficiency, and use of non-steroidal anti-inflammatory drugs (NSAIDs) within 14 days of enrolment. Patients were asked to abstain from NSAID use and the consumption of foods containing the spice turmeric during the study period, and their

general practitioners were asked not to prescribe NSAIDs. Written informed consent was obtained at trial enrolment from each patient at least 24 h after they were given the patient information leaflet. Demographic and baseline characteristics of patients are shown in Table 4.1.

Table 4.1: Patients' characteristics at enrolment

	Daily dose of *Curcuma* extract (mg)				
	440	880	1320	1760	2200
Sex: Male	3	3	2	1	3
Female	0	0	1	2	0
Age: (years) mean	67.3	61	60.3	53	63.7
(range)	(65 – 71)	(53 – 72)	(59 – 66)	(42 – 73)	(51 – 71)
WHO performance:					
0	2	2	2	3	2
1	1	1	1	0	1
Sites of measurable disease:					
Colorectum	1	0	0	0	0
Liver	2	2	2	2	3
Lung	1	0	0	0	1
Peritoneum	0	1	2	0	1
Other	0	0	0	1	0
Previous Chemotherapy:					
5-Fluorouracil	3	3	3	3	3
Other	2	3	2	2	2
Previous Radiotherapy	1	1	0	2	1
Previous Surgery	3	3	3	3	3
NSAID stopped for trial	2	0	0	1	0
CEA > 10 ng/ml	3	3	3	3	3
CA 19.9 > 37 iu	3	3	2	3	2

All patients were caucasian and all had undergone previous surgery. Three patients stopped NSAID medication 3 weeks before treatment with *Curcuma* extract.

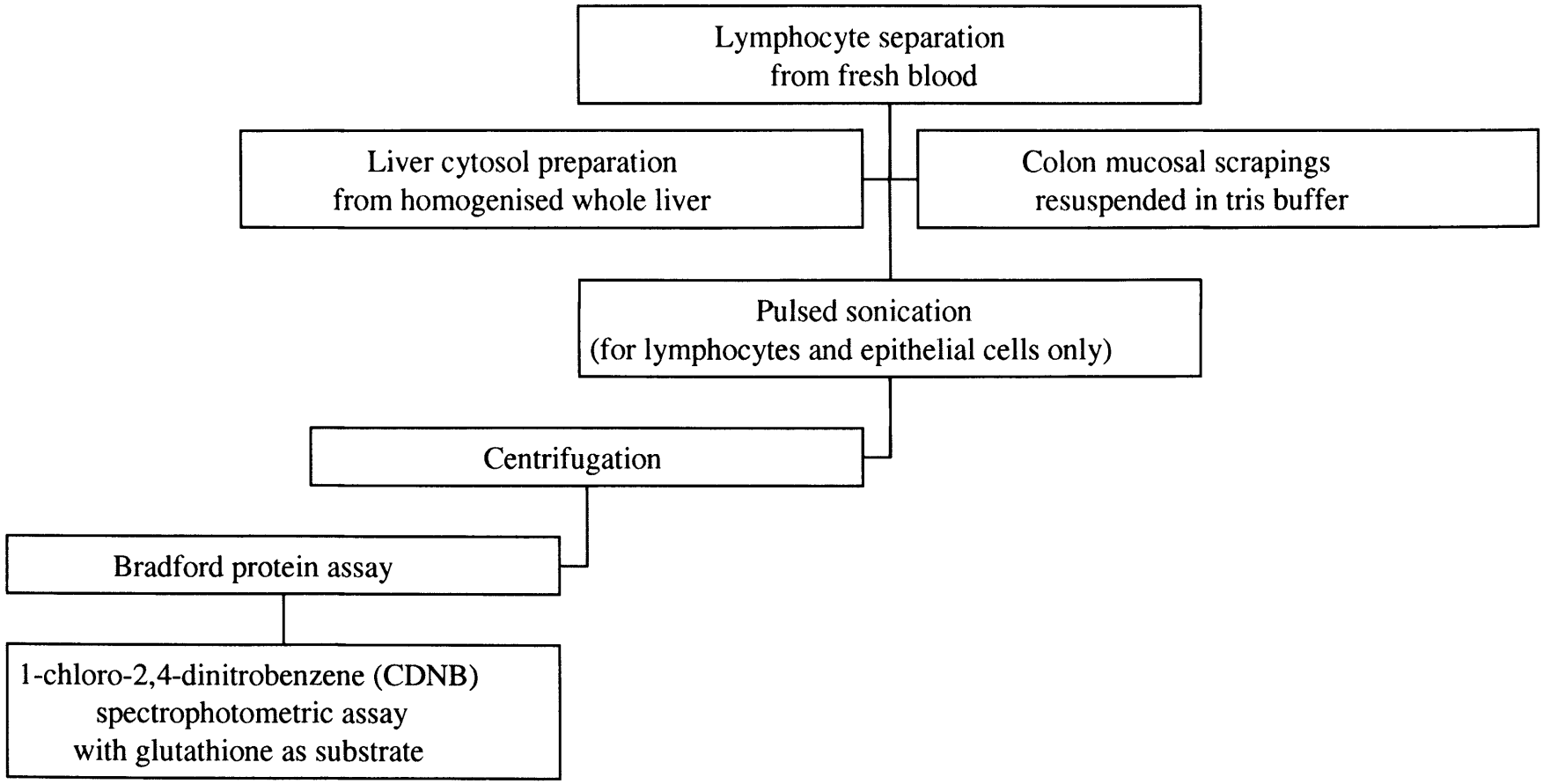
There were 3 patients per dose level. Following at least 2 h fasting, patients consumed 2, 4, 6, 8 or 10 capsules once daily with water. This scheme translates to doses of 440, 880, 1320, 1760 and 2200 mg of *Curcuma* extract per day containing 36, 72, 108, 144 and 180 mg curcumin, respectively. Treatment was continued until disease progression was established or consent was withdrawn. Blood, urine and faeces were collected on days 1, 2, 8 and 29 by Ms H. McLelland, Oncology Trials Unit, University Hospitals of Leicester. All blood, plasma, urine and faecal samples were protected from light and stored at -80^{0}C. Blood collection for pharmacokinetic analysis on the first treatment day was pre-dose and at 0.5, 1, 2, 3, 6 and 8 h post-dose, and samples were kept in standard tubes pre-treated with lithium-heparin. Full blood cell count and urea, electrolytes, liver and bone function were measured in venous samples, and physical examination was performed, at screening visit 7 – 14 days before treatment and on treatment days 1, 2, 8, 29 and monthly thereafter. Venous blood levels of the tumour markers carcinogenic embryonic antigen (CEA) and CA19.9 were measured by the Chemical Pathology Laboratory, University Hospitals of Leicester, before treatment and every month of treatment. Radiological assessment of target malignant lesions was performed by consultant radiologists at the University Hospitals of Leicester every 2 months by computed tomography (CT) or magnetic resonance imaging scan, in addition to monthly chest X-rays. Patients were asked to complete the EORTC quality of life questionnaire GLQ-C30 (version 2.0)[168] pre-treatment and monthly during treatment.

DNA extraction for blood samples for dose levels 3, 4 and 5 of the clinical trial were performed by Mrs S. Euden, University of Leicester. Analysis of PGE_2 levels for plasma samples from dose levels 3, 4 and 5 were performed by Ms K. Hill, University of Leicester. Extraction and HPLC/MS analyses for tissue levels of curcumin and its metabolites were performed by Mr C. Ireson and Ms K. Hill, University of Leicester.

4.4 MEASUREMENT OF GLUTATHIONE S-TRANSFERASE ACTIVITY

The following method was applied according to published papers[14,18,169], and modified to eliminate red blood cell contamination and minimise inter-day variability. In brief, 10 ml human blood or 6 ml rat blood was collected in standard lithium-heparin tubes. Lymphocytes were isolated by centrifugation, and washed in PBS. Erythrocyte/haemoglobin contamination of lymphocyte samples was found to affect the protein assay significantly (all GST results were expressed per mg lymphocytic protein). A red cell lysis step (10 min incubation in 0.15M ammonium chloride buffer at 37°C) and two further PBS washes were therefore introduced into the procedure for lymphocyte separation of all blood samples, based on standard protocols used by the Department of Immunology, University Hospitals of Leicester. This addition was shown not to affect results obtained using the CDNB assay. Following resuspension of lymphocytes in 10 mM Tris buffer pH 7.8 at 4°C, samples were stored at -80 °C. Once thawed on ice, samples were subjected to pulsed sonication for 30 s on ice and 10 min centrifugation at 3000 x *g* at 4°C. The total GST activity of the supernatant was determined at 37°C by the CDNB spectrophotometric assay[169] using GSH as substrate. Serial dilutions were used to determine the limit of quantitation of the assay, found to be about 10 nmol/min/mg protein. Inter-day variation was 10 - 20 nmol/min/mg protein, and its effect on results was minimised by analysing all the samples from one experiment or from one patient on the same day. One sample of rat liver cytosol was included in each assay as an internal standard. The methodological scheme for tissue samples is shown in Figure 4.1. All GST activity values were quoted as nmol CDNB conjugated with GSH per min per mg lymphocytic protein.

Figure 4.1: Methodological scheme of CDNB assay for measuring total GST activity



Rodent Studies

In Rat Experiment 1, F344 rats were fed a 2% curcumin diet for 14 days. Lymphocytes were separated from fresh blood and resuspended in 10 mM Tris buffer (pH 7.8) and frozen at -80^0C. Liver and rat colon mucosal scrapings were frozen immediately in liquid nitrogen and stored at -80^oC. Rat liver cytosol was prepared immediately by mechanical homogenisation in potassium chloride buffer (150 mM) and centrifugation. CDNB and protein assays were performed as described above.

Human Samples

Morning blood samples were collected from 4 healthy individuals on 3 separate occasions 2 - 4 weeks apart to assess intra- and inter-individual variability. Simultaneous collection in pre-treated lithium-heparin and EDTA tubes was compared. Adding increasing amounts of lithium-heparin or EDTA to aliquots of the same sample showed that both additives affected the assay. Standardised pre-treated 7.5 ml lithium-heparin tubes from the same batch were used for all future blood sampling in healthy volunteers and patients. In the clinical trial of *Curcuma* extract, blood samples were collected one week before commencement, immediately pre- and one hour post-dose on day 1, and then immediately pre- and 1 hour post-dose on days 2, 8, and 29. Blood samples were kept at room temperature, and lymphocytes separated within one hour of the blood being taken. Washed lymphocytes were resuspended in 10 mM Tris buffer (pH 7.8) and frozen at -80^0C. All samples from the same patient were analysed on the same day. A collaboration was established with Prof M. Pirmohamed of the Department of Clinical Pharmacology, University of Liverpool, who agreed to determine the genotypes of

patient blood samples for the major GST isozymes expressed in lymphocytes. The *GSTM1*, *GSTT1*, and *GSTP1* genotypes were determined by Prof M. Pirmohamed using PCR methods previously described[170,171]. These isozymes have been linked to the total GST activity of lymphocytes measured using CDNB as substrate, and have also been linked to various cancers[18,112,172,173].

4.5 URINARY POLYAMINES

The method for extraction, derivatisation and determination of polyamine concentration by HPLC was based on published papers[122,132,174,175] and is summarised here. Standard curves and inter-day variation were determined for acetylputrescine, N8-acetyl spermidine, N1-acetyl spermidine, putrescine, acetyl spermine, spermidine, and spermine, dissolved in water; both with and without solid phase extraction (see below). Urine samples over 24 h were collected in pre-treated bottles, and the entire sample was mixed by shaking. An aliquot of 5 ml was stored for creatinine estimation (by the Chemical Pathology Laboratory, University Hospitals of Leicester), and 100 ml stored at -80^{0}C for polyamine determination. To 1 ml of this sample was added 20 µl of 10 µM hexane-diamine internal standard, and 1 ml buffer (heptane-sulphonic acid pH 3.4). For solid phase extraction, the sample was applied to C-18 Sep-pak© cartridges and washed with buffer and with water. Polyamines were eluted with methanol, which was then evaporated. The polyamines were re-dissolved in acetonitrile, and dansylated by reaction with dansyl chloride at 70^{0}C. Remaining dansyl chloride was removed by adding an excess of L-proline, and the dansylated products separated by dissolution in cyclohexane. This was evaporated, and the dansylated polyamines re-dissolved in acetonitrile. A volume of 40 µl was injected for HPLC analysis with fluorescence detection, using a complex gradient of heptane sulphonic acid pH 3.4/acetonitrile/methanol and acetonitrile/methanol[175]. Peak areas detected by scanning fluorescence, set at excitation wavelength 342 nm and emission wavelength 520 nm, were compared to the peak area of the internal standard. Urine from healthy volunteers and colorectal

cancer patients was analysed following solid-phase extraction, and known amounts of standards added to determine yields. Similarly, 24-h urine samples were collected in Rat Experiment 1 the day before and on the 14th day of curcumin feeding.

A urine sample size of 1 ml was found to be the optimal amount for solid phase extraction, although interfering HPLC peaks were often present and made interpretation impossible for certain polyamines. Increasing the sample size to 2 ml was found to increase the number of interfering peaks causing difficulties in interpretation of the chromatogram. Reducing the sample size to 0.5 ml resulted in peaks being below the limit of detection (approximately 50 fmol polyamine). An example HPLC trace for 1 ml **human urine** is shown in Figure 4.2. Based on the standard curves produced following solid phase extraction, the concentration of individual polyamines in human urine was highly variable. Values obtained were often above or below the standard curves (range 50 pmol to 5 nmol total polyamine in 1 ml urine sample). **Rat urine** showed less variability, but peaks were often below the limit of detection and there were greater numbers of interfering peaks on the chromatogram (Figure 4.3). These peaks may have been due to sample contamination by faeces, despite solid phase extraction. On account of interfering peaks or large variations in the concentrations of individual polyamines in human urine, further method development was not attempted. It was felt that consistent and reliable results could not be obtained by this method and that the high degree of background variability in individual polyamines rendered the likelihood of observing a treatment effect extremely slim.

Figure 4.2: Chromatogram obtained for 1 ml human urine.

Following 24 h urine collection from a healthy volunteer, an aliquot of 1 ml was spiked with the internal standard, 1,8-hexane diamine, and then subjected to solid phase extraction, dansylation and analysis by HPLC, as described in section 4.5. Identification of polyamines was based on retention times of standards under identical conditions. Typical retention times (mins) are shown on the *x*-axis. Peak areas of individual polyamines were compared to the peak area of the internal standard. Interfering peaks, not shown on this chromatogram, were often present in analyses of other samples of urine from volunteers and patients.

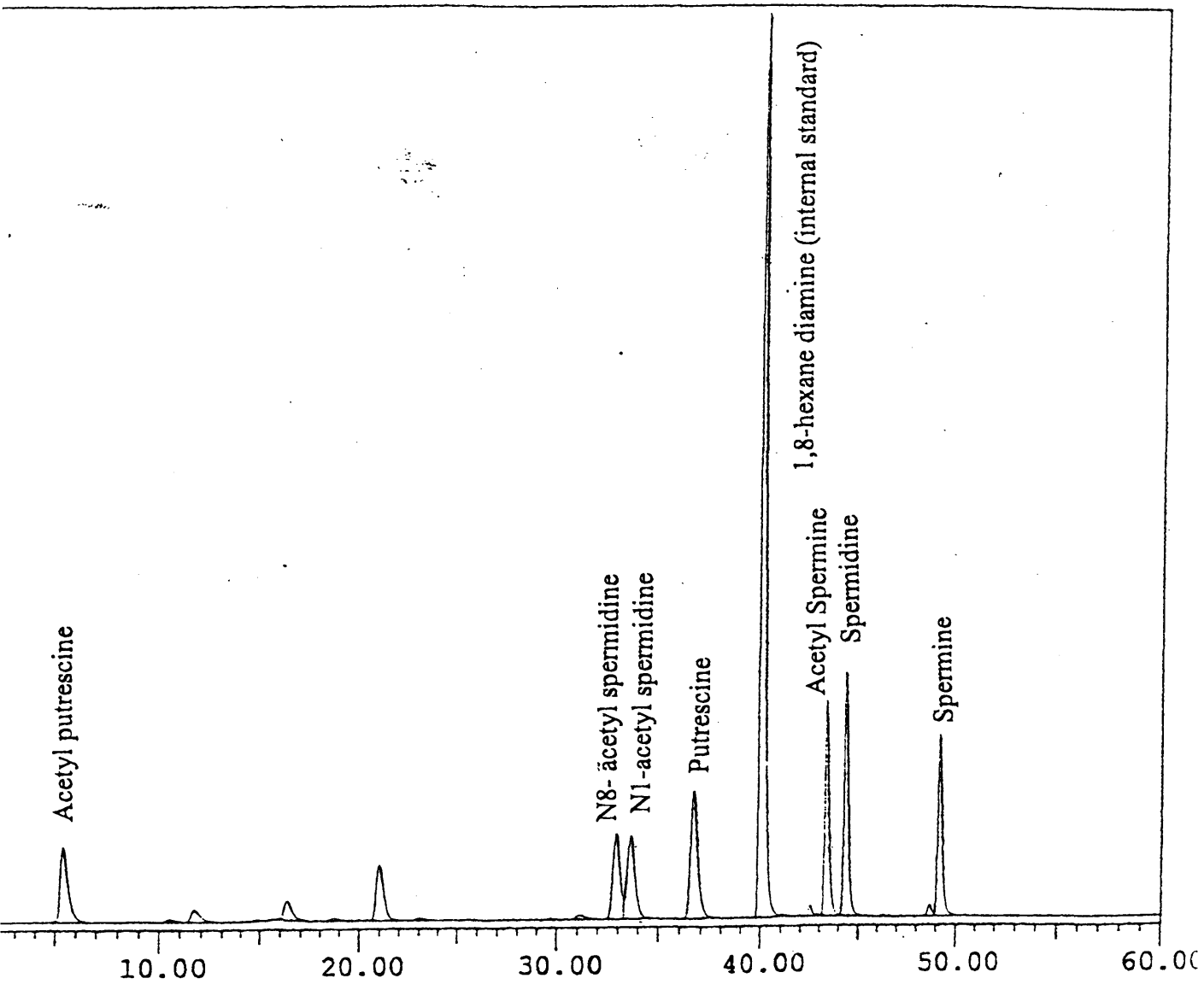
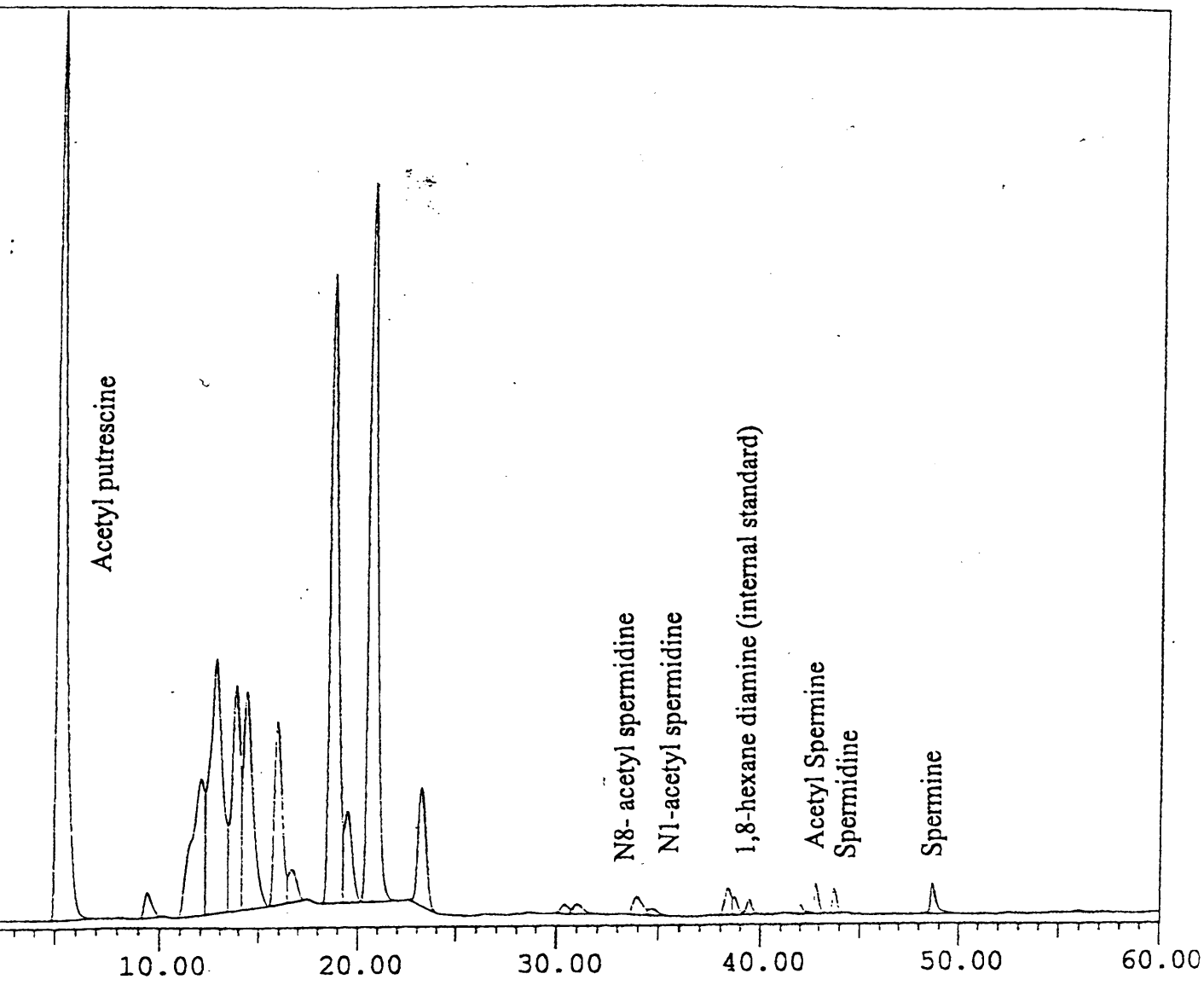


Figure 4.3: Chromatogram obtained for 1 ml rat urine.

Following 24 h urine collection from a control animal in Rat Experiment 1, an aliquot of 1 ml was spiked with the internal standard, 1,8-hexane diamine, and then subjected to solid phase extraction, dansylation and analysis by HPLC, as described in section 4.5. Provisional identification of polyamines was based on retention times of standards under identical conditions. Typical retention times (mins) are shown on the *x*-axis. Peak areas of individual polyamines were compared to the peak area of the internal standard. Interfering peaks, in addition to those shown on this chromatogram, were often present in analyses of samples of rat urine and were attributed to faecal contamination during sample collection



4.6 CYCLOOXYGENASE 2 PROTEIN LEVELS AND ACTIVITY

The method for measuring PGE_2 production as a measure of the inducibility of COX-2 activity *in vitro* has been published previously[139,140]. A blood sample (4.5 ml) was collected in lithium-heparin tubes from **healthy volunteers** on three separate occasions 2 – 4 weeks apart. This was mixed by inversion and 1.5 ml aliquots studied in triplicate for each treatment. In order to irreversibly inhibit platelet COX-1 activity, acetylsalicylic acid (200 μM) was added to all samples. At the same time, 1 to 20 μM concentrations of curcumin were added to certain samples, and mixed by gentle vortex. After a 30 min incubation at 37°C, LPS (10 μg/ml) was added to all samples except the controls, to induce COX-2 activity *in vitro* by the addition of this pro-inflammatory stimulus, and thus induce PGE_2 production. All samples were then mixed well by gentle vortex, and re-incubated at 37°C. After 4 h, 1 ml was removed from each sample, and the triplicates pooled for leukocyte separation by centrifugation through a density gradient. Leukocyte samples were lysed in buffer[57] and subjected to protein estimation by Bradford assay, before storage at -80 °C. COX-2 protein levels of these samples were assessed by Western blotting according to standard procedures[57], using 25 μg protein per lane; then stripping and re-probing the blot for actin. 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. After a further 20 h incubation at 37°C, the remaining 0.5 ml whole blood in each tube was centrifuged and the plasma from each tube stored separately at -80°C for PGE_2 measurement by

competitive enzyme immunoassay, with limit of detection approximately 30 pg/ml plasma.

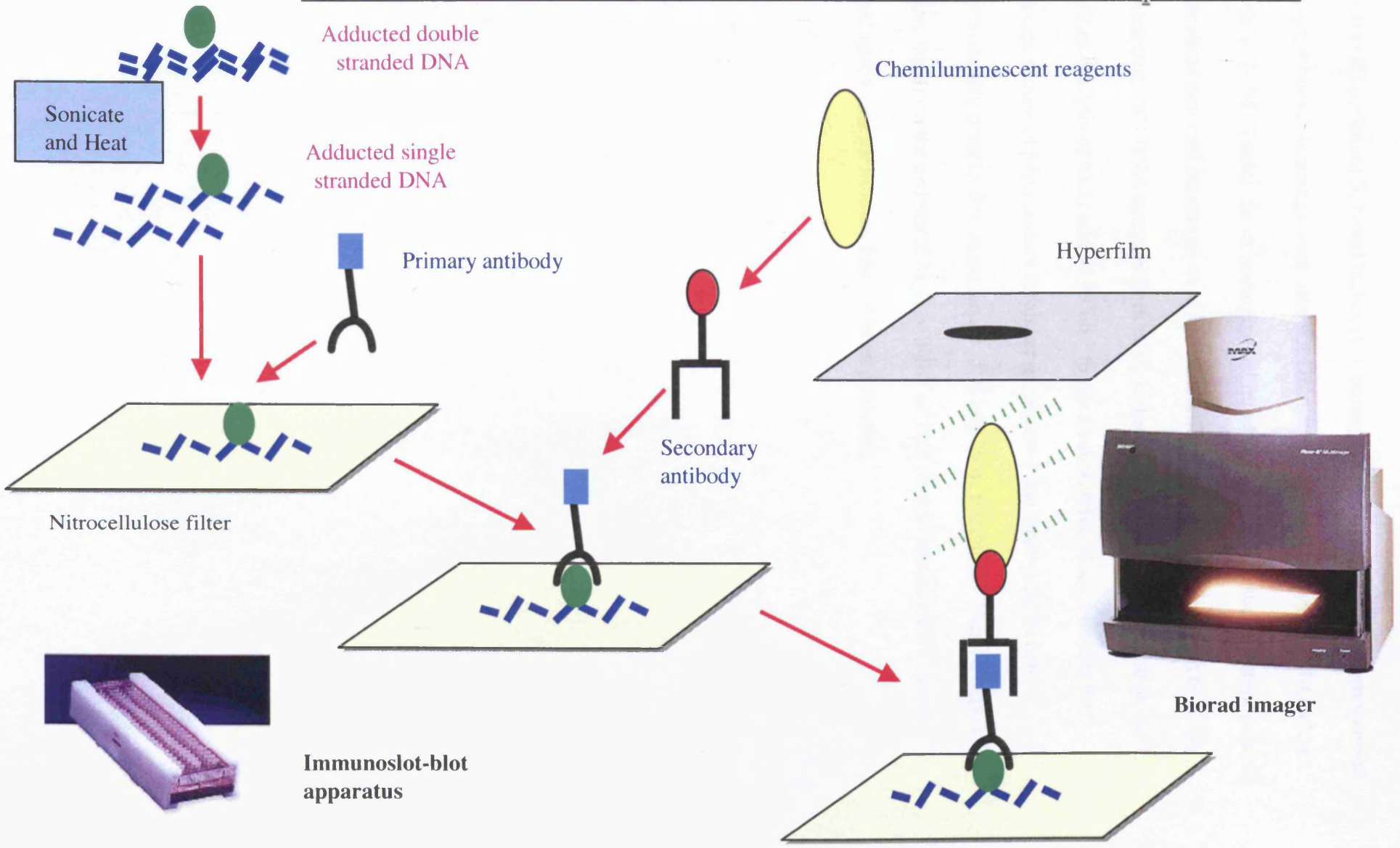
Having determined the inter-individual and intra-individual variation in blood from healthy volunteers, this method was used to compare blood samples from the screening visit and then immediately pre- and 1 h-post dose on treatment days 1, 2, 8 and 29 of the **clinical trial**. Curcumin was not added to the blood for these samples, to allow a direct comparison of the inducibility of PGE_2 production in samples taken pre- and post-dose. Although the total leukocytes obtained from 3 ml whole blood proved sufficient for satisfactory bands on Western blots for COX-2, they did not yield sufficient signal when re-probed (or initially probed) for actin, necessary for protein correction. Although practical considerations did not allow an increase in the amount of blood taken from patients, 12 ml was taken per sample from healthy volunteers, of which 10.5 ml was used for leukocyte separation. This increase in total leukocytes collected per sample yielded satisfactory blots for both COX-2 and actin proteins.

Similarly, 1 ml blood from each animal in **Rat Experiment 1** was used for the *ex vivo* assay of PGE_2 production, comparing controls against treated rats; the sample was taken on the 14th day of a diet containing 2% curcumin.

4.7 MDA AND M_1G ADDUCT LEVELS

MDA levels were assessed by colorimetric assay, with detection limit approximately 0.1 nmol/mg protein. The method principally measures the free MDA in cell lysates or homogenised tissue, without detecting base propenals[154,155]. Genomic DNA extraction was performed without addition of antioxidants, since they may give rise to artefactual formation of adducts[163]. Quantity and purity of DNA was assessed spectrophotometrically using the 260/280 nm ratio (range 1.6 to 1.9). M_1G levels were analysed by immunoslot blot, as previously described[176] using 1 μg of each sample/standard DNA in triplicate. Each blot consisted of 9 standards and 15 samples, all in triplicate. The limit of detection of the procedure was 5 adducts per 10^8 nucleotides. Discrepancies in the amount of DNA in each slot were corrected in the data analysis by staining the nitrocellulose filter with propidium iodide and performing UV light densitometry[176]. The method for the determination of M_1G adduct levels is represented in Figure 4.4.

Figure 4.4: Schematic representation of immunoslot blot procedure for measuring M_1G adduct levels



In **Rat Experiment 3**, blood leukocytes, homogenised liver tissue, and homogenised colon mucosal scrapings were used for DNA extraction and analysis of M_1G adduct levels. In **MIN mice**, fresh homogenised intestinal adenoma tissue and homogenised intestinal mucosal scrapings were used immediately for MDA estimation or DNA extraction. All DNA samples from cells, rodents and humans were stored at -80°C before analysis of M_1G adduct levels. In the **clinical trial**, blood samples for determination of M_1G adduct levels were collected one week prior to and immediately prior to dosing on days 1, 2, 8 and 29 of treatment. These timepoints were based on the estimated M_1G half-life of 12.5 days in mouse liver[177] since analogous data for humans has not been published.

4.8 STATISTICAL ANALYSES

Plots of residuals were used to ensure that variances were homogeneous, and that the residuals had a normal distribution. All results were subjected to repeated measures analysis of variance (ANOVA) or covariance, or linear regression analysis, using Minitab (version 13) software package. *Post hoc* comparisons were performed using Fisher's Exact Test or Dunnett's Test, as stated. Advice was provided by Dr. M. Festing, MRC Toxicology Unit. On account of the high degree of PGE_2 induction in blood by LPS, basal and LPS-induced PGE_2 values were analysed separately and a logarithmic transformation of all the LPS-induced values was performed. A $p < 0.05$ value was considered to be statistically significant.

CHAPTER 5

RESULTS

5.1 RELATIONSHIP OF COX-2, MDA AND M_1G IN CULTURED COLON CELLS

5.1.1 BASAL LEVELS OF COX-2, PGE_2, MDA AND M_1G

Expression of COX protein and PGE_2 production were compared with levels of intracellular MDA and M_1G adducts in five human-derived colon cell types, to test the hypothesis that COX activity is associated with formation of M_1G adducts in basal culture of cells. All the cells contained low but similar levels of COX-1 protein when corrected for protein loading (Figure 5.1). Detectable COX-2 protein was found only in HCA-7 cells and, to a slight extent, in HT29 cells under basal culture conditions (Figure 5.1). Measurable levels of M_1G adducts were detected in all five cell lines (Table 5.1) and the differences observed between SW480, HCA-7 and SW48 cells were statistically significant ($p < 0.005$ by ANOVA and Fisher's Test). DNA extracted from RKO cells by Dr J. Plastaras at Vanderbilt University, USA, was found to have M_1G adduct levels below the limit of detection of the immunoslot blot assay, compatible with levels previously described using LC/MS[159]. In the malignant cell types, MDA concentration correlated significantly with levels of M_1G adducts, although the non-malignant HCEC cells did not fit this correlation (Figure 5.2). There was no correlation of M_1G levels with COX-2 protein or PGE_2 production (Table 5.1).

Figure 5.1: COX expression in human-derived colon cell lines in basal culture, and after treatment of HCEC cells with PMA. SDS-PAGE Western blots show constitutive levels of COX-2 (72 kDa) and COX-1 (69 kDa) protein in HT29, SW480 SW48, and HCA-7 colorectal carcinoma cells, and non-malignant HCEC colon cells in basal culture; and in HCEC cells after incubation with 75nM PMA for 4 h. STD lane shows COX-2 and COX-1 standards. The blots were re-probed for α-tubulin (46 kDa) to control for equal protein loading and transfer. The results shown are typical of 3 separate experiments. For experimental details see 'Materials' and 'Methods' chapters.

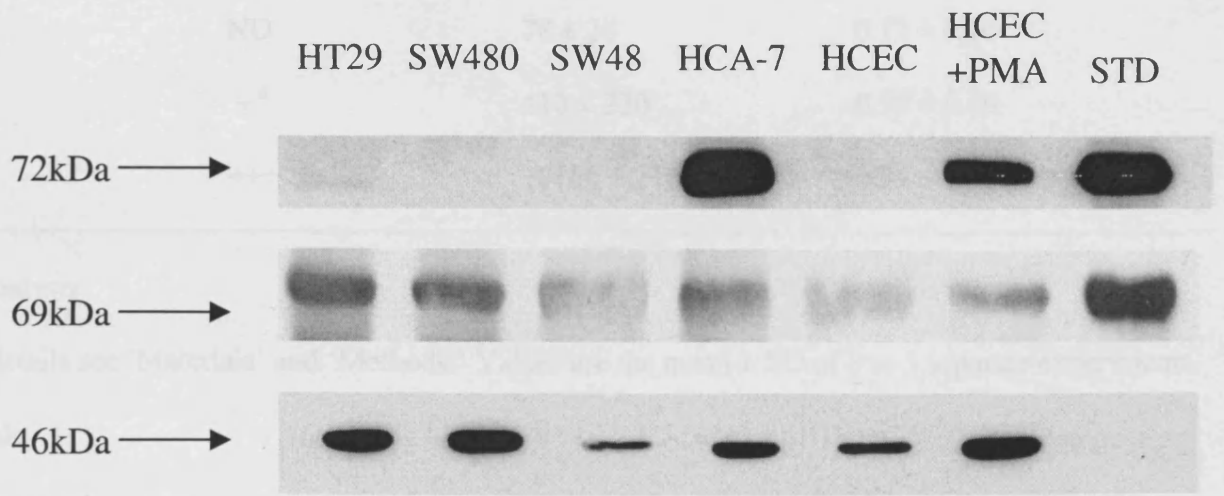


Table 5.1: Basal cellular levels of COX-2, PGE_2, MDA and M_1G adducts in five human colon cell lines.

Cell Type	COX2[a]	PGE_2 (pg/10^6 cells)[b]	MDA (nmol/mg protein)[b]	M_1G (adducts per 10^8 nucleotides)[b]
HCEC	ND[c]	31 ± 20	0.38 ± 0.12	101 ± 24
SW48	ND	122 ± 30	0.42 ± 0.18	148 ± 22
SW480	ND	78 ± 26	0.12 ± 0.10	77 ± 13
HT29	+[d]	410 ± 220	0.19 ± 0.10	94 ± 12
HCA-7	+++	10700 ± 2100	0.24 ± 0.10	123 ± 16

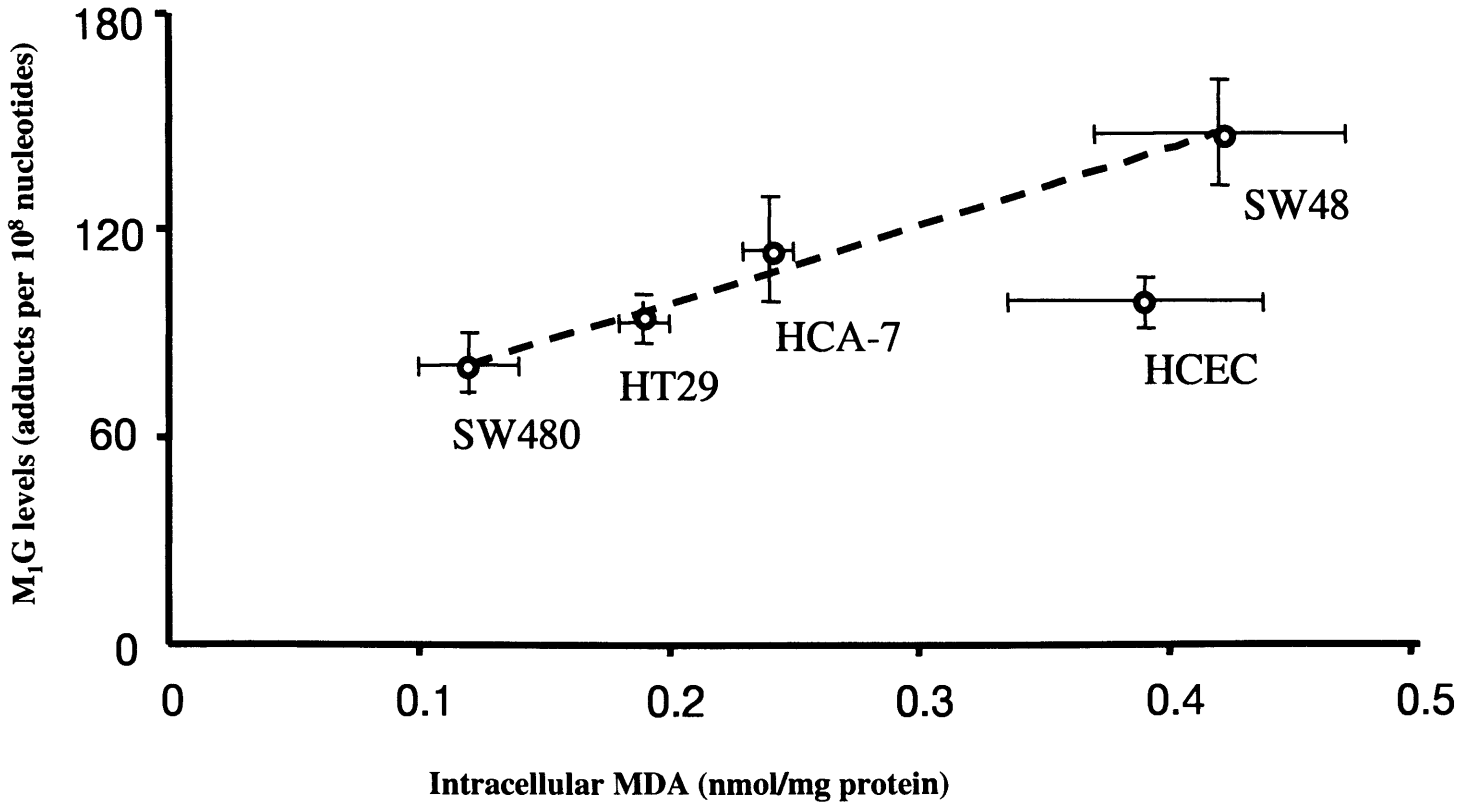
[a] By Western blot analysis

[b] For experimental details see 'Materials' and 'Methods.' Values are the mean ± SD of 3 to 5 separate experiments.

[c] ND = not detectable

[d] + detectable, +++ very strong band

Figure 5.2: Relationship between intracellular MDA and M_1G adduct levels in unstimulated culture of colon cell lines. The line of best fit ($r = 0.98$, $p < 0.001$) links values for the malignant cell types and excludes non-malignant HCEC cells. Values are the mean ± SD from 3 separate experiments. For experimental details see 'Materials' and 'Methods.'



5.1.2 PHARMACOLOGICAL MODULATION OF COX-2 AND M_1G.

COX-2 activity was induced in cells which did not constitutively express this isozyme, to look for potential changes in M_1G adducts levels. Incubation of HCEC cells with the tumour promoter PMA induced COX-2 expression (Figure 5.1) but did not affect COX-1 levels. Treatment with this phorbol ester also elicited a concomitant increase in PGE_2 production, which after 4 h was approximately 20-fold higher than control levels (Table 5.2). This increase was augmented to 50-fold in the presence of arachidonate. COX-2 induction was also accompanied by an increase in intracellular MDA, which after 4 h was approximately 3-fold over control values (Table 5.2). In contrast, M_1G adduct levels were unaffected by PMA treatment. PMA treatment of SW480 and HT29 cells also resulted in increases in COX-2, PGE_2 and MDA levels similar to those seen in HCEC cells, without significant changes in M_1G levels. Incubation of the COX-2 expressing HCA-7 cells with the selective COX-2 inhibitor NS-398 reduced PGE_2 levels from 10.7 ± 2.1 ng/10^6 cells (mean $\pm$ SD of $n = 3$) in control cells to 1.3 ± 0.6 ng/10^6cells after incubation for 24 h. Incubation of cells with NS-398 for up to 72 h did not alter levels of intracellular MDA or M_1G adducts.

Based on the findings of a study using malignant colon cells in culture[159], non-malignant HCEC colon cells were incubated with a high concentration of monomeric MDA in order to elicit formation of M_1G adducts. Incubation of HCEC cells with 1 mM NaMDA for 24 h caused an increase in M_1G levels from 79 ± 25 adducts per 10^8 nucleotides in control cells to 140 ± 34 adducts (mean $\pm$ SD of $n = 3$).

Table 5.2: Effect of treatment of HCEC cells with PMA on levels of cellular COX-2 protein, PGE_2 in the medium, intracellular MDA and M_1G adducts.

	Incubation time (h)		
	0	4	24
COX2 [a,b]	ND [c]	+++ [d]	+
PGE_2 ($pg/10^6$ cells) [b]	30 ± 16	745 ± 210	408 ± 170
MDA (nmol/mg protein) [b]	0.38 ± 0.12	1.07 ± 0.32	0.8 ± 0.36
M_1G (adducts per cell) [b]	4600 ± 670	5300 ± 820	4950 ± 1050

[a] Based on Western blot analysis

[b] For experimental details see 'Materials' and 'Methods.' Values are the mean ± SD of 3 separate experiments.

[c] ND = not detectable

[d] + detectable, +++ very strong band

5.1.3 DISCUSSION

These results describe the relationship in colon cells between MDA, M_1G adducts and COX activity, and thus contribute to our understanding of the role of COX-2 in colon cancer. They demonstrate for the first time that levels of M_1G adducts in colon cancer cells reflect constitutive intracellular levels of MDA, and confirm that M_1G levels can be increased significantly in colon cells by MDA treatment. Similar to COX-2 protein expression, there was considerable heterogeneity in constitutive M_1G adduct levels in colorectal cancer cells, ranging from below the limit of detection of the immunoslot blot assay (*i.e.* less than 5 adducts per 10^8 nucleotides) in RKO cells to 148 ± 22 adducts per 10^8 nucleotides in SW48 cells. However there appeared to be no correlation between constitutive COX-2 expression in cultured cells and levels of M_1G adducts. Similarly, inhibition and induction of COX-2 activity did not alter M_1G levels.

There are several possible explanations for this lack of association. The addition of exogenous MDA in high concentrations to non-malignant HCEC cells almost doubled M_1G adduct levels, to reach values similar to those observed constitutively in malignant SW48 and HCA-7 cells. However, this increase is not dramatic when compared to the size of changes in M_1G adduct levels in human leukocytes engendered by dietary modification. For example, mean M_1G levels in leukocytes of women were increased up to four-fold by the inclusion of polyunsaturated fatty acids in their diet[161]. The discrepancy between these findings and the relative insensitivity of M_1G levels in cells in culture towards challenge by MDA is consistent with the possibility that routes other than the enzymatic and non-enzymatic generation of MDA may be the principal sources of M_1G adducts. Little is known about such

sources, but oxidation of DNA by bleomycin has been shown to give rise to M_1G adducts *via* base propenal formation, without the involvement of lipid peroxidation[150]. The apparent insensitivity of M_1G adduct levels to changes in MDA concentration may also be related to rapid repair. M_1G adducts are susceptible to elimination by mammalian mismatch repair and nucleotide excision repair pathways[146].

Alternatively, the products of COX catalysis may be less efficiently converted to MDA in cultured cells than *in vivo*. It has been shown previously that PGH_2 can be converted to MDA by thromboxane synthases and certain cytochromes P450 (Step 2 in Figure 1.4 on page 32)[143,179]. Certain CYPs may be overexpressed in colon cancer tissue but poorly conserved in cells in culture[143]. However, breakdown of PGH_2 can also occur spontaneously, resulting in modest changes in MDA concentration. A twenty-fold increase in PGE_2 production was associated with a three-fold elevation in intracellular MDA. This result underlines the limitations of the *in vitro* model used here. The multiple pathways of MDA biotransformation *in vivo*, involving aldehyde dehydrogenases and reductases, cellular antioxidants, and glutathione S-transferase isoenzymes[180,181], may also be poorly represented in cultured cells.

These limitations, however, do not detract from three novel conclusions being made regarding the other elements of the pathway studied (Steps 1 and 3 shown in Figure 1.4). For the first time in non-malignant and malignant human colon cells, it has been demonstrated that increased COX-2 activity is associated with production of MDA. Despite increasing knowledge of alternative sources of M_1G adduct formation, basal levels of MDA correlated with those of M_1G adduct levels in

malignant colon cells grown in culture. Further to similar findings in malignant colon cells used in a previous study, exposure of non-malignant colon cells to MDA increased M_1G levels. In view of the convincing role of COX-2 in the pathogenesis of CRC[54] and of M_1G adduct levels as indicators of oxidative DNA damage[146,147], both merit investigation *in vivo* as biomarkers of colorectal carcinogenesis.

5.2 RAT COLON MUCOSA AND LIVER AS PHARMACOLOGICAL TARGETS FOR DIETARY CURCUMIN

5.2.1 INDUCTION OF HEPATIC GST BY DIETARY CURCUMIN

In **Rat Experiment 1**, animals were fed a diet supplemented with 2% curcumin for two weeks to investigate potential changes in biomarkers measured in colon mucosa, liver and blood. Based on mean food consumption per day and mean bw of the animals on day 14, the approximate daily dose ingested was 1.2 g curcumin/kg-bw. Dietary curcumin at this dose did not affect the animals' bw gain. Mean food consumption per day did not differ between the study groups. Levels of GST activity in the liver of curcumin-fed rats were elevated by 36% over those in control animals ($p < 0.01$ by ANOVA, Table 5.3). GST levels in colon mucosa and lymphocytes were marginally decreased in curcumin-fed rats, but the difference to controls was not significant. In order to study whether dietary curcumin led to altered COX-2 expression in blood cells, the concentration of PGE_2 in plasma was determined after induction with LPS added *in vitro*. Although there was 3-fold inducibility of PGE_2 production by LPS ($p < 0.005$ by ANOVA), there was no inhibition observed as a result of curcumin feeding. The inducibility in rat blood was markedly less than in human blood under the same conditions (see section 5.4.2 below).

Table 5.3: Effect of dietary curcumin[a] on GST levels in rat liver, colon mucosa and blood lymphocytes in Rat Experiment 1.

Tissue	Control	Curcumin-fed
Liver	634 ± 66 [b]	860 ± 73 *
Colon mucosa	84 ± 13	69 ± 16
Blood lymphocytes	20 ± 4	16 ± 4

[a] Animals were fed curcumin (2%) in the diet for 14 days.

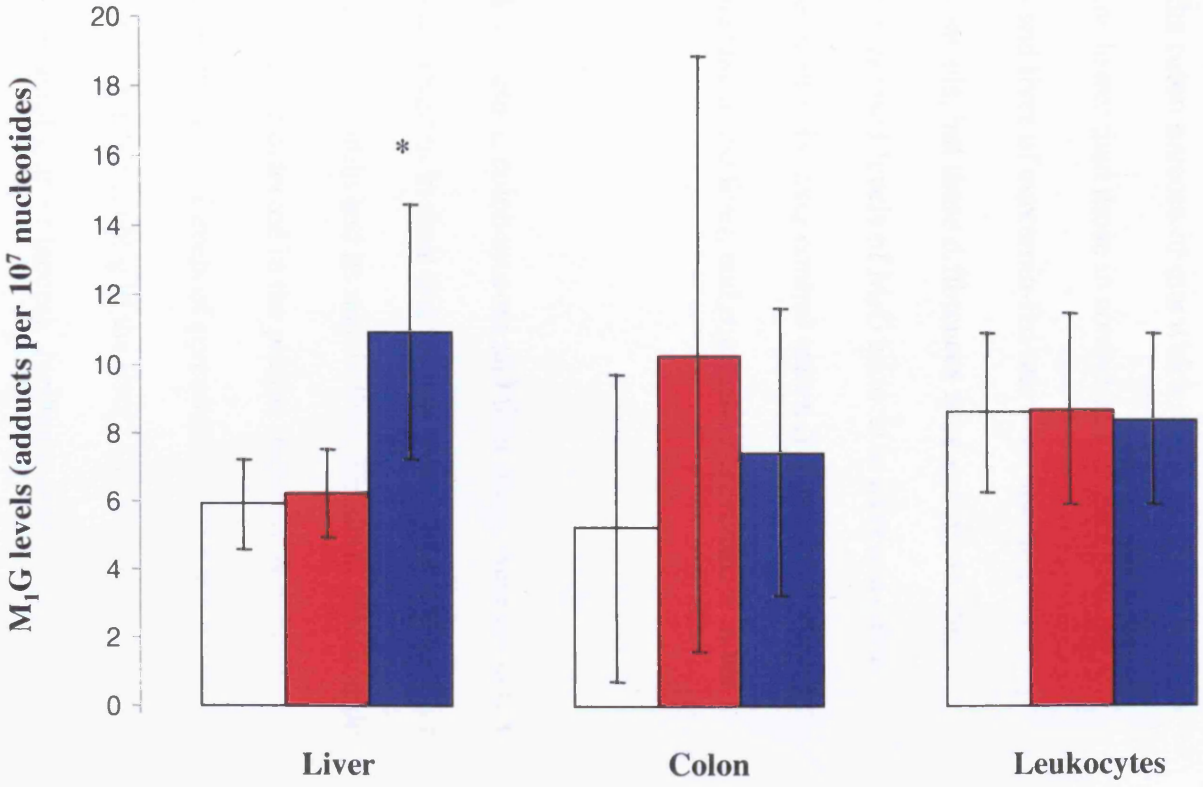
[b] Values, expressed as nmol/min/mg protein using CDNB as substrate, are the mean ± SD of 8 separate animals. For experimental details see 'Materials' and 'Methods.'

* Difference between control and treated animals is significant ($p < 0.01$, by ANOVA).

5.2.2 ALTERATION OF COLON MUCOSAL M_1G ADDUCT LEVELS

In **Rat Experiment 2**, animals ate a 10% fish oil diet for 26 days, since diets rich in poly- and di-unsaturated fatty acids have been shown previously to elevate levels of M_1G adducts in liver tissue or leukocytes[161,162]. Half the animals were fed 2.5 g/kg-bw curcumin from days 22 – 26, to study potentially preventive effects on dietary-induced changes in M_1G levels. A significant increase ($p < 0.05$ by ANOVA) in liver M_1G adducts was found for rats fed the fish oil and curcumin diet compared to the rats fed fish oil alone or control diet without addition of fish oil or curcumin (Figure 5.3). In whole homogenised colon, levels were found to be extremely variable. In preliminary experiments using control rats, greater reproducibility was found for colon mucosal scrapings than homogenised whole colon. Further experiments therefore analysed mucosal scrapings rather than homogenised whole colon. Fish oil alone did not increase levels of M_1G adducts in any of the tissues tested, compared to controls.

Figure 5.3: Average M_1G Levels for Curcumin Rat Experiment 2. Open bars denote control animals, **red** and **blue** bars animals which received dietary fish oil (10%) for 26 days, and **blue** bars animals which received curcumin (2%) for days 22 to 26 of the study diet. Values are mean ± SD of triplicate readings from 3 animals. **Star** indicates that the difference between control and treated animals is significant ($p < 0.01$ by ANOVA). For experimental details see 'Materials' and 'Methods.'

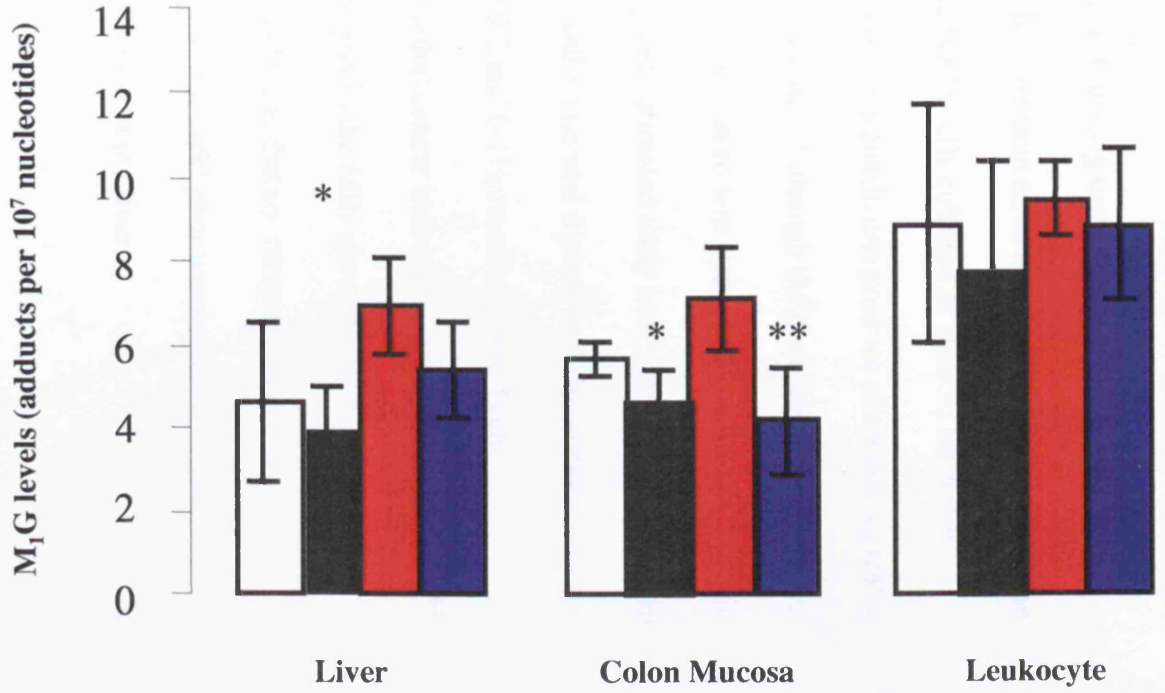


In **Rat Experiment 3**, animals were fed a diet supplemented with 2% curcumin for two weeks to investigate tissue levels of M_1G adducts. One group of rats received intragastric CCl_4 on day 10, which has been shown previously to induce lipid peroxidation and increase M_1G adduct levels in the liver[163,164]. Based on food consumption, the approximate mean daily dose ingested was 1.2 g curcumin/kg-bw. Levels of M_1G adducts in the colon mucosa of rats which received curcumin were moderately, but significantly, lower than those in control rats (Figure 5.4). M_1G adduct levels in leukocytes and liver of curcumin-fed rats were also slightly decreased as compared to controls, but these differences were not significant. Treatment of rats with CCl_4 increased levels of M_1G adducts in liver and colon mucosa by 49% and 25%, respectively, over control values (Figure 5.4). Dietary curcumin attenuated this increase in the liver, and completely prevented it in the colon.

In **Rat Experiments 1 and 3**, plasma, colon mucosa and liver tissue were collected from rats which had received curcumin in their diet for two weeks, and analysed for the presence of curcuminoids. Curcumin and its metabolites, curcumin glucuronide and curcumin sulphate, could not be detected in the plasma obtained by cardiac puncture or from the hepatic portal vein. Levels of curcumin were 1.8 ± 0.8 μmol/g tissue in the colon mucosa and 0.8 ± 0.3 nmol/g in the liver. Curcumin was also present in the faeces (8.6 ± 0.6 μmol/g dried faeces). Neither curcumin glucuronide nor curcumin sulphate was detected in tissues or faeces.

Figure 5.4: Effect of dietary curcumin on M_1G levels in liver, colon mucosa and blood leukocytes in Rat Experiment 3.

Open bars denote control animals, **black** and **blue** bars animals which received dietary curcumin (2%) for 14 days, and both **red** and **blue** bars animals which received carbon tetrachloride (0.5 ml/kg) *via* the *ig* route on the tenth day of the study diet. Values are mean ± SD of 8 animals. Stars indicate that the difference between control and curcumin-treated animals (*$p < 0.01$, by ANOVA), or between animals which received carbon tetrachloride either with or without curcumin (**$p < 0.005$, by ANOVA) is significant. For experimental details, see 'Materials' and 'Methods.'



5.2.3 DISCUSSION

Tissue Levels of Curcumin and Metabolites

To aid the interpretation of the pharmacodynamic results presented above, the chemical analytical results of an accompanying project must be considered. Curcumin has been shown to exert effects on growth, COX-2 expression and transcription factor activity in cells *in vitro* at concentrations of 5 μM or above[55-57]. The colon mucosal level of curcumin (1.8 μmol/g tissue) after 14 days feeding observed in Rat Experiments 1 and 3 is more than three hundred-fold higher than the minimal concentration shown to be active in cells cultured *in vitro*. This result demonstrates that dietary administration of curcumin can produce pharmacologically relevant drug concentrations in colon mucosa. Although the dose used in this study, 2% in the diet, has frequently been employed in rodent intervention studies[63,99,182]; it is at least ten times higher than the highest estimated daily human intake of curcumin as a dietary constituent[36] and does not reflect normal dietary use. In recent intervention studies, dietary levels of 0.2 and 0.1% curcumin have been demonstrated to protect rodents from colon cancer induced by azoxymethane[98,183,184] and to reduce numbers of intestinal polyps in the MIN mouse[29]. The pharmacokinetic results therefore intimate that dietary curcumin at doses considerably lower than that used here might yield pharmacologically efficacious levels in the colon mucosa, and perhaps also in the liver.

In Rat Experiments 1 and 3, dietary curcumin elevated hepatic GST levels, reduced colon mucosal M_1G adduct levels and decreased the elevation of M_1G adduct levels elicited by CCl_4, a powerful lipid peroxidative stimulus, in liver and colon mucosa. These potentially beneficial effects of curcumin were associated with tissue levels of

the unaltered drug of 1.8 μmol/g in the colon mucosa and 0.8 nmol/g in liver. The high concentrations of curcumin in the colon mucosa described here are consistent with the outcome of intervention studies in preclinical rodent models of colon cancer[29,63,98,99,183]. Although the decrease in M_1G levels in colon mucosa was modest, it underlines in principle that curcumin supplementation of the diet can achieve drug concentrations sufficient to decrease levels of DNA adducts formed as a corollary of oxidative DNA damage or lipid peroxidation. Curcumin glucuronide and curcumin sulphate were not found in liver or colon mucosa following dietary administration of curcumin, strongly suggesting that parent curcumin rather than either conjugate affects GST activities and M_1G adduct levels *in vivo*.

Tissue Levels of M_1G Adducts

The levels of M_1G adducts in the livers of animals in Rat Experiment 3 were approximately three times higher than those described previously in Sprague-Dawley rats,[163] and are more analogous to background levels found in normal human liver[163]. Differences may be related to the age, sex and strain of the animals used, or fat composition of the diets employed. M_1G adduct levels in rat leukocytes and colon mucosa have not been previously documented. CCl_4 has been shown to increase MDA and M_1G adduct levels in rat liver,[163,164] and increases in hepatic MDA levels have been attenuated by feeding rats 100 mg/kg curcumin for 4 weeks[185]. The results described here extend these findings, as they show that CCl_4 augments M_1G adduct levels in the colon mucosa, and that this increase can be prevented by dietary curcumin. These findings are pertinent in the light of a recent study in which oxidative DNA damage linked to lipid peroxidation was detected in biopsies from normal human colon[186]. The M_1G-lowering effect of curcumin provides a tentative

rationale for the regular use of dietary curcumin in the protection of the colon mucosa against oxidative damage, perhaps in premalignant conditions such as ulcerative colitis characterised by high levels of oxygen radicals[187].

GST Activity in Tissues

Liver GST activity was raised over controls following curcumin ingestion in the diet. The effects of curcumin on GST activity and expression are complex and may involve competitive enzyme inhibition[65] as well as indirect enzyme induction[188]. The finding in Rat Experiment 1 that 14 days of dietary curcumin induces GST activity in the liver is consistent with an earlier study of the same dose of curcumin in diet and water fed to 8-week old mice[63]. Similarly, mice that received curcumin dissolved in aqueous sodium carboxymethylcellulose *via ig* gavage for 15 days at a dose approximately one fifth of that used here were found to have significantly higher GST levels in liver compared to controls[64]. In contrast, a more recent study of 8-week old Sprague Dawley rats found that curcumin dissolved in corn oil given daily for 14 days *via* the *ig* route at various doses failed to induce hepatic GST at doses above 6% of the dietary dose used here[65]. This discrepancy may relate to differences between the studies in rodent species and strain, age of the animals, dose of curcumin and its route of administration. Compatible with earlier rodent studies of dietary curcumin[63,66], no significant alteration was detected in GST activity of colon mucosal tissue.

COX-2 Activity in Blood

In cells cultured *in vitro,* it has been shown previously that curcumin inhibits COX-2 expression by a mechanism involving interference with NF-κB activation and

inhibition of the IκB kinase complex[57]. In Rat Experiment 1, PGE_2 production, induced *ex vivo* as an indicator of leukocyte COX-2 activity, was not affected by dietary curcumin. The fact that curcumin administered in the diet did not affect COX-2 inducibility, GST activity or M_1G levels in rat blood leukocytes is consistent with the finding that this mode of administration yielded extremely low levels of parent curcumin in the plasma.

Summary

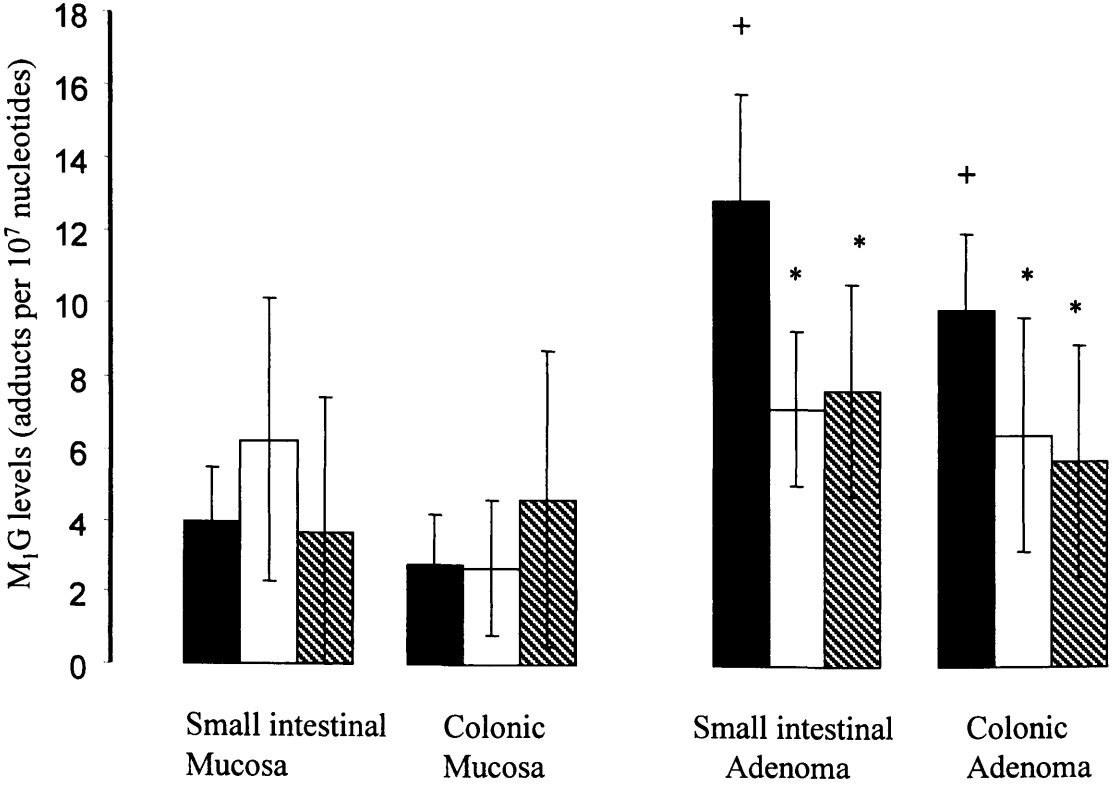
In conclusion, the results presented in this part of the project show that dietary administration of curcumin to rats produces pharmacologically active levels of unmetabolized curcumin in the colon mucosa and liver, capable of decreasing M_1G levels and elevating GST activity. These effects may be of value in cancer chemoprevention. Dietary admixture may be the preferable mode of administration for curcumin if used to prevent colon cancer. The chemopreventive efficacy of oral curcumin in the colorectum and the liver merits clinical evaluation.

5.3 SUPPRESSION OF M_1G LEVELS IN INTESTINAL ADENOMAS OF MIN MICE BY DIETARY CURCUMIN

5.3.1 MDA AND M_1G LEVELS IN ADENOMAS OF MIN MICE

It has been reported previously that COX-2 levels are elevated in adenomas compared to intestinal epithelium[28,137,138]. The hypothesis tested in this part of the project was that adenoma levels of MDA and M_1G adducts are elevated in association with raised COX-2 activity. Despite the potential link between COX-2 and MDA[143,179], the intestinal adenoma levels of MDA (mean 2.1 ± 0.9 nmol/mg protein, $n = 8$) were not significantly higher than those of normal mucosa (mean 0.9 ± 0.9 nmol/mg protein). In contrast, M_1G adduct levels were three-fold higher in adenoma tissue than intestinal mucosa (see Figure 5.5).

Figure 5.5: Levels of M_1G adducts in normal mucosa and intestinal adenomas in Min/+ mice. Values are mean ± SD of at least 8 animals showing controls (closed bars) and animals fed 0.1% (open bars) and 0.2% (hatched bars ▨) dietary curcumin. Crosses (+) indicate that adenoma values are significantly different from mucosal values for control animals (by ANOVA, $p < 0.001$). Stars (*) indicate that adenoma values of curcumin-fed animals are significantly different from adenoma values for control animals (by ANOVA, $p < 0.01$). For experimental details, see 'Materials' and 'Methods' chapters.



5.3.2 EFFECT OF DIETARY CURCUMIN ON ADENOMA M_1G LEVELS.

MIN/+ mice were fed a diet supplemented with either 0.1 or 0.2% curcumin for 14 weeks, to investigate potential changes in mucosal and adenoma levels of M_1G adducts in the small intestine and colon. Based on daily food consumption and the mean body weights, the approximate dose ingested by mice fed the 0.2% curcumin diet was approximately 300 mg curcumin/kg-bw. Dietary curcumin at this dose did not affect the animals' bw gain. Levels of M_1G adducts in intestinal mucosa of mice which consumed curcumin were not altered significantly compared to control animals, but adenoma levels were significantly lower (Figure 5.5) than those in control rats ($p = 0.006$ by ANOVA). Adenoma M_1G adduct levels in mice fed 0.1 and 0.2 % curcumin diets did not differ significantly.

In a project accompanying the work described here, HPLC analysis was performed on extracted samples of intestinal mucosa. In mice fed a 0.2% curcumin diet, the concentrations of curcumin in intestinal and colon mucosa were 507 ± 86 and 111 ± 23 nmol/g tissue respectively. A trace of curcumin sulphate was found in these tissues, but no other metabolites were detected.

5.3.3 DISCUSSION

These results allow four novel conclusions, relevant to the chemoprevention of colorectal cancer: i) M_1G adduct levels are significantly elevated in premalignant lesions in the MIN mouse model; ii) this effect does not appear to be mediated by tissue MDA concentration; iii) dietary curcumin can attenuate the high level of M_1G adducts in intestinal adenomas; and iv) this attenuation is likely to be attributable to curcumin rather than its metabolites or other curcuminoids.

The M_1G adduct levels of intestinal adenomas from control MIN mice used in this study are in the range found for normal human tissue and leukocytes[146,161,163]. We also measured liver M_1G levels in these animals, and found a lower level (about 2 adducts per 10^7 nucleotides) consistent with liver adduct levels found in F344 rats shown on Figures 5.3 and 5.4. The lack of association between adenoma M_1G adduct levels and MDA concentration is in accordance with the findings *in vitro* of Marnett and colleagues[150], which suggest that certain products of oxidative DNA damage may contribute to intracellular M_1G adduct formation to a greater extent than MDA itself. Base propenals are examples of such products that may mediate adduct formation[150]. Evidence for the formation of mutagenic M_1G adducts by base propenals, in the absence of MDA and lipid peroxidation, has been found by treating calf thymus DNA with propenal standards, measuring adduct formation by immunoslot blot and studying mutagenicity in a bacterial model[189]. Understanding the relative contribution of LPO and COX catalysis (both resulting in MDA) *versus* oxidative DNA damage (involving base propenals) in premalignant lesions is an important area of investigation in view of the high adenoma levels of M_1G detected in the study described here.

In contrast to Rat Experiments 1 and 3 described in the previous section, the doses used in the mouse studies described here, 0.1 and 0.2% of the diet, are more reflective of estimated daily human intake of curcumin as a dietary constituent[36]. In recent intervention studies, these dietary levels of curcumin have been shown to protect rodents from colon cancer induced by azoxymethane[98,183,184] and to reduce polyp numbers in the MIN mouse[29]. In an extension to the project described here,

MIN/+ mice consuming 0.2% dietary curcumin were found to contain significantly less intestinal adenomas than controls, whereas those consuming the 0.1% diet did not[c]. Indeed, the significant reduction measured in adenoma M_1G relative to controls at a dose of oral curcumin that did not prove efficacious at reducing polyp number raises the possibility that this molecular biomarker may offer greater sensitivity than the macroscopic measures currently used by the US Food and Drug Administration in the licensing of cancer chemopreventive drugs for patients with FAP (discussed in Appendix 2B).

Although there exists evidence for intestinal metabolism of curcumin in the rat and by human tissues *in vitro*[42,44,190], the results of the study described here suggest that at doses comparable to those in human diets, biological effects in the intestinal mucosa of this mouse model are likely to be due to unaltered curcumin rather than its metabolites. This is compatible with the results of studies of the COX-2 and NF-κB inhibiting potentials of curcumin *versus* its glucuronide and sulphate metabolites or its reduction products[45,191]. The high level of M_1G adducts in MIN mouse adenoma tissue appear susceptible to modulation by dietary curcumin at the doses used here, unlike the lower background intestinal mucosal level.

In summary, the results presented above show that dietary administration of curcumin to MIN mice produces high levels of unmetabolised curcumin in the intestinal mucosa, and is capable of decreasing M_1G levels in adenoma tissue. The cancer chemopreventive efficacy of oral curcumin in the intestinal tract of patients with premalignant gastrointestinal lesions merits evaluation. Investigation of the

[c] S. Perkins, unpublished data

clinical relevance of M_1G adducts to carcinogenesis is indicated, with a view to consideration of its use as a biomarker of drug efficacy in clinical trials of antioxidant chemopreventive agents such as curcumin.

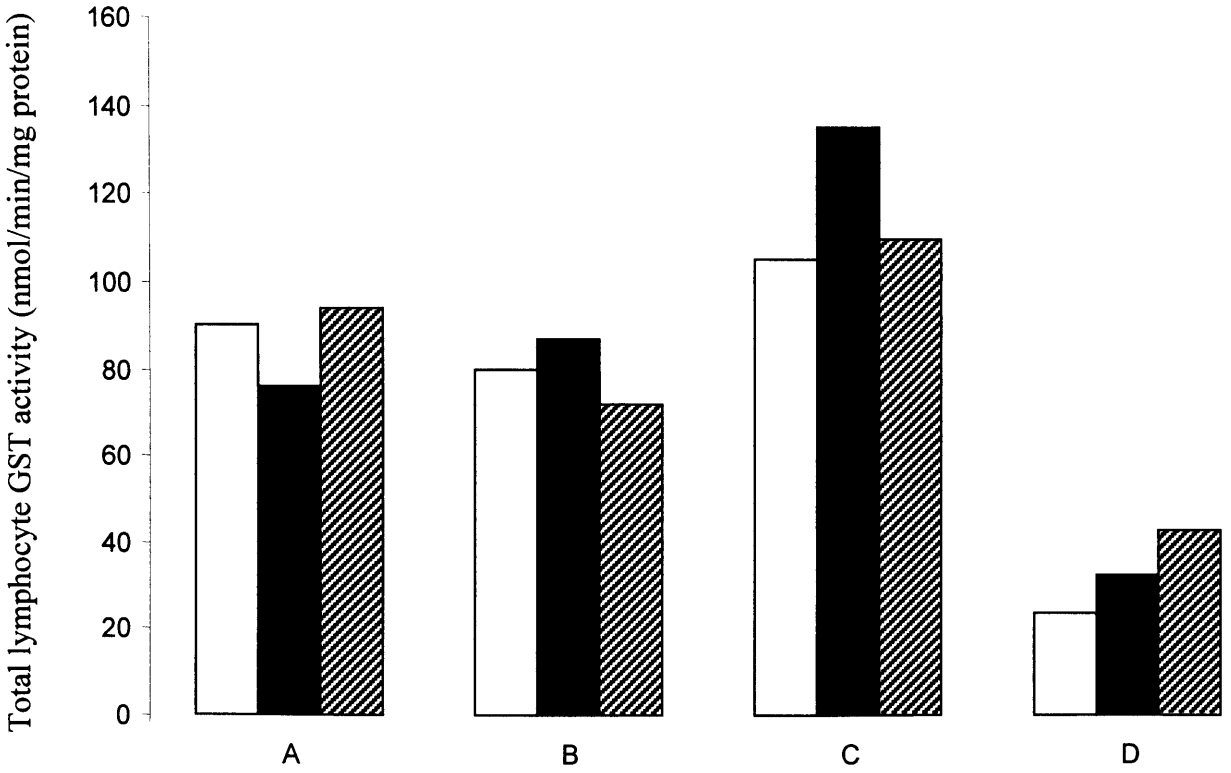
5.4 REPRODUCIBILITY OF GST AND CURCUMIN'S INHIBITION OF COX-2 ACTIVITY IN HUMAN BLOOD LEUKOCYTES

This section describes the results of experiments performed using blood from healthy volunteers, to assess the reproducibility of the biomarkers selected for the clinical trial and mechanistic effects of adding curcumin to blood *in vitro*.

5.4.1 LYMPHOCYTE GST LEVELS

In concordance with published papers[14,18], the results shown in Figure 5.6 suggested that lymphocytic total GST activity in human blood samples obtained 4 – 6 weeks apart from normal healthy individuals, showed a high degree of inter-individual variability (from 25 to 150 nmol/min/mg protein), but less intra-individual variability (less than 10 nmol/min/mg protein).

Figure 5.6: Total lymphocyte GST activity for human volunteers. Four volunteers (A, B, C and D) were bled at zero timepoint (open bars) and then 2 weeks (closed bars) and 4 weeks later (hatched bars ▨). The pooled SD, including all data points, is 10.1 nmol/min/mg protein. For experimental details see 'Materials' and 'Methods' chapters.



5.4.2 COX-2 AND PGE_2 LEVELS IN BLOOD FOLLOWING ADDITION OF CURCUMIN

In whole blood from different individuals on the same day, the inducibility of PGE_2 concentration in a 24 h incubation with LPS varied from approximately 25- to 75-fold (see Table 5.4). Reasonable reproducibility was found in samples taken from four individuals, taken 1 h apart each and performed in triplicate. Pre- and 1 h post-dose values were therefore used for comparisons in the trial samples. Each experiment was analysed on a single or at most two plates, and an internal standard was included on every plate analysed. Variation for repeat analyses of the same sample was less than 10%.

Table 5.4: Variability in basal and LPS-induced PGE_2 concentrations of plasma. PGE_2 concentration was measured at 24 h after treatment of blood from 4 healthy volunteers (mean ± SD of at least 3 readings per timepoint). All measurements shown are ng PGE_2 per ml plasma. **B** represents blood taken 1 h later than **A** from the same volunteer; both samples treated immediately. For experimental details see 'Materials' and 'Methods' chapters.

	Basal	Basal	LPS-induced	LPS-induced
	A	**B**	**A**	**B**
Volunteer 1	2.97 ± 0.29	2.19 ± 0.29	76.57 ± 4.22	131.50 ± 5.39
Volunteer 2	1.88 ± 0.03	2.28 ± 0.18	80.02 ± 8.63	99.02 ± 9.39
Volunteer 3	1.77 ± 0.45	2.90 ± 0.87	134.29 ± 16.49	130.32 ± 9.41
Volunteer 4	2.38 ± 0.56	2.03 ± 0.68	163.48 ± 6.32	144.35 ± 3.69

The hypothesis that curcumin alters stimulated PGE_2 production, as a measure of monocyte COX-2 activity[139], was tested. To that end, whole blood was incubated for 24 h with LPS (10 μg/ml) following a 30 min pre-treatment with curcumin (1 – 20 μM). As shown in Figure 5.7A, addition of LPS caused a marked increase in plasma PGE_2 level ($p < 0.001$ by ANOVA), compared to basal control values (mean 0.73 ± 0.06 ng/ml, $n = 6$). Pre-treatment with 1 μM curcumin caused a 24% inhibition of PGE_2 induction ($p < 0.05$ by ANOVA), relative to DMSO controls (mean 26.28 ± 6.10 ng/ml, $n = 3$), but the extent of this inhibition was reduced at 5 μM curcumin and abolished at 10 μM curcumin. In order to test the hypothesis that essential oils derived from *Curcuma spp.* could alter the ability of curcumin to modulate COX-2 activity, P54FP, a standardised formulation of *Curcuma* extract, was added to whole blood to give curcumin concentrations equivalent to those used in the experiments with curcumin. In addition to curcuminoids, these incubates contained ten-fold higher concentrations of *Curcuma* essential oils (see Chapter 4, section 4.3). The same degree of inhibition was observed (Figure 5.7A), with no enhancement of COX-2 inhibition over curcumin alone. For comparison, experiments were also performed with the selective COX-2 inhibitor NS-398. Pre-treatment with NS-398 (10 μM) caused 94% inhibition of LPS-mediated PGE_2 production, suggesting that changes in PGE_2 concentration were COX-2-mediated.

In order to make the results more relevant to the physiological concentrations of LPS observed in inflammation and cancer, parallel experiments were performed using 100-fold lower concentrations of LPS as a novel modification of the published method. Pre-treatment with 1 and 5 μM curcumin caused 41 and 22 % inhibition of

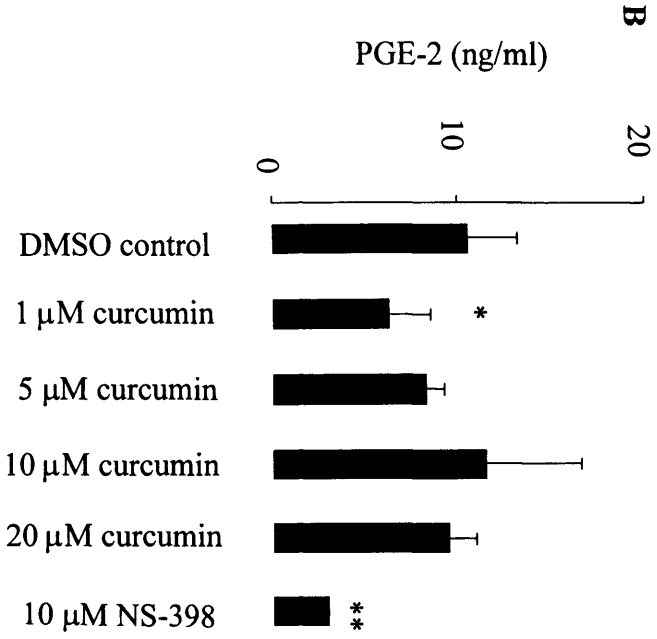
PGE_2 induction respectively ($p < 0.05$ by ANOVA for 1 μM curcumin), as shown in Figure 5.7B. Addition of equivalent concentrations of *Curcuma* essential oils, the non-curcuminoid constituents of P54FP, without the curcuminoid components, did not cause inhibition in PGE_2 production. Experiments studying the effect submicromolar concentrations of curcumin were limited by the inhibitory activity of the DMSO solvent used in this model system, and alternative solvents for curcumin were found to cause excessive leukocyte lysis.

In order to determine whether changes in the production of PGE_2 correlated with changes in COX-2 protein levels, the latter was measured in leukocytes by Western blot. Although undetectable in the absence of LPS, incubation of whole blood for 4 h with LPS (10 μg/ml) caused a marked induction of COX-2 protein (Figure 5.8). Pre-treatment with 1 μM curcumin caused a reproducible inhibition of this induction by approximately 24% relative to controls. Inhibition of COX-2 protein levels was not observed at higher curcumin concentrations. Incubation with NS-398 (10 μM) caused approximately 25% inhibition of LPS-mediated COX-2 protein induction, and it is conceivable that this effect may have been related to decreased PGE_2 concentrations which in turn downregulated COX-2 protein expression[192].

Figure 5.7: Effect of P54FP or curcumin on LPS-induced plasma PGE_2 levels.

Whole blood from healthy volunteers was incubated for 24 h in the presence of LPS (10 μg/ml in **A** and 0.1 μg/ml in **B**) following a 30 min pre-treatment with P54FP (**A**) or curcumin (**B**) at concentrations defined by curcumin content (shown on column labels). All results are the mean ± SD of 3 separate experiments. Stars indicate that difference to control values is significant (* $p < 0.05$, ** $p < 0.001$ as assessed by ANOVA and *post hoc* Dunnett's comparison).

For details of PGE_2 measurement, see 'Materials' and 'Methods' chapters.


B
PGE-2 (ng/ml)
0
10
20
DMSO control
1 μM curcumin
5 μM curcumin
10 μM curcumin
20 μM curcumin
10 μM NS-398
*
**

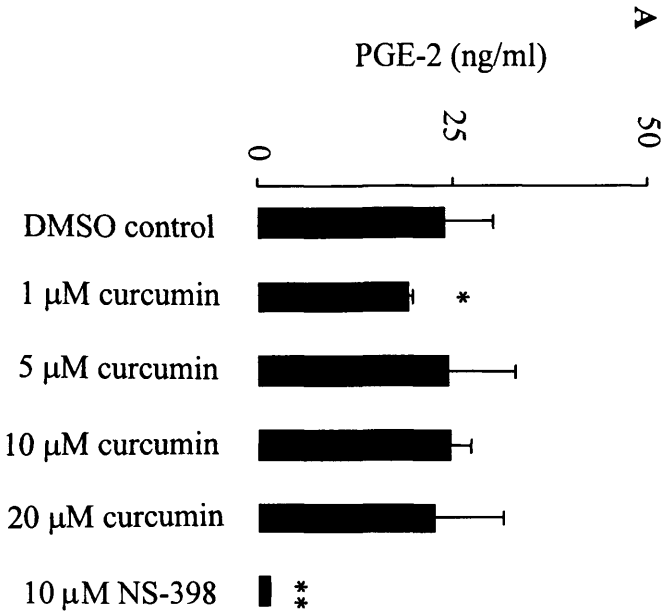
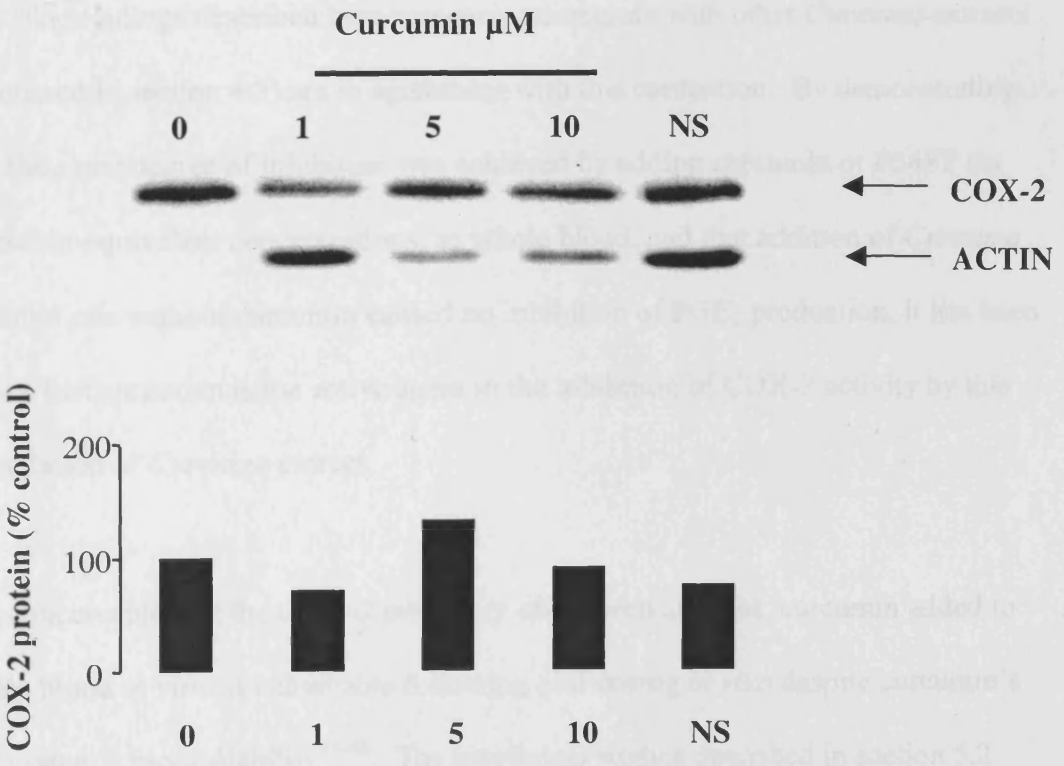

A
PGE-2 (ng/ml)
0
25
50
DMSO control
1 μM curcumin
5 μM curcumin
10 μM curcumin
20 μM curcumin
10 μM NS-398
*
**

Figure 5.8: Effect of curcumin on LPS-induced COX-2 protein levels in leukocytes. Whole blood from healthy volunteers was incubated for 3 h in the presence of LPS (10 μg/ml) following a 30 min pre-treatment with curcumin, prior to analysis of COX-2 protein in leukocytes. From left to right: Lane 1, DMSO control; lane 2, curcumin 1 μM; lane 3, curcumin 5 μM; lane 4, curcumin 10 μM; lane 5, NS-398 (10 μM). The Western blot is representative of two independent experiments. Bar chart shows means from both experiments, based on densitometry of COX-2 bands from Western blots normalised to actin. For experimental details see 'Materials' and 'Methods' chapters.



5.4.3 DISCUSSION

COX-2 is an important pharmacological target for NSAIDs, selective COX-2 inhibitors and polyphenolic agents derived from the diet (see Appendices 2C and 2D). Since COX-2 appears to play a pathogenic role in the carcinogenesis of many tissues[54], its pharmacological modulation has implications for cancer prevention and treatment. The results outlined above demonstrate that curcumin inhibits leukocyte COX-2 gene induction and concomitant PGE_2 production when added to human blood *in vitro*, and they suggest that measurement of this activity can be incorporated as a biomarker of drug efficacy in clinical trials.

Since its chemical structure was first characterised almost a century ago[37], curcumin has generally been considered the most active constituent derived from *Curcuma spp.* The findings described here comparing curcumin with other *Curcuma* extracts (discussed in section 4.3) are in agreement with this contention. By demonstrating that the same degree of inhibition was achieved by adding curcumin or P54FP (in curcumin-equivalent concentrations) to whole blood, and that addition of *Curcuma* essential oils without curcumin caused no inhibition of PGE_2 production, it has been shown that curcumin is the active agent in the inhibition of COX-2 activity by this formulation of *Curcuma* extract.

It is conceivable that the COX-2 inhibitory effect seen at 1 μM curcumin added to whole blood *in vitro* is achievable following oral dosing *in vivo* despite curcumin's low systemic bioavailability[45-48]. The preclinical studies described in section 5.2 suggest that curcumin concentrations in the 1-10 nmol/g range are attainable in rat colon mucosa following dietary admixture of powdered curcumin. Moreover, the

pharmacokinetic extensions of these studies (see Appendix 2E) suggest that curcumin's systemic bioavailability may be increased by an improved oral formulation, such as use of an amphiphilic solvent.

There are at least two possible explanations for the apparent lack of significant inhibitory activity on PGE_2 production at curcumin concentrations above 1 μM. Firstly, it has been shown previously that curcumin at 5 μM and above induces significant apoptosis in human colon cells[57,89] and blood neutrophils[193], both grown *in vitro*. The apoptotic process is associated with an increase in PGE_2 production[194] and cell lysis would result in increased release of PGE_2 into plasma, thus masking any decrease in PGE_2 production elicited by curcumin. On account of positive regulation of COX-2 transcription in non-apoptotic cells by PGE_2 released into the plasma by lysed cells[192], COX-2 protein levels in leukocytes may show the same trend as PGE_2 levels. Another possible explanation for lack of significant inhibition of COX-2 activity at higher curcumin concentrations relates to this compound's antioxidant properties[40]. Curcumin's inhibition of COX-2 transcription may be related to its potent scavenging of reactive oxygen species, known to activate NF-κB [195] and perhaps other elements upstream of COX-2 transcription. In one study of strand cleavage of phage DNA[73], curcumin (100 μM) has been shown to possess predominantly pro-oxidant activity, and it is not known at what lower threshold or under what physiological conditions this compound starts to show paradoxical pro-oxidant activity, with reduced capacity to scavenge reactive oxygen species.

Although the inhibition of LPS-induced COX-2 activity by 1 μM curcumin observed in this study was markedly less than that observed following incubation with 10 μM

NS-398, the diet-derived polyphenol may be the more 'innocuous' of the two potential cancer chemopreventive agents. The advancement of cancer chemoprevention requires the clinical development of drugs with a high therapeutic index, despite potential toxicity, for use in individuals at high risk of developing certain malignancies; it also requires development of innocuous agents for potential use more widely in low risk subjects[196]. Toxicity associated with NSAIDs may override the potential benefit from causing regression of colorectal adenomas[197], and the clinical side-effect profile of selective COX-2 inhibitors currently appears similar to that of traditional NSAIDs, although peptic ulceration is less likely than for the conventional agents[198]. Comprehensive data on the toxicity of oral administration of pure curcumin to humans is lacking, but early indications[47-50,199] and its history of dietary use as turmeric[36] would suggest that it is likely to be safe.

In conclusion, since COX-2 is an important target for cancer chemopreventive and chemotherapeutic drugs, the assessment of its pharmacological modulation *in vivo* or *ex vivo* may be a useful biomarker of efficacy. The finding that curcumin inhibits basal and LPS-induced COX-2 activity in human blood illustrates its potential for systemic efficacy at plasma concentrations as low as 1 μM. It is suggested that measurement of leukocyte COX-2 expression and PGE_2 production, as a measure of COX-2 activity, may be a useful and feasible proposition in future trials of curcumin and other transcriptional inhibitors of the isozyme.

5.5 EFFECTS OF ORAL *CURCUMA* EXTRACT IN PATIENTS WITH ADVANCED COLORECTAL CANCER

5.5.1 TOLERABILITY OF TREATMENT

Patients with advanced colorectal cancer ingested P54FP capsules once daily for up to 4 months at doses between 440 and 2200 mg *Curcuma* extract, containing between 36 and 180 mg curcumin. The treatment was well tolerated at all dose levels, and there was no dose-limiting toxicity. Two types of adverse events, both gastrointestinal, were possibly related to *Curcuma* consumption. One patient on 1320 mg *Curcuma* extract daily experienced nausea during the first month of treatment (NCI toxicity grade 1), which resolved spontaneously despite continuation of treatment. Two patients, on 880 and 2200 mg *Curcuma* extract daily, developed diarrhoea (NCI grades 2 and 1, respectively) four months and one month into treatment, respectively. Both patients withdrew from the study before the cause of the diarrhoea could be investigated.

5.5.2 PRE-TREATMENT GST AND M_1G LEVELS IN RELATION TO *GST* POLYMORPHISMS.

Leukocyte M_1G levels and lymphocyte total GST activity differed substantially between patients (Figures 5.9 and 5.10). Patients were genotyped for GST isoenzymes *GSTM1*, *GSTP1* and *GSTT1*. Two thirds of the patients lacked *GSTM1*, slightly more than the 40 - 60% proportion expected in healthy caucasians[112], with an even distribution across the five dose levels (Table 5.5). In patients who displayed null genotype for *GSTM1*, pre-treatment levels of leukocyte M_1G were 7.6 ± 4.3 per

10^7 nucleotides, 74% higher than those in patients expressing *GSTM1*, in whom adduct levels were 4.3 ± 2.6 per 10^7 nucleotides ($p < 0.001$ by ANOVA). Two patients were null for *GSTT1*. Their pre-treatment levels of leukocyte M_1G (mean levels pooling triplicate readings from both time points, 5.8 ± 1.6 adducts per 10^7 nucleotides) were marginally lower than those in patients expressing *GSTT1* (6.6 ± 4.3 adducts per 10^7 nucleotides; $p = 0.02$ by ANOVA). Leukocyte levels of M_1G did not correlate with total GST activity, active smoking status ($n = 2$), vegetarianism ($n = 2$) or age.

5.5.3 BIOLOGICAL EFFECTS OF ORAL *CURCUMA* EXTRACT.

In patients taking 440 mg *Curcuma* extract (36 mg curcumin) daily, lymphocyte GST activity decreased gradually with time from a pre-treatment mean GST value of 64 ± 19 nmol/min/mg protein (measured in triplicate at two timepoints one week apart for each patient) to 26 ± 13 nmol/min/mg protein on day 29 of *Curcuma* consumption ($p < 0.001$ by ANOVA, Figure 5.10). This decline was not observed at the higher dose levels. Retrospective subset analysis revealed that pre-treatment lymphocyte GST levels (53 ± 10 nmol/min/mg protein) in the 5 patients who subsequently exhibited stable disease radiologically for more than 3 months of treatment, were lower ($p = 0.001$ by ANOVA) than those in the other 10 patients (84 ± 25 nmol/min/mg protein). Leukocyte M_1G levels varied considerably between patients, with no significant difference between subsets of patients as defined by *GST* polymorphisms or radiological criteria. Consumption of *Curcuma* extract did not affect M_1G levels (Figure 5.9).

Figure 5.9: Leukocyte M_1G adduct levels for patients on *Curcuma* extract over a 5-week period. Each line represents one patient, each point is the mean of 3 - 5 readings from one sample taken pre-dose on each day (shown relative to first dose on day 1). For experimental details, see Materials and Methods chapters. The inter-patient variation is significant ($p < 0.001$ by ANOVA), but there is no significant intra-patient change with time. The pooled SD, including all data points, is 5.4 adducts per 10^7 nucleotides.

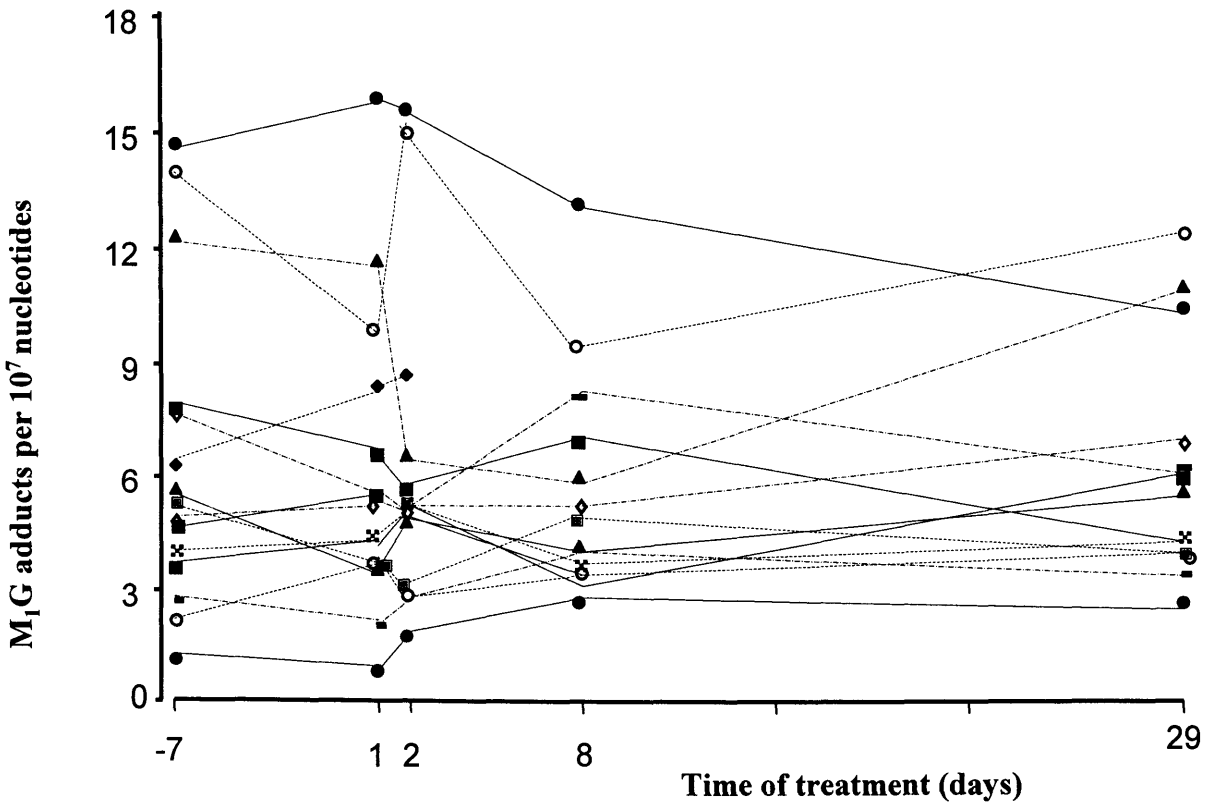


Figure 5.10: Mean lymphocyte GST levels in the clinical trial.

Patients ingested the following daily doses of *Curcuma* extract: 440 (closed circles •), 880 (concentric squares ◙), 1320 (diamonds ◆), 1760 (closed squares ▪) and 2200 mg (open circles o), containing 36, 72, 108, 144 and 180 mg curcumin, respectively. Each point represents means of values for 3 patients, calculated from triplicate readings for 2 samples per patient per day (immediately pre-dose and 1 h post-dose). Time is relative to first dose on day 1. The pooled SD, including all data points, is 10.9 nmol/min/mg protein.

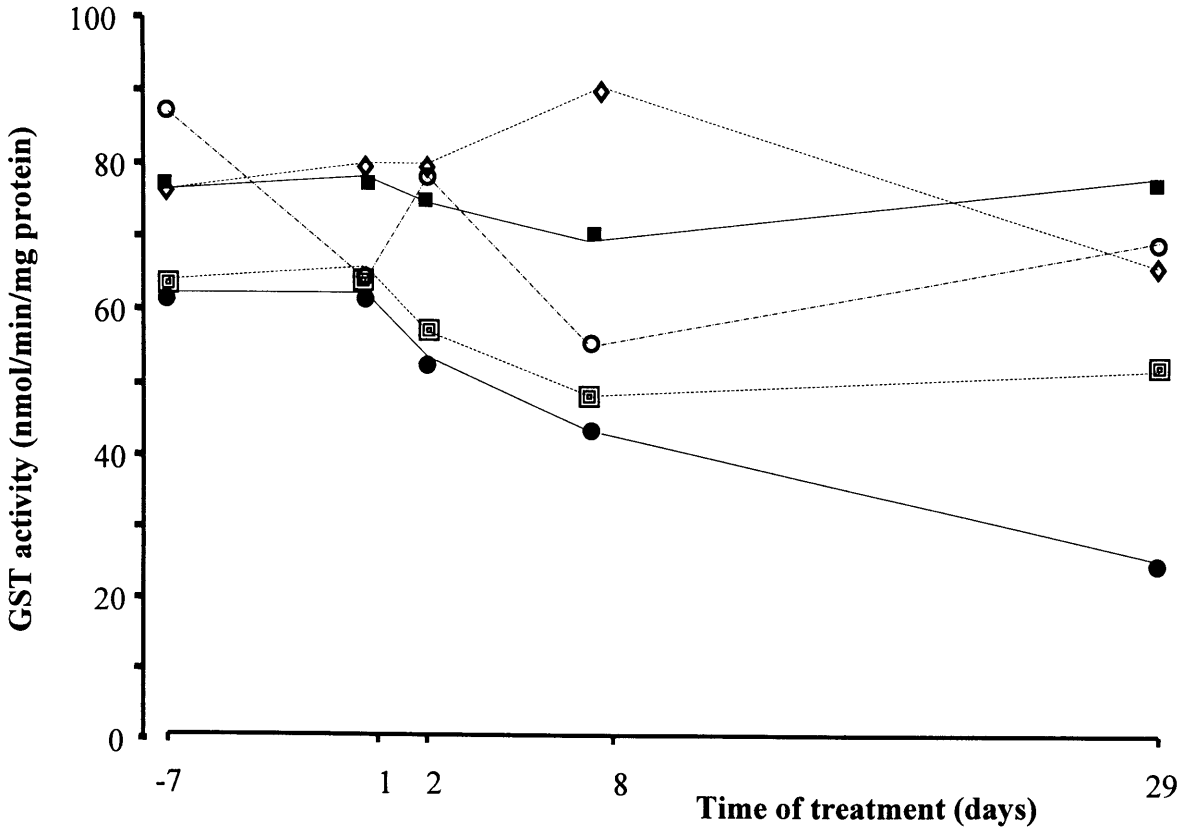

100
80
60
40
20
0
GST activity (nmol/min/mg protein)
-7
1
2
8
29
Time of treatment (days)

Table 5.5: *GST* Polymorphisms for 15 patients recruited.

Numbers represent the total number of patients from each dose level ($n = 3$). The dose level is shown by daily dose of total *Curcuma* extract (mg). For experimental details see 'Materials' and 'Methods.'

Dose Level	*GSTP1* Wild Type	*GSTP1* Heterozygous	*GSTP1* Mutant	*GSTM1* Null	*GSTT1* Null
440	1	2	0	2	1
880	1	2	0	1	1
1320	1	1	1	2	0
1760	1	2	0	2	0
2200	1	1	1	3	0

5.5.4 EFFECTS ON PGE_2 INDUCTION IN BLOOD FROM PATIENTS

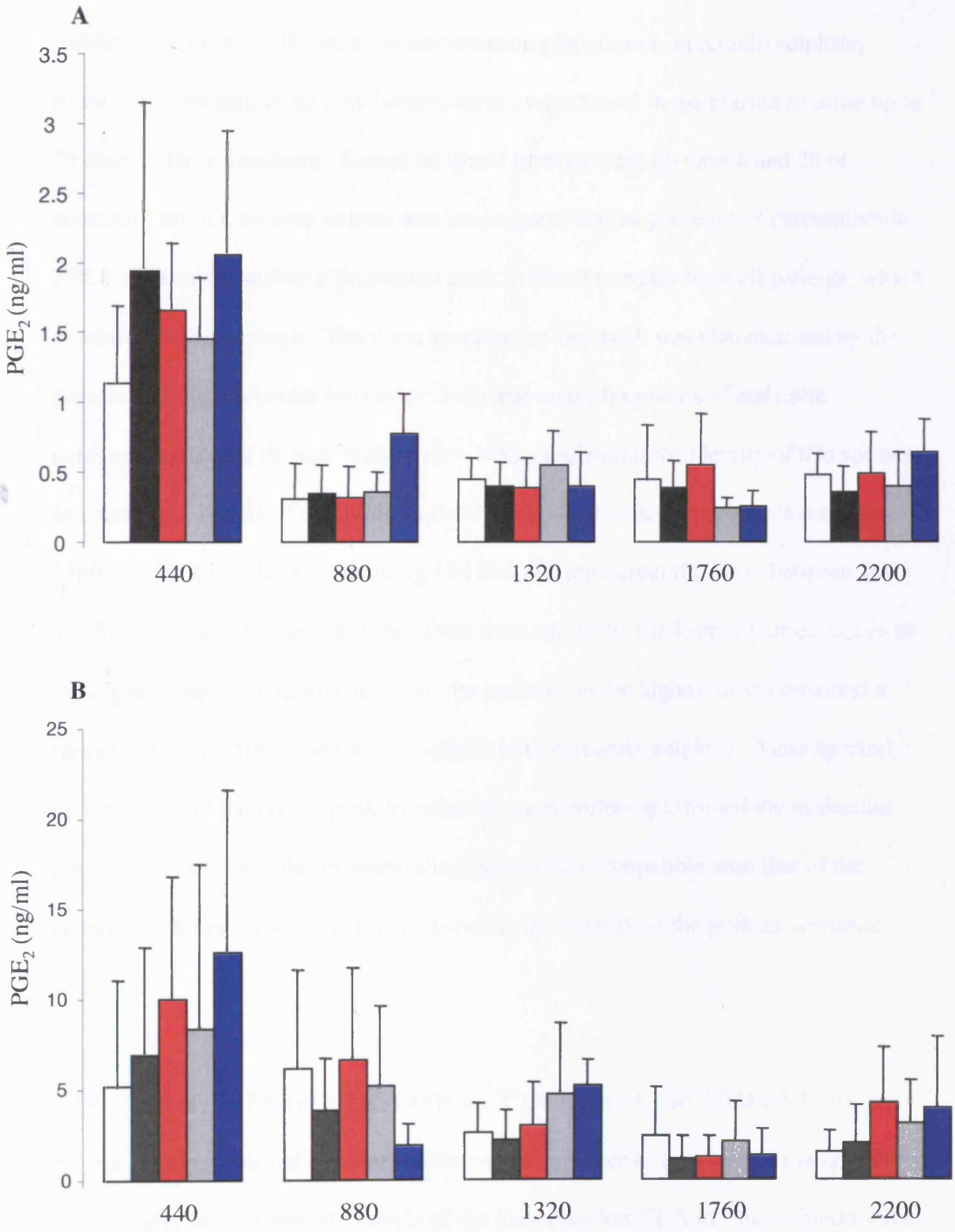
Blood samples were obtained for assessment of basal and LPS-induced PGE_2 concentrations at screening visit and immediately before and 1 h after each dose on treatment days 1, 2, 8 and 29. Comparison of basal blood PGE_2 values (ng/ml) with body mass index (BMI, kg/m^2) for patient samples was performed by regression fit analysis, and there was no statistical association despite such a relationship having been shown previously between BMI and rectal mucosal PGE_2 content in healthy volunteers with a history of resected colorectal polyps[200]. LPS-induced plasma PGE_2 values were significantly higher than basal PGE_2 values at all timepoints measured ($p < 0.001$ by ANOVA). Although LPS-induced PGE_2 values were approximately 20% lower in blood taken 1 h post-dose compared to pre-dose, this difference was not significant. Similarly, there was no significant difference in basal PGE_2 between pre- and post-dose values within each dose level. Time-dependent trends related to treatment were not identified in basal or LPS-induced PGE_2 values.

Since there was no significant difference between pre-and post-dose PGE_2 values, all timepoints after the first dose (*ie.* days 1, 2, 8, and 29) were combined to allow analysis of results from each dose level for treatment-related effects (Figure 5.11). Values obtained one week prior to treatment and immediately before dosing on day 1 were combined to give 'control' pre-treatment values for the same patients. Plasma PGE_2 levels differed between dose levels ($p = 0.006$ by ANOVA) and the decrease in PGE_2 with increasing dose level was confirmed by polynomial curve regression fit ($p < 0.005$). Nevertheless, a comparable difference between dose levels was also observed in 'control' plasma samples (Figure 5.11). Pooled values for LPS-induced

plasma PGE_2 from days 1, 2, 8 and 29 of treatment seemed to decrease in a dose-dependent fashion, but this trend was not significant ($p = 0.075$ by linear regression analysis).

Figure 5.11: Plasma PGE_2 levels measured in clinical trial of *Curcuma* extract.

Basal (**A**) and LPS-induced (**B**) PGE_2 levels in plasma of patients consuming the total daily doses of *Curcuma* extract shown by the labels on the *x*-axis. Blood was taken immediately pre-dose or 1 h afterwards on days 1, 2, 8 and 29. Whole blood was incubated for 24 h, in the absence (**A**) or presence (**B**) of LPS (10 µg/ml). Results are the mean ± SD of triplicate measurements pooled from 1 week pre-treatment combined with day 1 pre-dose (open bars), day 1 post-dose (**black** bars), day 2 pre- and post-dose (**red** bars), day 8 pre- and post-dose (grey bars) and day 29 pre- and post-dose (**blue** bars). For experimental details see 'Materials' and 'Methods' chapters.


A
3.5
3
2.5
2
1.5
1
0.5
0
PGE2 (ng/ml)
440
880
1320
1760
2200
B
25
20
15
10
5
0
PGE2 (ng/ml)
440
880
1320
1760
2200

5.5.5 LEVELS OF CURCUMINOIDS IN BLOOD AND EXCRETA

Neither curcumin nor its metabolites curcumin glucuronide, curcumin sulphate, hexahydrocurcumin or hexahydrocurcuminol were found in the plasma or urine up to 29 days of daily treatment. Faeces obtained from patients on days 8 and 29 of consumption of *Curcuma* extract was investigated for the presence of curcuminoids. HPLC analysis furnished a prominent peak in faecal samples from all patients, which co-eluted with curcumin. The mass spectrum of this peak was characterised by the presence of the molecular ion (m/z = 367) and major fragments of authentic curcumin including its base peak (m/z = 149), confirming the identity of this species as curcumin. Levels of curcumin in day 29 faecal samples from patients consuming 1760 and 2200 mg daily (containing 144 and 180 mg curcumin) were between 144 and 519 nmol/g dried faeces at the lower dose and 64 to 1054 nmol/g dried faeces at the higher dose. The faeces of one of the patients on the highest dose contained a species which on HPLC analysis co-eluted with curcumin sulphate. Mass spectral investigation of the HPLC peak by selected ion monitoring afforded the molecular ion of m/z = 447, and the fragmentation pattern was compatible with that of the authentic reference compound, corroborating the identity of the peak as curcumin sulphate.

5.5.6 CHEMOTHERAPEUTIC EFFICACY OF ORAL *CURCUMA* EXTRACT.

All the patients enrolled exhibited radiological evidence of progressive malignant disease prior to recruitment. Levels of the tumor marker CEA in venous blood were above the normal range in all patients, and those of CA19.9 were abnormal in 80% of patients. In one patient, who received 440 mg *Curcuma* extract (equivalent to 36 mg curcumin) daily, venous blood CEA levels decreased from a pre-treatment value

of 310 ± 15 to 175 ± 9 μg/l after two months of treatment. This patient experienced stabilisation of disease in the colon but progression in the liver, as demonstrated on CT scan. None of the other patients had measurable disease in the colon, since the primary tumor had been resected previously. Levels of CA19.9 did not change with treatment. Five patients exhibited stable disease on CT scan, three (on 440, 880, and 1760 mg *Curcuma* extract) for three months, and two (on 880 and 1320 mg *Curcuma* extract) for four months of treatment. Significant changes in quality of life variables were not recorded.

5.5.7 DISCUSSION

This study constitutes the first clinical evaluation of a standardised *Curcuma* extract in patients with cancer including pharmacodynamic and pharmacokinetic measurements. The results suggest two conclusions which may help to optimise the design of future clinical trials of curcumin or *Curcuma* extracts: i) oral administration of *Curcuma* extract for several months at doses up to 2.2 g daily, equivalent to 180 mg curcumin, appears safe within the limitations of a phase I study; ii) the systemic bioavailability of orally administered curcumin is low in humans.

Safety

The first conclusion regarding the apparent safety of *Curcuma* extracts is consistent with previous reports of clinical studies of curcumin and turmeric. Soni and Kuttan treated 10 volunteers with 500 mg curcumin daily for 7 days, and failed to observe clinical toxicity[108]. Two clinical trials, designed to study the efficacy of curcumin as an anti-inflammatory agent in the treatment of arthritis or postoperative

inflammation, found that daily doses of 1.2 to 2.1 g curcumin for 2 - 6 weeks did not cause adverse effects[52,107]. In a pilot study published in abstract form[48], tablets of turmeric extract containing 99.8% curcumin did not cause any treatment-related toxicity at doses as high as 8 g per day. Furthermore, a single dose of 50 to 200 mg of micronized curcumin formulated as capsules or sachets was administered to 18 volunteers without causing any detectable toxicity[49]. Clinical trials of oral curcumin incorporating larger subject populations will be required to establish the safety of chronic administration. Although certain communities in the Indian subcontinent consume up to 1.5 g dietary turmeric per person per day[36], curcumin constitutes only 2 - 8% of most turmeric preparations[38]. The acceptable daily intake of curcumin as a food additive has been defined by the World Health Organization as 0 - 1 mg/kg-bw[199]. Thus the largest dose administered in the study presented here (2.2 g *Curcuma* extract, containing 180 mg curcumin) exceeds that of dietary consumption.

Bioavailability

The finding that curcumin was only detectable in the faeces of patients, but not in plasma or urine, is in keeping with the low systemic bioavailability of orally administered curcumin seen in rodents[42-46] and suspected in humans[47-49]. In a previous study, following a single oral dose of 2 g, curcumin levels were transiently detectable in the serum of healthy volunteers[47]. In that study, co-ingestion of curcumin with the pepper constituent l-piperoylpiperidine, which is thought to inhibit xenobiotic glucuronidation, appeared to increase curcumin serum area under the plasma concentration-time curve (AUC) by a factor of 20. The presence of curcumin sulphate in the faeces of one patient at the highest dose level described here is

consistent with the suspicion that curcumin can undergo metabolic conjugation in the gut[43,45].

Glutathione S-Transferase

Three potential biomarkers of curcumin's systemic efficacy were evaluated in the pilot study described here. Lymphocyte GST activity decreased with time in the three patients who received the lowest dose of *Curcuma* extract. This decrease may have been associated with the treatment, but in the light of the small number of patients studied and the fact that GST activity was not decreased in patients on higher dose levels, the interpretation of this observation has to be tentative. Rats fed dietary curcumin at approximately 250 mg/kg-bw and above were found to have decreased hepatic GST activity compared to controls, and competitive enzyme inhibition by the curcumin molecule was thought to be responsible[65]. It is unlikely that this observation can be used to rationalise the decline observed in our patients, as the dose used in the rats was more than sixty-fold higher than that given to the patients. Lymphocyte GST activity, measured by the CDNB assay, has been shown to be independent of age and gender[201] and constant within subjects, as borne out by measurements on at least 3 occasions over a 2 - 4 week period in normal individuals and those at increased risk of developing colorectal cancer[18]. The observations made in the study reported here propose similar consistency for patients with advanced cancer. Whether or not lymphocyte GST activity correlates with colon mucosal GST levels in patients with colon cancer, as was demonstrated in individuals at risk of developing colon cancer[18], remains to be established.

M_1G Adducts

This study provides the first description of leukocyte M_1G levels in patients with colorectal cancer. The lower levels shown in Figure 5.9 are comparable to those previously reported in healthy volunteers, whilst the highest levels resemble those seen in humans on pro-oxidant diets[161]. M_1G adduct levels were unaffected by *Curcuma* consumption. It is conceivable that higher doses of curcumin, which may result in measurable plasma curcumin concentrations, are required to elicit effects on endogenous DNA adducts found in blood leukocytes. Nevertheless, the intra-individual reproducibility over time of M_1G adduct levels supports the potential suitability of this adduct as a biomarker of the systemic effects of curcumin or other chemopreventive antioxidants. The putative link between *GSTM1* null genotype and elevated leukocyte M_1G adduct levels, observed in the patients in this trial, is congruous with associations previously reported between *GSTM1* genotype and levels of aflatoxin B_1-induced DNA adducts[202], but is the first suggestion of such an association for an adduct formed by endogenous processes such as lipid peroxidation and oxidative DNA damage.

Cyclooxygenase Activity

This study represents the first report of the assessment of blood COX-2 activity induced *ex vivo* as a biomarker of the efficacy of oral administration of an agent capable of inhibiting the transcription of this isoenzyme and the first measurement of this biomarker in patients with cancer. The findings highlight potential pitfalls in measurement of this biomarker in clinical trials with small numbers of subjects. Wide variation in pre-treatment basal and LPS-induced PGE_2 values prevented the

interpretation of potentially significant treatment effects observed. This heterogeneity was attributable to inter-day variation within each patient and variation between patients, as has been observed previously for colon mucosal PGE_2 levels in a chemoprevention trial of ibuprofen in healthy individuals who had polyps previously resected[200]. In order to see treatment effects on blood PGE_2 production in the context of this variability, future prospective studies should stratify treated and control patients pre-treatment according to measures of basal and LPS-induced PGE_2 concentration on at least two occasions, as has been suggested for trials measuring rectal mucosal PGE_2 levels[202]. Such stratification of control and treatment groups by baseline PGE_2 levels could help balance subjects better and improve the chances of observing a treatment effect.

Chemotherapy

A third of the patients in this study experienced stable disease for 2 - 4 months, and in one further patient consumption of *Curcuma* extract may have been linked to stabilisation of the primary colon tumour without a cytostatic effect on liver metastases. In this patient, a significant decrease in the venous tumour marker CEA was observed after two months of therapy. Together these findings hint at the possibility that patients with colorectal cancer may benefit from consumption of *Curcuma* extract, but definite conclusions cannot be drawn from this type of small pilot study.

Conclusion

In summary, this pilot study of *Curcuma* extract in patients with colorectal cancer provides information which might help optimise the design of the future clinical evaluation of curcumin and other *Curcuma* extracts. Doses up to 2.2 g *Curcuma* extract, containing 180 mg curcumin, per day can be administered to patients with cancer for up to 4 months, and in this pilot study such treatment was safe. Clinical trials of *Curcuma* extracts as potential cancer chemopreventive agents should focus on the effects of such doses in target tissues, particularly colon epithelium. To investigate whether *Curcuma* extract may be linked to clinical benefit in patients with advanced cancer, future trials of *Curcuma* extracts as potential cancer chemotherapeutic agents may study systemic effects of higher dose levels. Measurements of GST activity, inducibility of COX-2 activity, and M_1G levels in leukocytes merit further exploration as potentially suitable biomarkers of pharmacological efficacy in this regard.

CHAPTER 6

GENERAL DISCUSSION

(1) POTENTIAL BIOMARKERS OF CANCER CHEMOPREVENTION BY CURCUMIN IN PRECLINICAL MODELS

Measurement of COX-2, PGE_2, MDA and M_1G levels in five human colon cell lines demonstrated relationships that exist between COX-2 activity and MDA production, and between MDA concentration and M_1G adduct levels. Modification of COX-2 activity, however, did not result in sufficiently large alterations in MDA concentrations to effect changes in M_1G adduct levels. This negative result is attributable to the limitations of the preclinical model system used (see section 5.1.3). A further example of the relationship between MDA and M_1G adduct levels has been found recently in human gastric mucosal biopsies, in which 200% increases in MDA concentration only resulted in approximately 30% increases in M_1G levels[203]. Although the novel mechanistic information obtained in this study regarding the relationships of these four biomarkers in non-malignant and malignant colon cells was valuable, the *in vitro* model system was not deemed suitable for studying the efficacy of curcumin in altering M_1G adduct formation.

Compatible with reports of earlier studies[64,66], dietary curcumin in high dose short-term feeding was found to induce GST activity in rat liver. For the first time, rat colon mucosal levels of the M_1G adduct were measured in the project described here, and they were found to be diminished by a diet containing curcumin. Dietary curcumin also prevented increases in colon mucosal M_1G adduct levels associated with chemically induced lipid peroxidation. These effects are compatible with potential for cancer chemoprevention in colon mucosal and hepatic tissues, and are

associated with biologically relevant levels of unaltered curcumin detected in these tissues following dietary consumption.

In the MIN mouse model, characterised by a defect in the gene underlying the human disease FAP, high levels of M_1G adducts were found in intestinal adenomas relative to surrounding normal mucosa. MDA levels in adenomas were not significantly higher than those of surrounding mucosa, so one may speculate that M_1G adduct formation in this pre-malignant tissue was occurring principally through MDA-independent pathways, such as oxidative DNA damage resulting in formation of base propenals[150,189]. M_1G adduct levels in adenomas were diminished significantly by a diet containing 0.1% or 0.2% curcumin, although intestinal mucosal levels were unaffected. The latter result is inconsistent with the findings in the normal rat model, although the basal colon mucosal M_1G level in the rats was higher than that in the mice and the proportion of curcumin was at least ten times greater in the diet fed to the rats than the one fed to the mice. Another notable difference was the timeframe of feeding: fourteen days in the rats compared to 14 – 16 weeks in the mice.

Considered in isolation and bearing in mind the limitations of this model system (discussed in section 1.1.3), the findings in MIN mice may prove relevant to the chemoprevention of adenoma development in patients, particularly colorectal polyps since curcumin levels in the colon mucosa were over ten-fold higher than those in the intestinal mucosa in the same mice. Since total intestinal adenoma number was not

modified dramatically by dietary curcumin at a dose of 0.1%[c], a greater understanding of the role of the M_1G adduct in colorectal carcinogenesis will determine the significance, perhaps subtle, of the findings in the mice used here. Unlike most other chemopreventive drugs studied in this model (discussed in [28]), curcumin at the doses used here demonstrated no toxicity in long-term dietary administration to MIN mice.

[c] S. Perkins, unpublished data

(2) CLINICAL TRIAL OF *CURCUMA* EXTRACT IN PATIENTS WITH COLORECTAL CANCER

As in previous pilot studies of curcumin or turmeric in other patient groups, oral administration of a standardised *Curcuma* formulation resulted in no toxicity definitely linked to the agent. The low systemic bioavailability of oral curcumin found in rodents appeared to be similar in humans on the basis of the absence of detectable curcuminoids in plasma. The discovery of curcumin sulphate in the faeces of one patient provided evidence for the hypothesis that curcumin may undergo intestinal metabolism in humans.

There were three *hints* that oral administration of *Curcuma* extract may have systemic biological activity. Firstly, stable malignant disease was demonstrated radiologically in five patients treated for 2 – 4 months, despite documented progressive malignant disease prior to trial enrolment. Secondly, one patient was found to have a significant decrease in the venous level of the tumour marker CEA after two months of treatment, although it had been rising pre-treatment. Neither of these findings can be interpreted in this type of small pilot study since the natural history of colorectal cancer can be variable in patients. Finally, lymphocyte GST activity decreased significantly with 29 days of treatment in patients receiving the lowest dose of *Curcuma* extract. Although induction of GST activity may be desirable in the prevention of the early stages of carcinogenesis (see Appendix 2D), in patients with advanced malignancy GST isozymes may be aberrantly overexpressed and linked with resistance to chemotherapy[112]. If changes in lymphocyte GST activity correlate with similar changes in tumour GST activity in

patients with advanced colorectal cancer, decreases in lymphocyte GST may be favourable in the treatment of malignancy. However, it is important to stress that the small number of patients at each dose level and the lack of a control group in this type of pilot study prevent conclusions being drawn from this preliminary finding. The only conclusion that can be reached regarding these three *hints* of possible biological activity is that these three biomarkers merit inclusion in future trials of curcumin with larger patient numbers and different primary endpoints.

With regard to the other biomarkers of curcumin's systemic activity measured in this study, no hints of treatment effects were observed but novel clinical information was discovered. Although leukocyte M_1G adduct levels in healthy individuals may fluctuate up to four-fold with dietary factors[161], they appeared relatively stable within each patient in this study over a 5-week period. This consistency would suggest that dietary factors remained relatively constant in the patient group studied. An association was tentatively drawn between leukocyte levels of this endogenous deoxyguanosine adduct and the *GSTM1* genotype in the same cells, as has been described for bulky DNA adducts formed by exogenous carcinogens[202]. Although other GST subclasses are known to detoxify the products of lipid peroxidation[112,204], GSTM1 has not previously been associated with the endogenous processes known to give rise to M_1G adducts.

In marked contrast, COX-2 activity induced in whole blood *ex vivo* was highly variable for each patient on different days, and even more variable between patients with advanced colorectal cancer. Pre-treatment stratification of patients in future studies according to their basal and inducible leukocyte PGE_2 production may reduce

variability and improve the chances of observing pharmacological effects. Despite the limitations of the *in vitro* model used for the study of curcumin's inhibition of leukocyte COX-2 activity when added to blood from healthy volunteers (*e.g.* the degree of apoptosis, which is not necessarily representative of the degree occurring *in vivo*), use of such models systems sets a precedent for preliminary studies of potential biomarkers before their incorporation into clinical trials. It is only when the investigator has some idea about the range of concentrations at which biological activity is observed and the magnitude of possible effects, that the size of the subject group can be planned in order to ensure that a clinical trial carries sufficient statistical power. Results from small pilot studies, such as the biomarkers measured as secondary objectives in the clinical trial described here, demonstrate the feasibility of measuring these indices and may offer suggestions of pharmacodynamic effects, useful in planning larger Phase I/II trials.

(3) OPTIMISATION OF FUTURE DEVELOPMENT OF CANCER CHEMOPREVENTIVE AGENTS

Although it was deemed necessary, as stated in Chapter 2, aim (2), to commence the clinical investigation of oral *Curcuma* extract at low doses, the findings of the pilot study described here do not exclude the possibility that oral administration of low doses of curcumin is capable of systemic effects relevant to the chemoprevention of cancer, as described in hepatic and mammary tissues of animals[82,205]. Curcumin's biological effects may also modify the behaviour of established malignancy. Further dose escalation of standardised oral *Curcuma* formulations in patients with cancer is suggested, until curcumin levels are measured systemically. Optimisation of oral bioavailability may require reformulation of curcumin, as suggested by the rodent studies presented in Appendix 2E.

At inception of this project, clinical trials of plant-derived putative chemopreventive agents in patients with cancer had been proposed in theory[206] but no reports of such trials had been published. More recently, Phase I studies of D-limonene, perillyl alcohol and green tea extracts have been published[207-209], studying pharmacokinetics and toxicity in patients with advanced solid tumours; thus adopting a rationale in choice of subject group similar to this project. International standardisation of chemopreventive trial design has been defined by the Chemoprevention Working Group of the American Association for Cancer Research as an essential prerequisite for the advancement of preventive agents[210]. Although the degree of inhibition of biomarker activity by curcumin may be less than that observed with similar concentrations of certain synthetic drugs such as celecoxib (*e.g.* see chapter 5.4.3),

the diet-derived polyphenol may still be of value in cancer chemoprevention. The advancement of cancer chemoprevention requires the clinical development of drugs with a high therapeutic index, despite potential toxicity, for use in individuals at the highest risk of developing certain malignancies; it also requires development of safer agents for potential use more widely in lower risk subjects[196].

Although the "point of failure" for the clinical development of oral curcumin as an agent directed at the prevention or therapy of cancer in tissues outwith the gastrointestinal tract may be its low systemic bioavailability, this pharmacokinetic profile should not necessarily inhibit its development as a chemopreventive agent against colorectal cancer. High levels of unaltered curcumin were measured in the colon mucosa of rodents following dietary administration, and if the same holds true for humans, biological effects may be observed in this target organ. In this regard, human studies incorporating intestinal tissue levels, and perhaps hepatic and biliary drug levels, will provide valuable pharmacokinetic information. Such studies may be performed in patients undergoing resection of primary or metastatic colorectal cancer. Dose de-escalation in the MIN mouse model has provided some preliminary data on the minimum dose of oral curcumin that reduces polyp numbers, and thus a provisional indication of the dose ranges that may be required for similar effects in patients. The measurement of biomarkers such as oxidative DNA damage and M_1G adducts may add sensitivity to exisiting macroscopic (*e.g.* adenoma number) or histological (*e.g.* adenoma morphology) techniques for assessing cancer risk and the efficacy of intervention. Measurement of different classes of complementary biomarkers, including pathological, cellular, biochemical, molecular, and genetic (described in Appendix 2D, see Table 3) may help to overcome some of the

limitations of models currently used (*e.g.* unlike patients with FAP, MIN mouse adenomas occur predominantly in the small intestine rather than the colon, and histological characteristics may differ between small and large intestinal adenomas, but no significant difference was detected in this study between M_1G levels of adenomas from different levels of the intestinal tract). Lack of mechanistic understanding of the exact relationship of biomarkers such as M_1G to human carcinogenesis is currently a limiting factor in such progress. Ultimately, certain biomarkers may advance from preclinical model systems to the judgement of successful intervention in patients, although target thresholds for a single biomarker should not be used in isolation, as discussed in section 1.1.2.

The overall aim of this project focussed on the pharmacodynamics of curcumin, but the biomarkers studied are relevant to the clinical development of other cancer chemopreventive agents which may ultimately prove superior to *Curcuma* extracts. Development and validation of biomarkers of the efficacy of chemopreventive intervention not only allows shorter, smaller and less expensive definitive clinical trials, but also allows the elimination of agents on the basis of negative results, in preference for alternative agents showing more promising activity in the preclinical screening methods applied *in vitro* and *in vivo*[211]. Pharmacodynamic effects such as inhibition of adduct formation by exogenous or endogenous carcinogens, inhibition of free radical formation and damage, enhancement of glutathione levels, and induction of glutathione S-transferase levels are examples of biomarkers cited by the Chemoprevention Working Group[210] as generally "invaluable in characterizing new agents." The study of complementary biomarkers, *e.g.* levels of COX-2 protein, PGE_2, MDA, M_1G adducts and the recently identified membranous PGE_2 synthase

(mPGES) enzyme in conjunction with GSH (necessary for mPGES activity) and GST isozymes known to metabolise MDA, is likely to provide more complete mechanistic understanding of cellular pathways *in vivo*.

Biomarkers relevant to cancer chemoprevention may increasingly be of use in the assessment of the anticancer efficacy of new chemotherapeutic agents[111]. Unlike traditional cytotoxics, many of these novel drugs do not cause tumour regression, so the use of current radiological measurements is not useful. Reliance on median survival as the only endpoint measured in trials of new chemotherapeutic agents *versus* existing chemotherapy regimes may be remarkably insensitive, and may not detect survival differences between study groups as large as two-fold[212]. Incorporation of carefully selected biomarkers from the earliest phases of clinical development of novel agents may help to overcome this insensitivity.

Such incorporation of biomarkers into the clinical development of novel agents must be judicious. Despite the relatively short history of cancer chemoprevention, the field is strewn with studies that either failed to adequately develop or validate biomarkers, or designs that did not adequately consider the biology of the endpoint. It is hoped that the combined prelinical and clinical approach fostered by this project may avoid such disappointments for curcumin or other agents that might prevent colorectal cancer in the future.

CHAPTER 7

APPENDICES

APPENDIX 1: List of abbreviations used

ANOVA	analysis of variance
AP-1	activator protein-1
APC	adenomatous polyposis coli
AUC	area under plasma concentration – time curve
BC	before Christ
bw	body weight
BMI	body mass index
CCl_4	carbon tetrachloride
CDNB	1-chloro-2,4-dinitrobenzene
CEA	carcinogenic embryonic antigen
COX	cyclooxygenase
CT	computed tomography
DMEM	Dulbecco's modified medium
DMSO	dimethylsulphoxide
EDTA	ethylenediaminetetra-acetic acid
EORTC	European Organization for Research and Treatment of Cancer
FAP	familial adenomatous polyposis
FCS	foetal calf serum
GST	glutathione S-transferase
h	hours
HCEC	human colon epithelial cells
HPLC	high pressure liquid chromatography
IKK	IκB kinases

iNOS	inducible nitric oxide synthase
JNK	c-Jun NH_2-terminal kinases
LC	liquid chromatography
LPO	lipid peroxidation
LPS	lipopolysaccharide
M_1G	malondialdehyde-deoxyguanosine adduct
MDA	malondialdehyde
min	minutes
MIN	multiple intestinal neoplasia
mPGES	membranous prostaglandin E_2 synthase
MS	mass spectrometry
NADPH	hydrogenated nicotinamide adenine dinucleotide phosphate
NCI	United States of America National Cancer Institute
NF-κB	nuclear factor-κB
NO	nitric oxide
NOS	nitric oxide synthase
NSAID	non-steroidal anti-inflammatory drug
ODC	ornithine decarboxylase
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PG	prostaglandin
PMA	phorbol 12-myristate 13-acetate
s	seconds
SDS	sodium dodecyl sulphate

ULN	upper limit of normal
WHO	World Health Organization

APPENDIX 2: Full papers published

APPENDIX 2A: Sharma RA. Cancer chemoprevention: A clinical reality. *J Royal Soc Med* 2000, **93**:518-520.

Cancer chemoprevention: a clinical reality

Ricky A Sharma MA MRCP

J R Soc Med 2000;93:518–520

In 1867 the Viennese surgeon Theodor Billroth claimed that cancer could be cured with the knife[1], but later review of his own results showed that this was seldom true. Even today, despite advances in surgery, radiotherapy and chemotherapy over the past century, cancer is an increasing cause of morbidity and mortality in most countries. In some, it has now overtaken heart disease as the commonest cause of death[2].

Although the increased funding for cancer research during the past 25 years has had negligible impact on cancer mortality rates, it has immensely increased our knowledge of the mechanisms whereby normal cells and tissues become malignant[3]. Pharmacological intervention to arrest or reverse such mechanisms is termed chemoprevention, and is well established in the prevention of other diseases such as dental caries, heart attacks and stroke. In this review I discuss the promising results already achieved in the chemoprevention of epithelial neoplasia, citing breast and colorectal cancers as examples. There already exists a case for establishing prevention clinics, run by oncologists in collaboration with clinical geneticists and molecular epidemiologists, to assess and advise individuals at high risk of developing certain well-characterized malignancies. Dietary intervention[4] will not be considered.

PRINCIPLES OF CANCER CHEMOPREVENTION

Cancer chemoprevention is the inhibition, retardation or reversal of carcinogenic processes by chemical means, and includes the treatment of patients who have undergone successful treatment of a primary malignancy but are at increased risk of a second[5]. This latter concept is designated 'tertiary' chemoprevention[6]. The prevention of carcinogenesis at a premalignant stage is 'primary', and prevention at an early phase of malignancy is 'secondary'.

Several effective chemopreventive agents were first developed for adjuvant chemotherapy; the preventive action of tamoxifen, for example, emerged when it was found to decrease breast tumours in the contralateral breast after surgery[7]. Such overlap between chemotherapeutic and chemopreventive properties implies an opportunity to conduct pilot studies of new chemopreventive agents in patients who already have cancer, and this has been done with the putative agents piroxicam[8], difluoromethylornithine (DFMO)[9], and perillyl alcohol[10].

Chemoprevention Research Group, MRC Toxicology Unit, University of Leicester, Leicester LE1 9HN, UK
E-mail: ras20@le.ac.uk

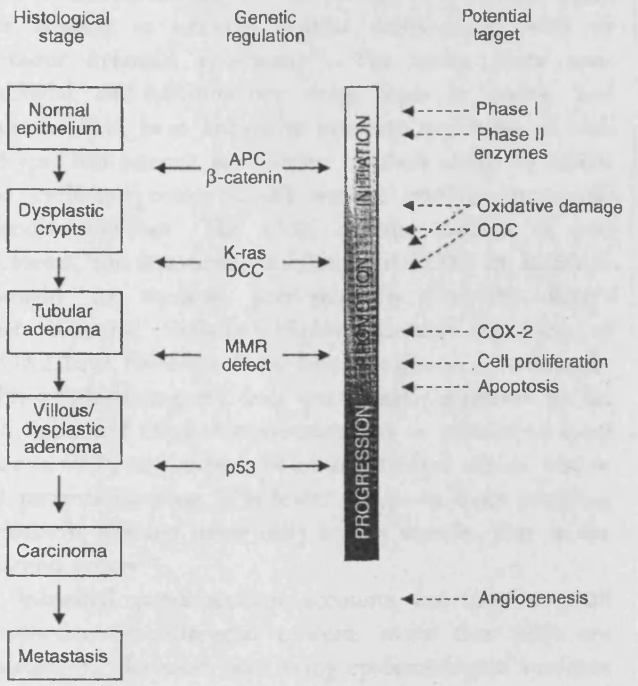


Figure 1 **Multistep model of colorectal carcinogenesis with targets for potential intervention.** APC=Adenomatous polyposis coli; COX2=cyclo-oxygenase 2; DCC=deleted in colorectal cancer; MMR=mismatch repair; ODC=ornithine decarboxylase [Modified from Ref. 11]

Although the development of malignancy is the result of a complex interaction between genetic and environmental influences, the multistep model of carcinogenesis[11] has provided a structure into which findings can be incorporated. Existing paradigms include clonal evolution[12] and epigenetic changes[4], and these too offer pointers to targets for chemopreventive agents. In colorectal neoplasia, carcinogenesis has been characterized in exceptional detail (Figure 1).

TAMOXIFEN CONTROVERSY

Opinions differ between America and Europe on the chemopreventive efficacy of two antioestrogenic agents, tamoxifen and raloxifene. The BCPT study in the United States involved 13 388 women at relatively high risk but free of detectable invasive breast cancer at study recruitment[13]. Tamoxifen reduced the subsequent development of breast cancer in the treated group as a

whole by 45%, and also seemed to prevent the progression of established preneoplastic lesions of the breast. These results led the US Food and Drug Administration to approve the use of tamoxifen 20 mg/day in women deemed at increased risk of breast cancer, albeit with consideration of its toxicities. Similar reductions in breast cancer were seen in a trial of raloxifene for postmenopausal osteoporosis; in this instance the women were not thought to be at excess risk of breast cancer[14].

By contrast, two smaller European trials of tamoxifen in healthy women not judged at high risk have not shown any protective effect[15,16]. In Europe, the International Breast Cancer Intervention study has now recruited more than 4000 women with at least a two-fold increased risk of developing breast cancer, and the results are awaited. Presumably the transatlantic differences of opinion on the preventive value of agents such as tamoxifen reflect differences in the study populations.

AVOIDANCE OF TOXICITY

In ancient times both Hippocrates and Galen warned that treatment for hidden cancer could hasten death[1]. Their wisdom was borne out in large-scale chemoprevention trials that may have increased cancer incidence in subsets of the population[17,18]. These negative results not only highlight the importance of gaining a detailed mechanistic understanding of putative chemopreventive agents before administering them to large groups who do not have cancer, but also emphasize the need to weigh up toxicity against benefit.

For example, although the vitamin A analogues isotretinoin[19] and retinol palmitate[20] have been shown to prevent second primary cancers in patients with malignancies of the lung and head and neck, the toxic effects are such that many patients stop taking them. Similarly the synthetic retinoid fenretinide decreased the risk of breast cancer in premenopausal women in a tertiary chemoprevention study, but night blindness and erythema proved prohibitive with high doses[21]. Some newer retinoids that selectively bind the three retinoid X receptors may lack these drawbacks; they appear highly chemopreventive in preclinical mammary models but do not possess the toxicity/teratogenicity profile of typical retinoids[22].

IS ASPIRIN THE ULTIMATE CHEMOPREVENTIVE?

In families with familial adenomatous polyposis (FAP), the presence of the *APC* gene defect confers a 100% lifetime risk of colorectal cancer[23]. At present affected individuals are advised to undergo regular colonoscopy, with or without eventual colectomy[24]. For many years non-steroidal anti-inflammatory drugs such as aspirin and sulindac have been known to promote regression of such polyps, and interest has focused on their ability to inhibit the cyclo-oxygenase (COX) enzyme involved in prostaglandin synthesis. The COX enzyme consists of two isoforms, and irreversible inhibition of COX1 by aspirin is thought to account predominantly for the drug's gastrointestinal toxicity. Highly selective inhibitors of COX2 have therefore been developed, such as celecoxib. This anti-inflammatory drug was recently approved by the US Food and Drug Administration as an adjunct to usual care in FAP, on the basis of an unpublished clinical trial in 83 patients showing 28% fewer polyps in those receiving celecoxib, 400 mg twice daily for six months, than in the placebo group[25].

Inherited predisposition accounts for only a small proportion of colorectal cancers: more than 80% are sporadic[23]. The most convincing epidemiological evidence for decreased incidence of total colorectal cancer as a result of pharmacological intervention exists for aspirin. Retrospective epidemiological studies suggest a decrease of up to 50% in regular aspirin users[26,27], although there may be a delay of a decade or so before benefit[28]. There are hopes that natural polyphenols such as turmeric extracts[29] and newer selective COX2 inhibitors[25] will offer similar chemopreventive efficacy with less toxicity.

The development of aspirin as a cancer chemopreventive agent raises the issue of indirect biomarkers for efficacy, analogous to sphygmomanometry or blood cholesterol measurement in cardiovascular disease. As judged by 'preneoplastic biomarkers' such as colon epithelial prostaglandins (Table 1), the

Table 1 **Types of biomarker**

Type of biomarker	*Stage of disease*	*Description*	*Application*
Risk biomarker	No disease detectable	Genetic predisposition, medical history, lifestyle factors, exposure	Useful to molecular epidemiologists to assess risk of developing cancer
Preneoplastic 'surrogate' biomarker	Initiation, promotion, progression of carcinogenesis	Biological alterations representing early and intermediate stages of carcinogenesis	Identification of carcinogenesis and efficacy of chemoprevention
Tumour markers	Established neoplasia; metastasis	Associated with particular cancers	Diagnosis and treatment

chemopreventive dose of aspirin may be low as 81 mg/day[30]. Biomarkers such as gluthathione S-transferase activity, relevant to the effects of many chemopreventive agents including aspirin[31], may be measurable as a simple blood test indicating events in the colon[32]. Finally, genetic 'risk biomarkers' may also prove important in selection of individuals for large-scale chemoprevention trials[33].

CONCLUSION

Effective agents have already been identified in the brief history of cancer chemoprevention. Retinoids, tamoxifen and raloxifene have entered clinical practice in the prevention of epithelial malignancies. Aspirin, highly selective COX2 inhibitors, and certain natural compounds offer promise in preventing colorectal cancer. The success of future chemoprevention trials will depend on selection of suitable individuals, use of sensitive biomarkers and avoidance of toxicity.

REFERENCES

1 Cantor D. Cancer. In: Bynum WF, Porter R, eds. *Companion Encyclopedia of the History of Medicine*, Vol. 1. London: Routledge, 1993:552

2 Tattersall MHN, Thomas H. Recent advances: oncology. *BMJ* 1999;**318**:445–8

3 Sporn MB, Suh N. Chemoprevention of cancer. *Carcinogenesis* 2000;**21**:525–30

4 Gescher AJ, Pastorino U, Plummer SM, Manson MM. Suppression of tumour development by substances derived from the diet—mechanisms and clinical implications. *Br J Clin Pharmacol* 1998;**45**:1–12

5 Sporn MB, Newton DL. Chemoprevention of cancer with retinoids. *Fed Proc* 1979;**38**:2528–34

6 De Flora S, Balansky R, Scatolini L, *et al.* Adducts to nuclear DNA and mitochondrial DNA as biomarkers in chemoprevention. In: Stewart BW, McGregor D, Kleihues P, eds. *Principles of Chemoprevention*. Lyon: IARC, 1996:291–301

7 Chemoprevention Working Group. Prevention of cancer in the next millennium. *Cancer Res* 1999;**59**:4743–58

8 Israel L, Breau JL, Morere JF, Boaziz C. Evaluation of the antiprostaglandin agent piroxicam in 87 patients with advanced cancer. *Sem Hôp* 1993;**69**:1293–7

9 Carbone PP, Douglas JA, Larson PO, *et al.* Phase I chemoprevention study of piroxicam and α-difluoromethylornithine. *Cancer Epid Biomarkers Prev* 1998;**7**:907–12

10 Ripple GH, Gould MN, Bailey HH. Phase I clinical and pharmacokinetic study of perillyl alcohol administered four times a day. *Clin Cancer Res* 2000;**6**:390–6

11 Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990;**61**:759–67

12 Nowell PC. The clonal evolution of tumour cell populations. *Science* 1976;**194**:23–8

13 Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J Natl Cancer Inst* 1998;**90**:1371–88

14 Cummings SR, Eckert S, Krueger KA, Grady D, Costa A, Jordan VC. The effect of raloxifene on risk of breast cancer in postmenopausal women. *JAMA* 1999;**281**:2189–97

15 Veronesi U, Maisonneuve P, Costa A, Robertson C, Boyle P. Prevention of breast cancer with tamoxifen. *Lancet* 1998;**352**:93–7

16 Powles T, Eeles R, Ashley S, Viggers J, Davey J. Interim analysis of the incidence of breast cancer in the Royal Marsden Hospital tamoxifen randomised chemoprevention trial. *Lancet* 1998;**352**:98–101

17 The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group. The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. *N Engl J Med* 1994;**330**:1029–35

18 Omenn GS, Goodman GE, Thornquist MD, Balmes J, Cullen MR, Glass A. Risk factors for lung cancer and for intervention effects in CARET, the beta-carotene and retinol efficacy trial. *J Natl Cancer Inst* 1996;**88**:1550–9

19 Hong WK, Lippman SM, Itri LM, Karp DD, Lee JS, Byers RM. Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck. *N Engl J Med* 1990;**323**:795–801

20 Pastorino U, Infante M, Maioli M, *et al.* Adjuvant treatment of stage I lung cancer with high-dose vitamin A. *J Clin Oncol* 1993;**11**:1216–22

21 Veronesi U, de Palo G, Marubini E, Costa A, Malone WF, Sporn MB. Randomised trial of fenretinide to prevent second breast malignancy in women with early breast cancer. *J Natl Cancer Inst* 1999;**91**:1847–56

22 Gottardis MM, Bischoff ED, Shirley MA, Heyman RA. Chemoprevention of mammary carcinoma by LGD1069, an RXR-selective ligand. *Cancer Res* 1996;**56**:5566–70

23 Midgely R, Kerr D. Colorectal cancer. *Lancet* 1999;**353**:391–9

24 Giardiello FM, Hamilton SR, Krush AJ, Piantadosi S, Hylind LM, Celano P. Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. *N Engl J Med* 1993;**328**:1313–16

25 Smigel K. Arthritis drug approved for polyp prevention blazes trail for other prevention trials. *J Natl Cancer Inst* 2000;**92**:297

26 Thun MJ, Namboodiri MM, Health CW Jr. Aspirin use and reduced risk of fatal colon cancer. *N Engl J Med* 1991;**325**:1593–6

27 Giovannucci E, Rimm EB, Stampfer MJ, Colditz GA, Ascherio A, Willett WC. Aspirin use and the risk for colorectal cancer and adenoma in male health professionals. *Ann Intern Med* 1994;**121**:241–6

28 Collet J-P, Sharpe C, Belzile E, Boivin J-F, Hanley J, Abenhaim L. Colorectal cancer prevention by non-sterodial anti-inflammatory drugs: effects of dosage and timing. *Br J Cancer* 1999;**81**:62–8

29 Plummer SM, Holloway KA, Manson MM, *et al.* Inhibition of cyclooxygenase 2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF-kB activation via the NIK/IKK signalling complex. *Oncogene* 1999;**18**:6012–20

30 Krishnan K, Ruffin MT, Brenner DE. Colon cancer chemoprevention: clinical development of aspirin as a chemopreventive agent. *J Cell Biochem* 1997;**28/29** (suppl):148–58

31 Van Lieshout EMM, Tiemessen DM, Roelofs HMJ, Peters WHM. Nonsteroidal anti-inflammatory drugs enhance glutathione S-transferase theta levels in rat colon. *Biochim Biophys Acta* 1998;**1381**:305–11

32 Szarka CE, Pfeiffer GR, Hum ST, Everley LC, Balshem AM, Moore DF. Glutathione *S*-transferase activity and glutathione *S*-transferase μ expression in subjects with risk for colorectal cancer. *Cancer Res* 1995;**55**:2789–93

33 Hittelman WM, Papadimetrakopoulou V, Naggar AKE, *et al.* High genetic instability is associated with poor therapeutic outcome following biochemopreventive treatment of advanced premalignant lesions of the larynx and oral cavity. *Proc Am Assoc Cancer Res* 2000;**41**:223

APPENDIX 2B: Sharma RA, Gescher A, O'Byrne KJ, Steward WP. Familiar drugs may prevent cancer. *J Postgrad Med*, in press.

Postgrad Med J 2001;77:492–497

REVIEWS

Familiar drugs may prevent cancer

R A Sharma, A J Gescher, K J O'Byrne, W P Steward

Oncology Department, University of Leicester, Leicester Royal Infirmary, Leicester LE1 5WW, UK
R A Sharma
K J O'Byrne
W P Steward

MRC Toxicology Unit, University of Leicester
A J Gescher

Correspondence to:
Dr Sharma
ras20@le.ac.uk

Submitted 10 October 2000
Accepted 15 February 2001

Abstract

Despite positive results in large scale chemoprevention trials, many physicians are unaware of the potential cancer preventive properties of drugs in common usage. The antioestrogen tamoxifen and the selective cyclo-oxygenase-2 inhibitor celecoxib have been licensed in the USA for the chemoprevention of breast and colorectal cancers respectively in selected high risk individuals. Similarly, folate and retinol have been shown to decrease the incidence of colorectal cancer and squamous cell carcinoma of the skin respectively in large scale intervention trials. Other retinoids have proved efficacious in the tertiary chemoprevention of cancers of the breast and head/neck. Epidemiological evidence also exists in favour of aspirin, non-steroidal anti-inflammatory drugs, and angiotensin converting enzyme inhibitors preventing certain cancers. Phytochemicals may represent less toxic alternatives to these agents. Although some of these drugs are available without prescription and most are not yet licensed for use in cancer chemoprevention, physicians and students of medicine should be aware of this accumulating evidence base. Practitioners should be amenable to patient referral to discuss complex issues such as risk estimation or potential benefit from intervention.

(*Postgrad Med J* 2001;77:492–497)

Keywords: cancer chemoprevention; tamoxifen; folate; retinoids; cyclo-oxygenase

Cancer incidence and mortality continues to increase, and it has now overtaken heart disease as the commonest cause of death in Britain and Ireland.[1] Although tamoxifen may have attenuated the rising mortality rates from breast cancer,[2] chemotherapy has displayed a disappointing lack of impact on the prognosis from solid malignancies in general. Alternative strategies (see fig 1) have developed since the "war on cancer" was first announced by US President Nixon in 1971. One involves pharmacological intervention to arrest, inhibit or reverse carcinogenesis, and is termed cancer chemoprevention.[3] Its definitions are shown in fig 2.

Two drugs have already been licensed in the USA for use in cancer chemoprevention (see below) and results of large European clinical trials are eagerly awaited.[4] Despite the absence of licensed drugs in countries such as Britain, physicians are often asked questions about cancer prevention by patients' relatives and "high risk" individuals, not least because of the frequently held fears evoked by this common disease and its media coverage. Such questions pertain to some of the drugs in common clinical usage for other diseases. Unlike the prevention of cancer, primary and secondary prevention are well established in other diseases such as dental caries, heart attacks, and stroke, and play a prominent part in medical education. It should be noted that any physician directly or indirectly involved with cancer screening is increasingly likely to encounter such questions, since many of the tests used will detect high risk patients with premalignant disease.[4]

The aim of this review is to inform practising physicians of positive large scale chemoprevention trials, and why these drugs may prevent cancer. From our experience, patients tend to ask about specific drugs, and we will therefore

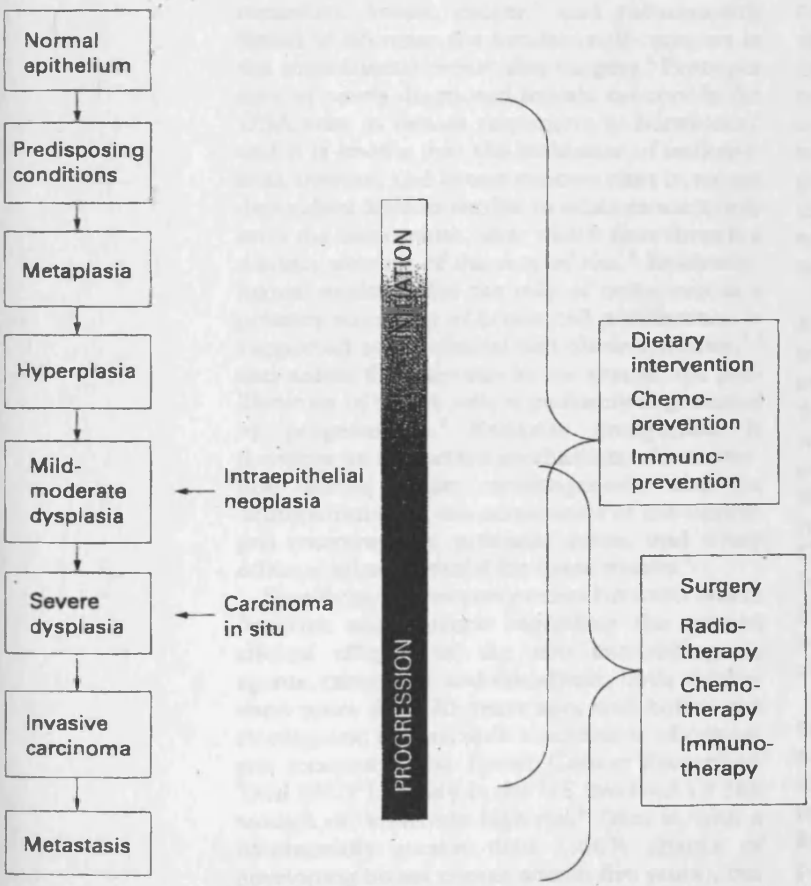


Figure 1 Simplified diagram of intervention strategies employed in the prevention and treatment of cancer. Carcinogenesis is shown using the multistep model (modified from Fearon and Vogelstein[57]). Dietary intervention is exemplified by UK Government advice on red meat intake based on epidemiological data.[1] Reviews on immunoprevention (for example, vaccination) and advances in the treatment of cancer have been published recently.[1 58] For overlap between chemoprevention and chemotherapy, see fig 2.

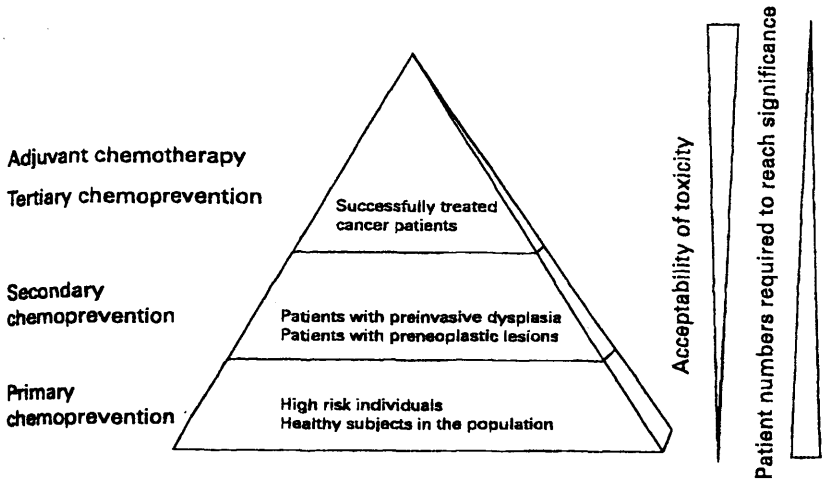


Figure 2 The levels of cancer chemoprevention, as defined by subject group (modified from Gescher et al[40] and De Flora et al[6]). Drug toxicity is least acceptable in primary chemoprevention, where large numbers of subjects are required for results to reach statistical significance.[40]

divide the text by agent rather than by disease. For organ based reviews of cancer chemoprevention, the reader is advised to read the key references shown in box 2.

Tamoxifen

Successful chemopreventive agents may be developed from their proven role in palliative or adjuvant chemotherapy. An example is the oestrogen analogue tamoxifen, which was initially found to improve survival in patients with metastatic breast cancer,[5] and subsequently found to decrease the incidence of tumours in the contralateral breast after surgery.[6] Forty per cent of newly diagnosed female cancers in the USA arise in tissues responsive to hormones,[7] and it is known that the incidence of endometrial, ovarian, and breast cancers rises in an age dependent fashion similar to other cancers only until the menopause, after which time there is a distinct slowing of the rate of rise.[8] Epidemiological evidence for the role of oestrogen as a primary stimulant of breast cell proliferation is supported by preclinical and clinical studies,[7 8] and unlike the response in the uterus, the proliferation of breast cells is probably augmented by progestogens.[7] Receptor antagonism is therefore an important mechanism of intervention during breast carcinogenesis, and the demonstration of the occurrence of the oestrogen receptor-β in prostate, colon, and ovary offers similar potential for these tissues.[9]

Significant controversy exists between North America and Europe regarding the proved clinical efficacy of the two antioestrogenic agents, tamoxifen and raloxifene, both synthesised more than 20 years ago, well before the cloning and mechanistic elucidation of oestrogen receptors. The Breast Cancer Prevention Trial (BCPT) study in the US involved 13 388 women of "relatively high risk" (that is, with a substantially greater than 1.66% chance of developing breast cancer within five years), but free of detectable invasive breast cancer at study recruitment.[10] Risk was determined using a mathematical model previously published,[11] and included factors such as age (any woman 60 or more years old was eligible), benign breast disease, nulliparity, and family history. Tamoxifen reduced relative risk for subsequent development of breast cancer in the treated group as a whole by 45%, and may also have prevented the progression of established preneoplastic lesions. These results led the Food and Drug Administration to approve the use of tamoxifen (20 mg/day) by women deemed at increased risk of developing breast cancer, albeit with consideration of its toxicities. Tamoxifen causes hot flushes, vaginal bleeding or discharge, fluid retention and amenorrhoea, increases risk for venous thromboembolic events[10] and can cause endometrial hyperplasia, dysplasia, and carcinoma.[12] Extrapolating from data on its effect in the adjuvant chemotherapy setting, one might speculate that tamoxifen continues to reduce the incidence of primary breast cancer for at least five years after treatment stops.[2] However, caution is advised since no reduction in mortality has yet been demonstrated in the BCPT study, perhaps because this drug may prevent only oestrogen receptor positive tumours,[13] which may respond to hormonal manipulation even once carcinoma is established.

Results favouring the prevention of breast cancer have also been published in a trial of raloxifene in postmenopausal women with known osteoporosis, but not considered at increased risk of breast cancer.[14] A larger breast cancer prevention trial of raloxifene is ongoing, enrolling 20 000 women and due for publication in 2006. Raloxifene, licensed in most countries only for the chemoprevention of osteoporosis, is generally better tolerated than tamoxifen. It may also cause hot flushes and peripheral oedema, and does increase risk for thromboembolism,[15] but may not possess the endometrial stimulatory properties of tamoxifen.[12 14]

In contrast to the BCPT study, two smaller European trials of tamoxifen in healthy women, with lower average risk factors, have not demonstrated any protective effects.[16 17] In the Italian study,[16] post-hysterectomy patients were included, and the British study[17] allowed patients to continue supplemental oestrogen therapy; the mean age of participants for both trials was lower than the BCPT study. Consequently, in Europe the results of the International Breast Cancer Intervention study (IBIS) are awaited, in which more than 4000 women with at least a twofold increased risk of developing breast cancer have been recruited.

The controversy regarding the benefit of agents such as tamoxifen in preventing breast cancer in the US compared with Europe illustrates the importance of subject selection in large scale chemoprevention trials. It is feasible that high risk subgroups, perhaps with particular molecular defects (see below) or a certain receptor status in preneoplastic lesions, must be studied for statistical significance to be demonstrated. Indeed such individuals may have the most favourable benefit-toxicity ratios.

Box 1: Key points

- There is increasing epidemiological evidence that certain drugs may decrease the incidence of cancer.
- Two drugs have been licensed in the USA for the chemoprevention of breast and colorectal cancers in selected high risk individuals.
- Folate and retinol have been shown to decrease the incidence of colorectal and skin squamous cell cancers respectively in large scale intervention trials.
- Aspirin, NSAIDs, and ACEIs may also reduce cancer incidence, as may certain phytochemicals.
- Practitioners should be amenable to referral of individuals in whom there is objective evidence of high risk that they may develop certain cancers.

Box 2: Five key references

1. Chemoprevention Working Group. Prevention of cancer in the next millennium: report of the chemoprevention working group to the American Association for Cancer Research. *Cancer Res* 1999;**59**:4743–58.
2. Lippman SM, Lee JJ, Sabichi AL. Cancer chemoprevention: progress and promise. *J Natl Cancer Inst* 1998;**90**:1514–28.
3. Kelloff JG, Crowell JA, Hawk ET, *et al.* Strategy and planning for chemopreventive drug development: clinical development plans II. *J Cell Biochem* 1996;**26S**:54–71.
4. Sharma RA, Manson MM, Gescher A, *et al.* Colorectal cancer chemoprevention: biochemical targets and clinical development of promising agents. *Eur J Cancer* 2001;**37**:12–22.
5. Sporn MB, Suh N. Chemoprevention of cancer. *Carcinogenesis* 2000;**21**:525–30.

Aspirin and NSAIDs

Retrospective epidemiological studies suggest a decreased incidence of cancers of the oesophagus, stomach, colon, and rectum in regular users of non-steroidal anti-inflammatory drugs (NSAIDs).[18] The most convincing evidence exists for regular usage of aspirin, which may reduce the incidence of colorectal cancer by up to 50%,[19 20] although there may be a delay of approximately one decade before the benefits of daily usage are seen.[21] Colorectal adenomas have been regarded as the quintessential precursor lesions of cancer since the 1970s,[22] and are present in a third of the general population by the age of 50 years and approximately half the population by the age of 70 years.[23] Mutations in the *APC* gene, first described in the inherited syndrome of familial adenomatous polyposis (FAP), are found in 80% of all colorectal adenomas and carcinomas.[24] Although FAP accounts for only 0.5% of all colorectal cancers, this disease may therefore represent a useful model of the more common sporadic form of this cancer.

In individuals with FAP, the presence of the *APC* gene defect confers a nearly 100% lifetime risk of developing colorectal cancer.[24] At present such individuals are advised to undergo regular colonoscopy with or without colectomy. It has been known for many years that aspirin and NSAIDs, such as sulindac, can cause regression of FAP polyps, although adenomas do recur and regrow when treatment is curtailed.[25] The postulated mechanism is their ability to inhibit the cyclo-oxygenase (COX) enzyme involved in prostaglandin synthesis. COX consists of two isoforms, and aspirin's irreversible acetylation of COX-1 is thought to account predominantly for its gastrointestinal toxicity.[26] Highly selective inhibitors of COX-2, such as celecoxib, have therefore been developed. COX-2 has been implicated in the pathogenesis of human cancers of the colorectum, breast, head/neck, lung, pancreas, stomach, and prostate.[26] Celecoxib, originally licensed in Europe and North America for the treatment of arthritis, was recently approved by the US Food and Drug Administration as an adjunct to usual care for patients with FAP. This decision was based on the results of a double blind, placebo controlled trial in 83 patients with FAP, which demonstrated that 400 mg of celecoxib twice daily for six months resulted in polyp incidence 28% lower than placebo.[27] Other "surrogate" biomarkers, such as prostaglandin synthesis or COX-2 expression, were not measured. Such biomarkers allow prediction of effects on cancer mortality based on scientific hypotheses, for example colorectal cancer develops in adenomas,[22] COX-2 levels in adenomas represent carcinogenic progression,[26] and suppression of COX-2 levels may represent efficacy of intervention.[4]

The long term safety of selective COX-2 inhibitors such as celecoxib remains unknown, but early indications are that their side effect profile is similar to that of traditional NSAIDs, although peptic ulceration appears to be less likely than for the older agents.[28] These agents are likely to maintain selectivity for COX-2 over COX-1 at the dose range used in the published study.[27] Irreversible inhibition of COX-1 by aspirin accounts for its antiplatelet effect since these cells do not possess nuclei and are

Box 3: Useful websites for monitoring chemoprevention trials ongoing

- http://clinicaltrials.gov/
- http://cancernet.nci.nih.gov/cgi-bin/srchcgi.exe
- http://www.cdc.gov/cancer/index.htm
- http://iarc.fr/pageroot/UNITS/CHP.HTM
- http://cancerindex.org/clinks4t.htm

Questions (see answers on p 496)

1. Which two drugs have been licensed by the US Food and Drug Administration for the chemoprevention of breast and colorectal cancers respectively; in which highly selected individuals?
2. According to the Nurses' Health Study, how much folate must be taken as a supplement for how long in order to decrease the incidence of colorectal cancer?
3. (A) In the pathogenesis of which human cancers has COX-2 been implicated? (B) Which drugs inhibit the COX enzyme?
4. Why are biomarkers useful in cancer chemoprevention trials?
5. (A) In what disease does differentiation therapy with all-*trans* retinoic acid lead to complete remission in the vast majority of patients? (B) Which skin cancer might retinol prevent?
6. In which dietary components are the following putative cancer chemopreventives found: EGCG, curcumin, genistein, resveratrol?

unable to resynthesise the enzyme. A low dose of aspirin is therefore equally efficacious in cardiovascular chemoprevention as larger doses, and lasts for the duration of each platelet life in vivo. This is fortunate since the likelihood of peptic ulceration, haemorrhage, and renal impairment do increase with dose.[29] In contrast, aspirin inhibits COX-2 by competitive inhibition and one might expect that if this is the mechanism of its cancer chemopreventive efficacy, higher doses should be more beneficial. One epidemiological study suggests that this may be the case,[30] but measurement of prostaglandin levels in a target tissue such as colon epithelium has suggested that a dose of aspirin as low as 81 mg/day may be sufficient for cancer chemoprevention.[31] The duration of use also appears critical, and there may be a "latency" period of a decade or more before statistically significant trends are seen in colorectal cancer incidence. It should also be noted that, unlike selective COX-2 inhibitors, aspirin exhibits other biological actions such as inhibition of peroxidase enzyme activity[32] and induction of apoptosis in cancer cells.[27] We conclude that although only one selective COX-2 inhibitor has been licensed for cancer chemoprevention in FAP patients in the USA, the more toxic drug aspirin may have more diverse efficacy in preventing sporadic cancer.

Folate

Folate supplementation is well established in the chemoprevention of fetal neural tube defects during pregnancy. Consequently, many foods are fortified with folic acid for its potentially beneficial effects. Large amounts of folate in the diet also appear to be protective against the development of colorectal adenomas.[33] Folate is central to methyl group metabolism, and as such may influence both methylation of DNA and the available nucleotide pool for DNA replication and repair.[34] DNA hypomethylation is an early step in colon carcinogenesis.[35] Vitamin B_{12} is a cofactor in this pathway, and the *val/val* polymorphism of the *methylenetetrahydrofolate reductase* (*MTHFR*) gene may also influence the association between folate intake and the development of carcinoma from adenoma.[36 37]

The degree of benefit from taking folate supplements may be greater than that from its consumption in the diet. The Nurses' Health Study began in 1976 and followed 121 700 married, registered female nurses of ages 30–55 years prospectively by questionnaire. In one subgroup, supplementation with folate was protective against colorectal cancer, with the greatest risk reduction among women taking daily doses of more than 400 μg folate; however, this reduction became statistically significant only after 15 years of use.[38] It is also becoming apparent that the protective role of folate supplementation may be greatest for those genetically predisposed to colorectal cancer, and that the benefit conferred by *MTHFR* genotype may be offset by a methyl deficient diet.[37]

Retinoids

Like folate, retinol (vitamin A) is available in the diet, particularly from green leafy vegetables, liver, eggs, and milk. Retinol is the precursor of all physiologically occurring retinoids, and is required for normal vision and reproduction. The oxidation products of retinol are essential for the maintenance of normal epithelial differentiation (reviewed in Hansen *et al*[39]).

It has been hypothesised that retinoids, at optimal or supraphysiological levels, inhibit the development of epithelial carcinogenesis. This activity is utilised in acute promyelocytic leukaemia, in which treatment with all-*trans* retinoic acid leads to complete remission in up to 95% of patients.[40] In the Skin Cancer Prevention-Actinic Keratosis trial, 25 000 IU of retinol was taken daily and primary prevention of squamous and basal cell carcinomas of the skin were the two endpoints measured.[41] The trial involved 2297 subjects deemed to be at moderate risk, and the treatment was found to prevent squamous cell carcinoma significantly. A drawback of this study is the fact that increasing dietary ingestion of retinol is unlikely to deliver more retinol to skin; it merely leads to an accumulation of retinyl esters in liver tissue. It has therefore been proposed that direct administration of retinoids to target tissues may be more effective chemoprevention than oral supplementation.[39]

It is vital that any agent under consideration for chemoprevention in healthy high risk individuals over prolonged periods of time should not cause more harm than benefit.[4] Although the retinoids, isotretinoin[42] and retinol palmitate,[43] have been demonstrated to prevent second primary cancers in patients with malignancies of the lung and head/neck,

Answers

1. Tamoxifen has been licensed for the primary chemoprevention of breast cancer in high risk individuals after consideration of its toxicities. Risk can be determined mathematically, using factors such as age, benign breast disease, nulliparity, and family history. Celecoxib, the selective COX-2 inhibitor, has been licensed as an adjunct to standard treatment of patients with familial adenomatous polyposis. This represents secondary chemoprevention (see fig 2).
2. In one subgroup of this large trial, supplementation with folate was protective against colorectal cancer, reaching signficance in women taking daily doses of more than 400 μg folate for 15 years.
3. (A) COX-2 has been implicated in the pathogenesis of human cancers of the colorectum, breast, head/neck, lung, pancreas, stomach, and prostate. (B) Aspirin, NSAIDs, selective COX-2 inhibitors, and certain phytochemicals (for example curcumin, resveratrol) inhibit the COX enzyme.
4. If cancer mortality acts as the only endpoint, chemoprevention trials must study time periods of 5–15 years in order to reach significance, with no indication of beneficial or detrimental effects. "Surrogate" biomarkers allow prediction of the efficacy of intervention based on scientific hypotheses of carcinogenesis.
5. (A) Acute promyelocytic leukaemia. (B) Squamous cell carcinoma of the skin.
6. They are found in tea, the spice turmeric, soya, and wine respectively.

compliance is often problematic on account of toxicity. Similarly the synthetic retinoid, fenretinide, significantly decreased the risk of breast cancer in premenopausal women in a tertiary chemoprevention study (see fig 2), but night blindness and erythema proved prohibitive at higher doses.[44] Newer retinoids that selectively bind to retinoid X receptors appear highly chemopreventive in preclinical models of epidermal and mammary carcinogenesis, and do not possess the toxicity/teratogenicity profile of the classical retinoids.[9]

Others

Treatment of hypertensive patients with captopril, an angiotensin-I converting enzyme inhibitor (ACEI), is associated with a reduced risk of developing malignancy, particularly lung and breast cancers.[45] This may relate to the ability of ACEIs to inhibit angiogenesis, the formation of new blood vessels vital to growth of cancers beyond 1–2 mm^3.[46] As well as inhibiting chemotaxis of capillary cells, captopril is a free sulfhydryl donor which can lead to the generation of antiangiogenic compounds in vitro.[47] Captopril also inhibits matrix metalloproteinase activity, integral to the neovascularisation process.[48]

Finally, certain phytochemicals offer a minimally toxic form of intervention during carcinogenesis with similar mechanisms to those described above.[49] An example is provided by curcumin, a potent antioxidant derived from the spice turmeric. This compound appears innocuous in doses up to 8 g/day,[50] yet possesses biological activity in micromolar concentrations very similar to that of aspirin.[51 52] In addition, it appears to inhibit other processes linked to carcinogenesis, such as lipid peroxidation[53] and angiogenesis.[54] Other such putative chemopreventive agents include epigallocatechin gallate (EGCG) in green and black tea, the isoflavone genistein found in soya, resveratrol from wine, and micronutrients such as selenium and vitamin D_3.[55 56] Both curcumin and EGCG have been shown to affect processes pivotal to cell signalling, such as kinases, cell cycle regulatory proteins, and downstream elements of cellular signalling cascades crucial for cell proliferation (reviewed in Gescher *et al*[49]). A convergence is thus developing between the targets identified for cancer chemopreventive agents and those for chemotherapeutic drugs. It is conceivable that modification of these targets may prove more efficacious in intervention when fewer cellular components are malfunctioning (chemoprevention) than treatment when dysregulation of many pathways is already established (chemotherapy).

Conclusions

Physicians should be aware of the potential cancer chemopreventive properties of commonly used drugs. Tamoxifen was found to reduce breast cancer development in high risk women in one large controlled intervention study, and the results of a similar study in Europe are awaited. Consideration of its toxicities must be weighed up against the cancer risk for each individual. Raloxifene, already used in the chemoprevention of osteoporosis, may offer a less toxic alternative to tamoxifen. A large prospective intervention study has found that folate supplementation may decrease the incidence of colorectal cancer after 15 years of daily use. NSAIDs and celecoxib have been shown to cause regression of adenomas, considered the premalignant lesions in this disease. Retrospective epidemiological surveys have suggested that aspirin may significantly reduce the incidence of colorectal cancer after at least nine years of daily usage. As demonstrated by studies of celecoxib and folate, consideration of risk ratios and genetic predisposition is increasingly important in recruitment and subset analysis of chemoprevention trials. Retinoids have been shown to be efficacious in the primary prevention of skin squamous cell carcinoma, and the tertiary chemoprevention of cancers of breast and head/neck, but toxicity has proved limiting. ACEIs and phytochemicals may also prevent certain cancers, and the latter may represent less toxic

alternatives to conventional drugs. There are complex issues in assessing one's risk of developing cancer and the potential benefit from intervention; practitioners should be amenable to patient referral should individuals wish to discuss these issues with specialist oncologists.

The authors are supported by the UK Medical Research Council, a research fellowship from the University Hospitals of Leicester and the Institute of Cancer Studies, Leicestershire and Rutland.

1 Tattersall MHN, Thomas H. Recent advances: oncology. *BMJ* 1999;318:445–8.
2 Jordan VC. Tamoxifen: a personal retrospective. *Lancet Oncol* 2000;1:43–9.
3 Sporn MB, Newton DL. Chemoprevention of cancer with retinoids. *Fed Proc* 1979;38:2528–34.
4 Sharma RA, Manson MM, Gescher A, *et al.* Colorectal cancer chemoprevention: biochemical targets and clinical development of promising agents. *Eur J Cancer* 2001;37:12–22.
5 Cole MP, Jones CT, Todd ID. A new anti-oestrogenic agent in late breast cancer. *Br J Cancer* 1971;25:270–5.
6 Cuzick J, Baum M. Tamoxifen and contralateral breast cancer. *Lancet* 1985;ii:282.
7 Henderson BE, Bernstein L, Ross R. Etiology of cancer: hormonal factors. In: DeVita VT, Hellman S, Rosenberg RA, eds. *Cancer: principles and practice of oncology.* 5th Ed. Philadelphia: Lippincott-Raven, 1997: 219–29.
8 Henderson BE, Ross RK, Pike MC. Endogenous hormones as a major factor in human cancer. *Cancer Res* 1982;42: 3232–9.
9 Sporn MB, Suh N. Chemoprevention of cancer. *Carcinogenesis* 2000;21:525–30.
10 Fisher B, Costantino JP, Wickerham DL, *et al.* Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J Natl Cancer Inst* 1998;90:1371–88.
11 Gail MH, Brinton LA, Byar DP, *et al.* Projecting individualized probablities of developing breast cancer for white females who are being examined annually. *J Natl Cancer Inst* 1989;81:1879–86.
12 Bergman L, Beelen MLR, Gallee MPW, *et al.* Risk and prognosis of endometrial cancer after tamoxifen for breast cancer. *Lancet* 2000;356:881–7.
13 Early Breast Cancer Trialists Collaborative Group. Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet* 1998;351:1451–67.
14 Cummings SR, Eckert S, Krueger KA, *et al.* The effect of raloxifene on risk of breast cancer in postmenopausal women. *JAMA* 1999;281:2189–97.
15 Hulley SB, Grady D, Bush T, *et al.* Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopusal women. *JAMA* 1998;280:605–13.
16 Veronesi U, Maisonneuve P, Costa A, *et al.* Prevention of breast cancer with tamoxifen. *Lancet* 1998;352:93–7.
17 Powles T, Eeles R, Ashley S, *et al.* Interim analysis of the incidence of breast cancer in the Royal Marsden Hospital tamoxifen randomised chemoprevention. *Lancet* 1998;352: 98–101.
18 Langman MJS, Cheng KK, Gilman EA, *et al.* Effect of anti-inflammatory drugs on overall risk of common cancer: a case-control study in a general practice research database. *BMJ* 2000;320:1642–6.
19 Giovannucci E, Rimm EB, Stampfer MJ, *et al.* Aspirin use and the risk for colorectal cancer and adenoma in male health professionals. *Ann Intern Med* 1994;121:241–6.
20 Thun MJ, Namboodiri MM, Heath CW Jr. Aspirin use and reduced risk of fatal colon cancer. *N Engl J Med* 1991;325: 1593–6.
21 Collet J-P, Sharpe C, Belzile E, *et al.* Colorectal cancer prevention by non-steroidal anti-inflammatory drugs: effects of dosage and timing. *Br J Cancer* 1999;81:62–8.
22 Muto T, Bussey HJR, Morson BC. The evolution of cancer of the colon and rectum. *Cancer* 1975;36:2251–70.
23 Williams AR, Balasoriya BA, Day DW. Polyps and cancer of the large bowel: a necropsy study in Liverpool. *Gut* 1982;23: 835–42.
24 Goss KH, Groden J. Biology of the adenomatous polyposis coli tumor suppressor. *J Clin Oncol* 2000;18:1967–79.
25 Giardiello FM, Hamilton SR, Krush AJ, *et al.* Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. *N Engl J Med* 1993;328:1313–16.
26 Prescott SM, Fitzpatrick FA. Cyclooxygenase-2 and carcinogenesis. *Biochim Biophys Acta* 2000;1470:M68–78.
27 Steinbach G, Lynch PM, Phillips RKS, *et al.* The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. *N Engl J Med* 2000;342:1946–52.
28 Are rofecoxib and celecoxib safer NSAIDs? *Drug Ther Bulletin* 2000;38:81–6.
29 Roderick PJ, Wilkes HC, Meade TW. The gastrointestinal toxicity of aspirin—an overview of randomised controlled trials. *Br J Clin Pharmacol* 1993;35:219–26.
30 Rodriguez LAC, Huerta-Alvarez C. Reduced incidence of colorectal adenoma among long-term users of nonsteroidal antiinflammatory drugs: a pooled analysis of published studies and a new population-based study. *Epidemiology* 2000;11:376–81.
31 Ruffin MT, Krishnan K, Rock CL, *et al.* Suppression of human colorectal mucosal prostaglandins: determining the lowest effective aspirin dose. *J Natl Cancer Inst* 1997;89: 1152–60.
32 Marnett LJ. Aspirin and the potential role of prostaglandins in colon cancer. *Cancer Res* 1992;52:5575–89.
33 Ferranoni M, La Vecchia C, D'Avanzo B, *et al.* Selected micronutrient intake and the risk of colorectal cancer. *Br J Cancer* 1994;70:1150–5.
34 Potter JD. Colorectal cancer: molecules and populations. *J Natl Cancer Inst* 1999;91:916–32.
35 Feinberg AP, Vogelstein B. Hypomethylation of ras oncogenes in primary human cancers. *Biochem Biophys Res Comm* 1983;111:47–54.
36 Chen J, Giovannucci E, Kelsey K, *et al.* A methylenetetrahydrofolate reductase polymorphism and the risk of colorectal cancer. *Cancer Res* 1996;56:4862–4.
37 Chen J, Giovannucci E, Hankinson SE, *et al.* A prospective study of methylenetetrahydrofolate reductase and methionine synthase gene polymorphisms, and risk of colorectal adenoma. *Carcinogenesis* 1998;19:2129–32.
38 Giovannucci E, Stampfer MJ, Colditz GA, *et al.* Multivitamin use, folate, and colon cancer in women in the Nurses' Health Study. *Ann Intern Med* 1998;129:517–24.
39 Hansen LA, Sigman CC, Andreola F, *et al.* Retinoids in chemoprevention and differentiation therapy. *Carcinogenesis* 2000;21:1271–9.
40 Huang ME, Ye YC, Chen SR, *et al.* Use of all-trans retinoic acid in the treatment of acute promyelocytic leukaemia. *Blood* 1988;72:567–72.
41 Moon TE, Levine N, Cartmel B, *et al.* Effect of retinol in preventing squamous cell skin cancer in moderate-risk subjects: a randomised double-blind controlled trial. *Cancer Epidemiol Biomarkers Prev* 1997;6:949–56.
42 Hong WK, Lippman SM, Itri LM, *et al.* Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck. *N Engl J Med* 1990;323:795–801.
43 Pastorino U, Infante M, Maioli M, *et al.* Adjuvant treatment of stage I lung cancer with high-dose vitamin A. *J Clin Oncol* 1993;11:1216–22.
44 Veronesi U, de Palo G, Marubini E, *et al.* Randomised trial of fenretinide to prevent second breast malignancy in women with early breast cancer. *J Natl Cancer Inst* 1999;91: 1847–56.
45 Lever AF, Hole DJ, Gillis CR, *et al.* Do inhibitors of angiotensin-I-converting enzyme protect against the risk of cancer? *Lancet* 1998;352:179–84.
46 Folkman J. Clinical applications of research on angiogenesis. *N Engl J Med* 1995;333:1757–63.
47 Gately S, Twardowski P, Stack MS, *et al.* The mechanisms of cancer mediated conversion of plasminogen to the angiogenesis inhibitor angiostatin. *Proc Natl Acad Sci U S A* 1997;94:10868–72.
48 Volpert OV, Ward WF, Lingen MW, *et al.* Captopril inhibits angiogenesis and slows the growth of experimental tumors in rats. *J Clin Invest* 1996;98:671–9.
49 Gescher AJ, Sharma RA, Steward WP. Cancer chemoprevention by dietary constituents: a salutary tale of failure and promise. *Lancet Oncol* 2001 (in press).
50 Cheng AI, Lin JK, Hsu MM, *et al.* Phase I chemoprevention clinical trial of curcumin (abstract). *Tumor Biology* 1998; 558a.
51 Plummer SM, Holloway KA, Manson MM, *et al.* Inhibition of cyclooxygenase 2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF-kB activation via the NIK/IKK signalling complex. *Oncogene* 1999;18:6013–20.
52 Yin M-J, Yamamoto Y, Gaynor RB. The anti-inflammatory agents aspirin and salicylate inhibit the activity of IkB kinase-β. *Nature* 1998;396:77–80.
53 Rao CV, Rivenson A, Simi B, *et al.* Chemoprevention of colon carcinogenesis by dietary curcumin, a naturally occurring plant phenolic compound. *Cancer Res* 1995;55: 259–66.
54 Arbiser JL, Klauber N, Rohan R, *et al.* Curcumin is an in vivo inhibitor of angiogenesis. *Mol Med* 1998;4:376–83.
55 Kelloff JG, Crowell JA, Hawk ET, *et al.* Strategy and planning for chemopreventive drug development: clinical development plans II. *J Cell Biochem* 1996;26S:54–71.
56 Manson MM, Gescher A, Hudson EA, *et al.* Blocking and suppressing mechanisms of chemoprevention by dietary constituents. *Toxicol Lett* 2000;112:499–505.
57 Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990;61:759–67.
58 Forni G, Lollini PL, Musiani P, *et al.* Immunoprevention of cancer: is the time ripe? *Cancer Res* 2000;60:2571–5.
59 De Flora S, Balansky R, Scatolini L, *et al.* Adducts to nuclear DNA and mitochondrial DNA as biomarkers in chemoprevention. In: Stewart BW, McGregor D, Kleihues P, eds. *Principles of chemoprevention.* IARC Scientific Publications No 139, 1996: 291–301.
60 Sharma RA. Cancer chemoprevention: a clinical reality. *J R Soc Med* 2000;93:518–20.

APPENDIX 2C: Gescher A, Sharma RA, Steward WP. Cancer chemoprevention by dietary constituents: A salutary tale of failure and promise. *Lancet Oncol* 2001, **2**: 371-379.

Cancer chemoprevention by dietary constituents: a tale of failure and promise

Andreas J Gescher, Ricky A Sharma and William P Steward

Although the results of clinical intervention trials of β-carotene to prevent lung cancer, and of dietary augmentation with fibre or fruit and vegetables to reduce the occurrence of colonic polyps have so far been negative, a structured path for the development of diet-derived constituents as cancer chemopreventive agents is emerging. Putative agents are identified on the basis of epidemiological and preclinical mechanistic studies. Some examples of promising diet-derived chemopreventive agents are folate, curcumin, genistein, and tea catechins. Long-term supplementation of the diet with folate seems to lower the risk of colorectal cancer. Curcumin in the spice turmeric, genistein in soya, and catechins in tea have tumour-suppressing properties in rodent models of carcinogenesis, and they interfere with cellular processes involved in tumour promotion and progression. Kinases, telomerase, cyclooxygenase-2, triggers of apoptosis, and transcription factors AP1 and nuclear factor κB are among the cellular targets. The investigation of dietary constituents should follow a structured design, incorporating parallel preclinical studies of the food source and the isolated agent in terms of efficacy, toxicity, biological mechanisms, and pharmacokinetics. Either the food source or the isolated agent should be selected for further development on the basis of dose-efficacy and toxicity data. Pilot clinical trials on the pharmacokinetics and mechanism-based markers of efficacy of the selected intervention should precede phase I–III development in suitable populations.
Lancet Oncol 2001; **2:** 371–79

One of the questions that cancer patients most frequently ask their oncologists is "Can my diet make a difference?" Answers to this question tend to be vague. Any recommendations must be based on international epidemiological studies of increased risk or protection conferred by diets, because results from intervention trials of pure constituents derived from the diet have so far been inconclusive. Governments in the UK and other Western countries now tread very carefully after the recent disputes about genetically modified foods and prion-infected meat.

Another reason for patients' questions about diet is the publicised role of dietary modification in the prevention of cardiovascular disease, which remains the major cause of death in most developed countries. Both cardiovascular disease and cancer are chronic illnesses, with a latency of many years before clinical diagnosis. It is therefore reasonable to postulate that dietary components may prevent cancer from reaching its invasive and metastatic stages. Alternatively, constituents of the diet may reduce the risk of second primaries or modify the behaviour of established cancer.

Figure 1. Dietary sources of cancer chemopreventive agents: (left to right) turmeric (curcumin), soya (genistein) and green tea (tea catechins).

In this review, some of the evidence supporting the cancer-chemopreventive efficacy of pure dietary constituents is discussed, with the aim of showing how future developments might help health professionals to answer questions about diet and its supplementation. Results of recent clinical studies illustrate a change in emphasis in cancer chemoprevention, towards mechanism-driven hypotheses. Phytochemicals such as epigallocatechin gallate (EGCG) in green tea, the curry constituent curcumin, and the soya isoflavone genistein (Figures 1 and 2) are thought to be the agents of the cancer-chemopreventive properties of their dietary sources. These phytochemicals, and micronutrients such as folate, exemplify innocuous diet-derived agents with interesting modes of action. This review does not attempt to review cancer chemopreventive agents comprehensively, because detailed information can be found in other recent publications, for example those by Singh and Lippman[1] and Kelloff.[2] Instead, it aims to outline

AJG, RAS, and WPS are at the Department of Oncology, University of Leicester, Leicester, UK.
Correspondence: Professor A Gescher, MRC Toxicology Unit, Hodgkin Building, University of Leicester, Leicester LE1 9HN, UK.
Tel: +44 (0) 116 252 5618. Fax: +44 (0) 116 252 5616.
Email: ag15@le.ac.uk

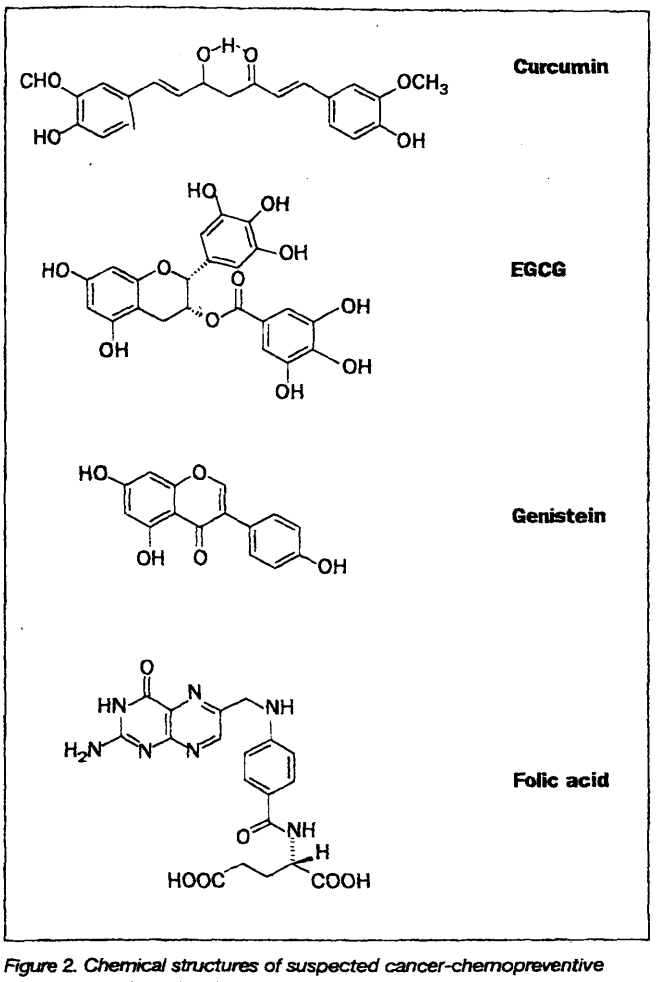


Figure 2. Chemical structures of suspected cancer-chemopreventive constituents of the diet. EGCG, epigallocatechin gallate.

principles that are generally applicable to the evaluation and development of dietary constituents as chemopreventive agents.

Mechanisms of chemoprevention

Chemoprevention can be defined as the use of natural or synthetic chemicals, to reverse, suppress, or prevent the process of carcinogenesis.[3] Figure 3 illustrates the different levels of chemoprevention and defines which individuals may benefit.[4,5] Carcinogenesis results from the accumulation of multiple sequential mutations and alterations in nuclear and cytoplasmic molecules, culminating in invasive neoplasms. These events have traditionally been separated into three phases: initiation, promotion, and progression. They are increasingly linked to biochemical events. Initiation, which is rapid, involves carcinogen binding and damage to DNA. In the promotion phase, which is generally reversible, tumour promoters, acting as mitogens, induce clones of initiated cells to expand. Promotion is a consequence of the functional loss of regulatory proteins and cellular checkpoints important for proliferation and apoptosis. Progression defines the stage in which phenotypically and genotypically altered cells develop irreversible macroscopic and microscopic changes. Both promotion and progression phases are of long duration, probably many years. The temporal sequence implied in this scheme is now thought to be overly simplistic and somewhat misleading. Nevertheless, this model allows convenient categorisation of chemopreventive agents into those that can block initiation and those that suppress promotion and progression.[4] Early solid cancers are generally detected as intraepithelial neoplasia or carcinoma *in situ*, which correspond to the promotion and progression stages. 'Anti-promotion' and 'anti-progression' agents are therefore of particular clinical interest. Ultimately, such agents prevent the growth and survival of cells already committed to becoming malignant. How might they do this?

Pleiotropism of diet-derived agents

Figure 4 highlights mechanistic targets that have been suggested for diet-derived chemopreventive agents. Mechanisms for preventing promotion and progression involve inhibition of events controlled by cascades of cellular signal-transduction molecules. Normally, these cascades are triggered by hormones or growth factors, and in carcinogenesis they are either faultily expressed or activated by tumour-promoting stimuli. Many of the substances which are pivotal in cell signalling, and thus attractive targets for cancer chemoprevention, are kinases. Curcumin, a major constituent of the spice turmeric, inhibits the activities of protein kinase C, epidermal growth factor receptor kinase, oncoprotein tyrosine kinase p185 (neu), mitogen-activated protein kinases, and c-Jun terminal kinase.[5-9] Such inhibition is not necessarily the corollary of direct enzyme interaction, but can reflect interference with activating elements upstream of the kinase. Reactive oxygen species have been implicated as causative stimuli in the carcinogenic process (for example, their involvement in cell-cycle progression induced by the oncogene *RAS*).[10] Cell-cycle regulatory proteins and checkpoints are downstream elements of cellular signalling cascades crucial for cell proliferation. They are also affected by curcumin[11] and EGCG.[12] Tea catechins, curcumin, and genistein have antioxidant properties and can therefore neutralise the detrimental effects of reactive oxygen species, a property that is thought to contribute to their anticarcinogenic activity. Another important reactive species with signalling potential is the messenger molecule, nitric oxide (NO). Curcumin was found to inhibit expression of the gene for NO synthase in livers of mice stimulated by lipo-polysaccharades.[13]

Cyclo-oxygenase 2 (COX2) is an important modulator protein in the eicosanoid pathway, where it catalyses the conversion of arachidonic acid to prostaglandins. This enzyme has recently received much attention as a target for chemopreventive agents, because it is overexpressed in various malignant diseases[14] Selective inhibitors of COX2, such as celecoxib, competitively inhibit this isoenzyme; their long-term safety is being evaluated in the clinical management of arthritis. A short-term randomised phase II study of celecoxib in patients with familial adenomatous polyposis coli (FAP) elicited much excitement, because the treatment was associated with significant regression of polyps.[15] In previous short-term studies of the non-selective COX inhibitor sulindac, in patients with FAP, adenomas

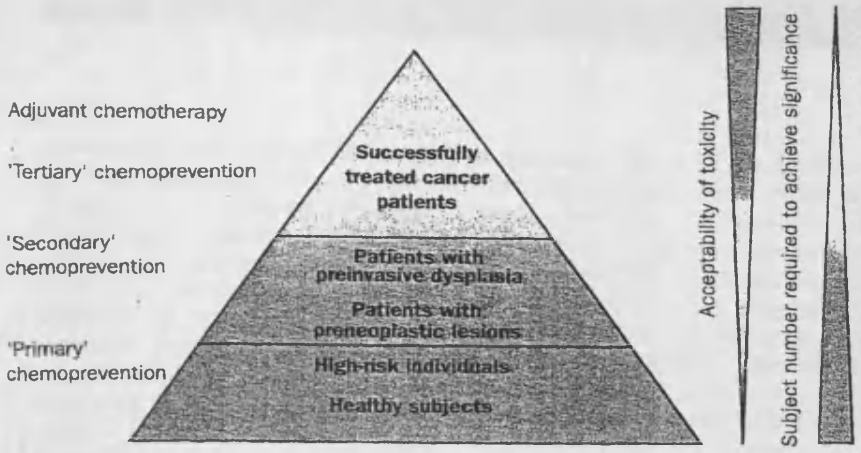


Figure 3. Levels of chemoprevention and characteristics of individuals who may benefit from chemoprevention (modified from reference 78).

increased in size and number on discontinuation of treatment.[16] Long-term treatment with COX-inhibiting drugs is therefore likely to be necessary to interfere with premalignant conditions effectively; this issue is particularly pertinent in clinical intervention trials in which polyp characteristics are used as surrogate biomarkers of chemopreventive efficacy. Whether celecoxib causes adverse effects that might militate against longer-term administration remains to be seen. Curcumin, which shares chemical structural features with aspirin, inhibits COX-2 at the transcriptional level in cells *in vitro*.[17] Although curcumin displays low oral bioavailability, concentrations inhibitory to COX-2 (greater than 10^{-6} mol/L) are attainable in the colonic mucosa of rats, after dietary administration.[18] However, the doses needed to achieve such tissue concentrations far exceed those normally consumed as turmeric in the diet.[19] Aspirin, salicylate, and curcumin share an ability to inhibit the IκB kinase signalling complex, which activates the transcription factor NF-κB in response to cytokines such as tumour necrosis factor α.[17,20] Aspirin, unlike curcumin, can irreversibly acetylate the COX protein, thus inhibiting its peroxidase activity.[21] Curcumin also inhibits signalling pathways involving the kinase JNK and the transcription factor AP1.[22,23] These events are important in the induction of expression of the gene for COX-2 by inflammatory and tumour-promoting stimuli,[24] so curcumin is likely to inhibit transcription of the gene at more than one level. Barnes and colleagues have proposed that the chemopreventive efficacy of oestrogen-receptor-modulating soy constituents, such as genistein, should be compared with that of tamoxifen in women at high risk of developing breast cancer.[25] By analogy, the balance between chemopreventive efficacy and toxicity during long-term treatment will ultimately determine whether dietary supplementation with food-derived agents such as curcumin or selective inhibitors of COX-2, offer greater potential in the chemoprevention of colon cancer. If both strategies prove differentially effective, accurate assessment of the risk of developing cancer becomes critical, so that the optimal intervention strategy can be selected.[26]

Unchecked telomerase activity has been associated with the genetic instability of transformed cells, and EGCG and other tea catechins can inhibit this enzyme.[27] Another target of chemopreventive agents is angiogenesis, the formation of new blood vessels in preneoplastic tissue. Tea catechins[28] and curcumin[29,30] are both antiangiogenic. Eventually, interference with cellular signalling pathways by agents such as curcumin and EGCG, and consequent down-regulation of mitogenic transcription factors, may induce apoptosis of preneoplastic and transformed cells, and thus curtail their survival.[31,32] Many of the targets outlined above – angiogenesis, kinases, transcription factors, apoptosis, and telomerases – are intrinsically related to the 'hallmarks' of cancer,[33] a small number of molecular, biochemical, and cellular traits shared by most and perhaps all types of human cancer. Not surprisingly, these targets are also under investigation as targets for new molecular or 'antisignalling' chemotherapeutic anticancer drugs, currently of great interest to cancer pharmacologists. Both chemopreventive agents and antisignalling drugs increase apoptosis, inhibit kinases and telomerase, and decrease transcription-factor activity and angiogenesis. Consequently, novel antisignalling drugs warrant clinical evaluation as potential cancer-chemopreventive agents, if they lack adverse effects. Indeed, such agents may prove more effective in intervention when fewer cellular components are malfunctioning (chemoprevention) than treatment when all the 'hallmarks' of cancer are established (chemotherapy).

Preclinical models of chemopreventive efficacy

How does one test whether an agent or a diet has chemopreventive efficacy? The Division of Cancer Prevention of the US National Cancer Institute adopts an exemplary but costly approach, in which single dietary constituents are developed side by side with the food source

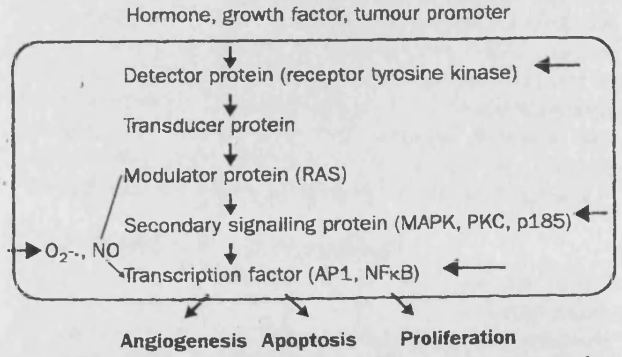


Figure 4. Simplified scheme of cellular signalling cascade and targets for cancer-chemopreventive dietary constituents. Solid line marks the cell membrane, blue arrows denote targets, specific examples of targets in brackets.

until parallel dose-response efficacy is demonstrated.[34] This approach integrates mechanistic studies and biochemical screening *in vitro* with the subsequent evaluation of safety and efficacy in rodents *in vivo*. Initially, substances or food extracts are subjected to tests *in vitro*. These tests use biochemical correlates of chemopreventive efficacy, such as inhibition of kinases, induction of differentiation and apoptosis, protection from mutagenic alterations, and reversal of biochemical changes elicited by tumour promoters. If similarly effective, the potentially more toxic purified agent's development ceases, in preference to investigation of the food source. However, if the purified agent proves more potent, it goes on to development *in vivo*, with a view to treating higher-risk populations in whom toxic effects may be more acceptable. The scope, success, and pitfalls associated with *in vitro* screening have been described by Pezzuto.[35] The method for isolation and laboratory assessment of entirely novel cancer-chemopreventive agents is exemplified by a study of brown rice, which brought to light the potential chemopreventive activity of tricin, an agent which is chemically related to quercetin, a flavone contained in onions and red wine.[36]

There are two types of rodent models in which the activity of chemopreventive agents can be investigated. In chemical models, cancer resembling the human counterpart is induced by exposure to high doses of genotoxic carcinogens. 'Transgenic' models are designed to incorporate the genetic features of carcinogenesis, and affected animals spontaneously develop tumours. Study of colorectal cancer provides a suitable example of use of both types of model. Azoxymethane is commonly used to induce neoplasia (chemical model), and Min (multiple intestinal neoplasia) mice and Msh2-/- mice are examples of genetic models. Min mice carry a heterozygous germline mutation at codon 850 of the *Apc* gene, and the wild-type *Apc* allele is not found in polyps taken from these animals. This model resembles the syndrome of FAP in human beings; although that disease accounts for only 0.5% of all colorectal cancers, defects in this gene are present in 80% of sporadic colorectal cancers.[37] Msh2-/- mice lack the *Msh2* gene, one of several mismatch repair genes that ensure accurate replication of the genome during the cell cycle. Germline mutations in this gene have been implicated in hereditary non-polyposis colorectal cancer and somatic mutations in about 15% of sporadic colorectal cancers.[38]

There are disadvantages with both types of model. Chemical models have the drawback that tumour development differs from that in human beings. Relatively high doses of carcinogens are used in these studies, and the mechanism is very different from the multifactorial aetiology of sporadic cancer. In addition, the molecular genetics of carcinogen-induced tumours in rodents are significantly different from those observed in human beings. In genetic models, phenotypic manifestations also differ from those seen in human disease. Min or Msh2-/- mice develop small intestinal adenomas, whereas in people with colorectal cancer, polyps of the colon and rectum are considered the premalignant lesions. Another disadvantage of the Min model is that the mice do not develop advanced malignant disease because they rapidly die as a consequence of the number and size of polyps. Therefore, such models better reflect early and accelerated stages of carcinogenesis, analogous to inherited types of cancers associated with FAP and hereditary non-polyposis colorectal cancer, and less the sporadic form of colorectal cancer that accounts for the vast majority of clinical cases. As such, genetic models allow scrutiny of similarities in the pathogenesis of sporadic and inherited cancers, such as the roles of gene hypermethylation, DNA repair, and the contents of the colonic lumen in the causation of colorectal cancer.[39]

There is good evidence for the chemopreventive activity of curcumin and tea catechins in rodent models. Curcumin inhibited the development of azoxymethane-induced colon adenocarcinomas in rats, whether the compound was administered during the initiation/post-initiation[40] or the promotion/progression stages of the disease.[41] It also inhibited forestomach cancer in mice, induced by the carcinogen benzo[a]pyrene.[42] Curcumin reduced adenomas in Min mice when added to the diet throughout their lives.[43] The chemopreventive activity of the tea, perhaps mediated predominantly through its major constituent EGCG, has been demonstrated in rodent models of carcinogen-induced skin, lung, and gastrointestinal-tract cancers.[44]

Min mice can be crossed with Msh2-/- mice to yield animals that show an accelerated intestinal adenoma phenotype with numerous dysplastic aberrant crypt foci in the colon. Folate supplementation started before the establishment of neoplastic foci significantly decreased the number of small-intestinal adenomas and colonic aberrant crypt foci. By contrast, when folate supplementation was not started until after the establishment of neoplastic foci, the incidence of adenomas increased rather than decreased.[45] This finding suggests that the conditions of chemopreventive regimens, especially the timing of treatment, have to be optimum for efficacy, and it highlights the possibility that the stage of the carcinogenic process at which intervention occurs is a determinant of unexpected detrimental, as well as expected beneficial, activity. This may help to explain the surprising findings in clinical trials with agents such as β-carotene. Another important determinant of the success or failure of intervention, which can be modelled preclinically, is the potential tissue selectivity of chemopreventive chemicals. This issue was clearly illustrated in studies in rats, showing that although genistein may prevent mammary carcinoma,[46] it seemed to increase tumorigenesis in a chemical model of colon carcinogenesis.[47]

Single agents or combinations?

Combination of cytotoxic agents has brought about important advances in the treatment of lymphomas, childhood tumours, and germ-cell neoplasms. The rationale for combination chemotherapy takes account of the multifactorial nature of cancer, which suggests that the simultaneous use of drugs with different mechanisms of action should be more effective. Can a similar rationale be applied to chemoprevention? A recent paper suggests that it can. The combination of the non-steroidal anti-inflammatory drug sulindac with a newly developed specific inhibitor of epidermal-growth-factor-receptor tyrosine kinase led to a striking decrease in the development of

adenomas in the Min mouse model, and the effect of the combination was much more pronounced than that of each agent individually.[48] Such combinations of pathway-specific chemopreventive agents affect different cellular proliferative and apoptotic control mechanisms, but they bear the risk of synergistic toxicity. Combinations therefore need to be tested initially in preclinical models. For example, piroxicam combined with difluoromethylornithine (DFMO), ellagic acid or 16-α-fluoro-5-androsten-17-one was first tested in rats,[49] which paved the way for the clinical evaluation of the combination of piroxicam with DFMO in human beings.

The use of single chemopreventive diet-derived constituents with pleiotropic activities might seem to offer a natural way to the same end, since they affect several targets of chemoprevention simultaneously. Alternatively, use of carefully chosen combinations of discrete dietary agents, formulated as 'nutriceuticals', might be beneficial to exploit the synergy between them. For example, the ability of EGCG to induce apoptosis and inhibit the growth of malignant PC9 cells was synergistically enhanced by epicatechin (a tea polyphenol), sulindac, or tamoxifen.[50] Similarly, the combination of EGCG and curcumin was synergistic in its effect on the growth of premalignant and malignant oral epithelial cells.[51] Nutritionists might argue that the human diet contains constituents with different chemopreventive mechanisms in the food matrix, thus offering natural combinations that cannot be bettered. This argument is supported by the recent observation that the oxygen-radical-scavenging capacity of an extract of fresh apples that inhibits tumour-cell proliferation is much higher than expected on the basis of its content of vitamin C alone.[52] A major argument for the use of chemopreventive agents in the natural food matrix is the expectation that there will be no adverse effects, based on the extended experience of long-term consumption, which might help to gauge the likelihood of unwanted effects in human beings more easily than for novel synthetic compounds. However, this expectation may be unrealistic, as highlighted by a report that implicates soya consumption in a variety of detrimental effects, which received publicity in the British press in 2000.[53] The debate about the best way to use dietary cancer-chemopreventive constituents, in the form of foodstuffs or as formulated isolated agents, is likely to continue for some time. A greater understanding of the mechanisms of the biological activity of dietary constituents will be pivotal to improving the chances of success for cancer-chemo-preventive strategies.

Chemoprevention trials

Epidemiological data suggesting that cancer is preventable by intervention with chemicals are based on time trends in cancer incidence and mortality, geographic variations and the effect of migration, identification of specific causative factors, and the lack of simple patterns of genetic inheritance for the majority of human cancers. Epidemiological studies of special diets are limited, and controlled trials of specific dietary constituents did not start until during the past decade. The clinical assessment of substances for their chemopreventive characteristics presents a host of challenges that are different from those encountered in clinical trials of new cancer chemotherapeutic agents. In particular, the substance under test has to be toxicologically innocuous, because it is likely to be administered to healthy people for long periods.

The design of clinical chemoprevention trials continues to evolve, but a few generalities about each phase can be defined.[54] In phase I studies, the dose-related safety of drugs is determined; this research includes pharmacokinetic investigations. Initial doses and schedules should be based on toxicity and efficacy data from preclinical models. In phase II trials, a randomised, masked, placebo-controlled design is used to evaluate dose-response and common toxic effects likely to result from long-term administration (ie 3 months or longer). Several dose levels are evaluated in these trials, which should incorporate measurement of previously validated surrogate biomarkers. Phase II trials can be done in individuals with premalignant lesions or patients cured of an initial cancer but at risk of developing a second primary tumour. If safety and efficacy are judged to be satisfactory in these trials, the agent proceeds to randomised, prospective, large-scale phase III clinical trials. These are the ultimate tests of drug efficacy, measuring the incidence of primary tumours as well as surrogate biomarkers, in relation to dose and toxicity. The longer times involved necessitate assurances of reproducibility of the formulation and long-term compliance by patients.

Unsuccessful trials

The initial optimism regarding cancer chemoprevention by dietary constituents has been dampened by the outcome of recent large trials that failed to detect any benefit. Two of these trials explored the potential of β-carotene to decrease incidence of lung cancer in about 50 000 individuals.[55] The outcome of these trials made newspaper headlines, because the results suggested that in high-risk groups of smokers, and workers occupationally exposed to asbestos, the intervention increased, rather than decreased, the risk of developing the disease. Subgroup analyses of these two trials showed that the risk of lung cancer was highest among individuals who continued to smoke at least 20 cigarettes per day and among those in the highest quartile of alcohol consumption. A conceivable explanation is that β-carotene suppresses tumours only in individuals in whom the initiating stimulus has been removed, but not in those who are still exposed to it, although the underlying mechanism is unknown. The results of these trials underline the importance of acquiring an understanding of the mechanisms underlying anticarcinogenicity, potential adverse effects, and the pharmacology of novel chemo-preventive agents in preclinical models, before they are subjected to extensive clinical evaluation. The agents in these large trials were chosen primarily on the basis of epidemiological data, a strategy that, in isolation, may have flaws.

Despite the negative outcome of the β-carotene studies, they provided valuable general insights into the clinical design and execution of multiagent chemoprevention trials. In the light of the problems raised by these trials, and the enormous cost and effort associated with large trials, the

development of chemopreventive agents should incorporate small pilot studies. These can be designed to validate specific mechanism-based biomarkers of efficacy, to identify adverse effects, to select the best candidates for specific intervention programmes, or to monitor efficacy in the short or medium term. Pilot studies have been part of the US National Cancer Institute's prevention research programme for most of the past decade. The lessons learned from the clinical trials of β-carotene have modified the approach adopted in the development of the red tomato constituent lycopene, another carotenoid with putative chemopreventive properties, especially with respect to prostate cancer. In contrast to the paucity of pharmacological knowledge on β-carotene when clinical trial of that agent started, there is now much information on the mechanisms of chemopreventive activity and pharmacology of lycopene.[56,57] These data will be taken into account in the design of forthcoming clinical evaluation. Especially interesting to nutritionists is the finding that consumption of lycopene in a lipid-rich matrix, which reflects the traditional use of many Mediterranean tomato-based diets, seems to increase amounts of bioavailable agent in the serum over that achieved in the absence of fats.[58]

Are the difficulties in obtaining encouraging results in clinical intervention studies of such agents confined to the 'nutriceutical approach' – ie to trials in which selected agents are given as supplements? Are trials of whole diets rather than dietary constituents more likely to yield results showing clearcut benefit? The recent, well designed, prospective trials hint at the puzzling possibility that the answers to both these questions may be "no". Increased consumption of fibre, vegetables, and fruit is thought to lower the risk of colorectal cancer, and this is supported by several cohort and case-control studies.[59] However, diets fortified with large amounts of wheat-bran fibre[60] or fruit and vegetables[61] were found not to reduce the development of colorectal adenomas.

Promising trials

Despite these disappointments, research in clinical cancer chemoprevention has yielded encouraging results. Dietary constituents have intriguing properties that strongly suggest the potential for beneficial effects. Results for folate, which is already established in the chemoprevention of fetal neural-tube defects, are promising. Epidemiological studies suggest a lower incidence of colorectal cancer among individuals with the highest intake of dietary folate, whereas people with diets low in folate, or with high alcohol intake, seem to have an increased risk of colorectal adenomas and carcinomas.[62] Large amounts of folate in the diet apparently protect against the development of colorectal adenomas, and the degree of benefit seems to be greater among people who take folate supplements. In the Nurses' Health Study, supplementation with folate was protective against colorectal cancer, with the greatest risk reduction among women taking daily doses of more than 400 μg folate; this reduction reached statistical significance only after 15 years of use.[63] Furthermore, the protective role of folate supplementation may be greatest for individuals who are genetically predisposed to colorectal cancer.[64] Understanding of the underlying mechanism involved is complicated by the fact that folate supplementation decreases the degree of DNA hypomethylation in the rectal mucosa, but both hypomethylation and hypermethylation of specific gene promoter regions in the DNA are characteristic of colorectal cancer.[39,65] Results such as these indicate the importance of considering population subsets and genetic polymorphisms in statistical analyses.

Epidemiological studies have suggested positive correlations between consumption of green tea and a lower incidence of gastric and oesophageal cancers.[66,67] There is no comparable evidence for curcumin consumption, although Asian countries in which turmeric is a major dietary component have low incidence rates for colorectal cancer. Both tea catechins and curcumin are currently, or have recently been, under clinical evaluation. Such studies are usually done in healthy volunteers, but for agents such as curcumin, which has antitumour and chemopreventive activities, phase I chemoprevention studies in patients with established malignant disease may be possible.

Clinical pharmacology

Arguably, clinical pharmacology is the 'cinderella' of the disciplines associated with diet-derived chemopreventive agents. An important fact to recognise is that most of the promising effects of curcumin and tea catechins on cells seen in the laboratory require concentrations above 10^{-5} mol/L, and these high concentrations are unlikely to be achievable when the agents are given to human beings. This is particularly important in the case of curcumin, since the dietary levels that elicit chemopreventive efficacy in rodent models range from 0.1 % to 2.0 %, which translate into daily doses of about 150–3000 mg/kg. The upper end of this dose range is unlikely to be feasible in human beings. Whether such high doses are really necessary to achieve a beneficial effect is currently under investigation. There are some examples of suspected cancer chemopreventive agents that can exert biological effects at low concentrations. The effect of curcumin on expression of the gene for NO synthase in rodents is one example;[13] a dose as low as 100 μg/kg affected gene expression in the liver.

The oral bioavailibility of curcumin in rodents,[68] and probably also in human beings,[69] seems to be very low, which may relate to efficient intestinal metabolism. Curcumin metabolites do not seem to have high pharmacological potency. These findings, if borne out in pilot studies in human beings, might mitigate against use of this agent in the prevention of malignant disease in tissues removed from the gastrointestinal tract. Both curcumin[68] and tea catechins[70] undergo extensive enterohepatic recirculation, but unlike the former, tea polyphenols such as EGCG are well absorbed and rapidly distributed in rodents.[50,70] The bioavailability of tea catechins may also be satisfactory in human beings.[71] Dietary constituents with poor systemic bioavailability in preclinical pilot studies could be considered for the chemoprevention of cancers in the gastrointestinal tract, but not in other tissues. Alternatively, even if they have poor systemic bioavailability, agents may be targeted pharmaceutically to the desired site of action. Such an approach has been adopted with the

glucocorticoid budenoside and with retinoid 13-*cis* retinoic acid, which have been formulated as aerosols in the chemoprevention of experimental lung cancer.[72,73] The extension of this approach to diet-derived phytochemicals will be a formidable pharmaceutical challenge.

In the biophase, the curcumin molecule not only acts as a potent antioxidant, but also as a pro-oxidant. The balance of these properties depends on the presence of metal ions. When copper(II) ions are present, curcumin can lead to the formation of reactive oxygen species.[74] Therefore, at high ingested doses and consequent substantial concentrations of curcumin in organs accessible to it, its beneficial antioxidant properties may be masked by the unwanted corollary of pro-oxidation. A related paradox has been described for the micronutrient vitamin C.[75]

Shureiqi and Brenner recently pointed out that the number of papers published since 1990 on chemoprevention in general has increased exponentially, whereas the number of papers specifically on clinical chemoprevention has remained more or less constant.[76] It is therefore not surprising that little quantitative pharmacokinetic information is available *in vivo* in human beings that can be related to knowledge of the biological effects of these agents in cells *in vitro* and in animals. Pilot studies are needed to help test mechanistic hypotheses in the 'translational' setting. This type of approach is exemplified by a report of a recent study of green tea in healthy volunteers, who consumed 0.6 g or more of standardised tea extract.[77] Concentrations of prostaglandin E_2 were lowered by about 50% in the rectal mucosa, and the decrease was greatest 4 h after consumption, coinciding with amounts of tea catechins of about 0.5 nmol/mg protein in homogenised rectal-biopsy tissue and of 0.1 nmol/mL in plasma. It is unclear how the tea constituents lowered prostaglandin E_2 concentrations. Despite the lack of mechanistic explanation for the link between pharmacokinetics and pharmacodynamics, this type of pilot study can help to validate putative biomarkers clinically and may optimise the design of subsequent clinical trials. Ideally, translational pilot studies should provide basic pharmacokinetic information at several dose levels, in relation to mechanism-based pharmacodynamic effects in target tissues or in blood, before phase I studies. Examples of such effects include changes in activities of COX2, telomerase, cell-cycle regulatory proteins, and transcription factors AP1 or NF-κB, in markers of apoptosis and angiogenesis, and in sequelae of antioxidation, such as amounts of oxidised forms of DNA.

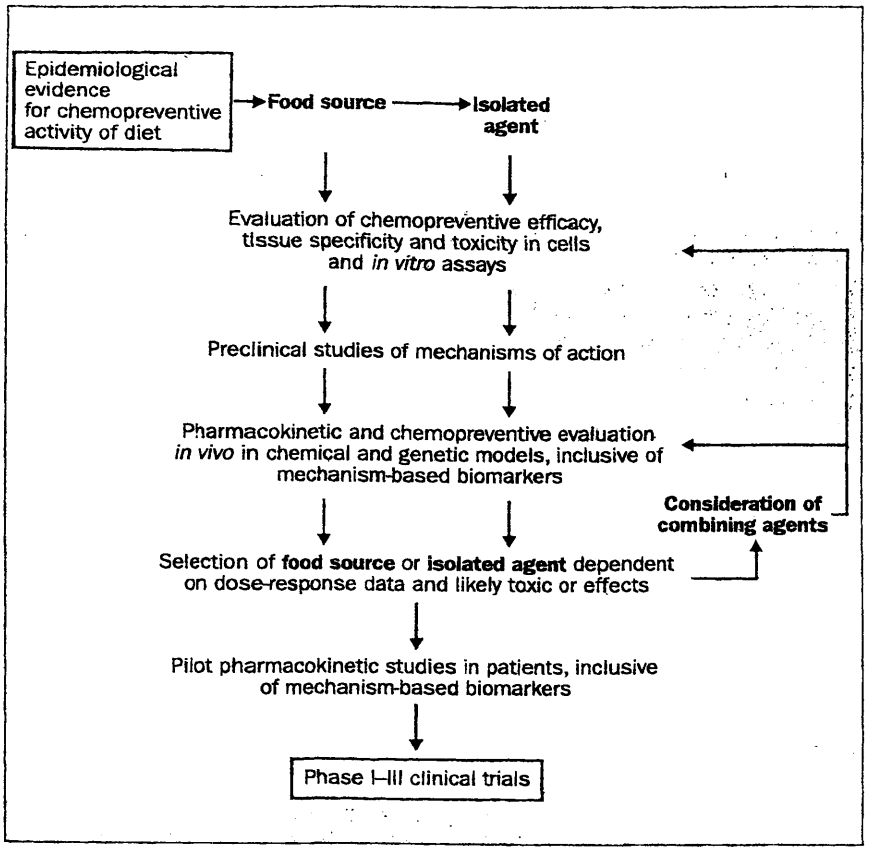


Figure 5. Path for optimum evaluation of dietary constituents as chemopreventive agents (modified from references 34, 54).

Conclusions

There are currently no definite answers to questions posed by patients and at-risk individuals to oncologists, concerning diet or dietary supplements. Nevertheless, the increasing amount of literature on diet-derived cancer chemopreventive agents is starting to allow inferences to be made, which will contribute to the scientific basis of sound advice. Dietary recommendations based on convincing epidemiological evidence, such as eating several helpings of fruits and vegetables every day, are generally considered safe. Supplementation with micronutrients or nutriceuticals is an attractive alternative in terms of ease of administration and reproduciblity of dose (eg folate supplementation, which may reduce the risk of colorectal cancer in the long term). Agents such as curcumin and tea catechins are in early clinical investigation. Almost every month, new insights into the mechanisms by which dietary constituents may prevent cancer are published, and targets such as COX2, telomerase, and cell-cycle regulatory proteins cause excitement among pharmacologists. Yet more robust mechanistic insights are required, that will allow characterisation of the effects operative at physiologically relevant concentrations. We also need more knowledge of the concentrations of chemopreventive agents and their metabolites that prevent cancer in rodents, and how such concentrations relate to those achievable in target tissues in human beings. Dietary constituents should be developed in a structured design, as summarised in Figure 5. Preclinically, the food source and

Search strategy and selection criteria

Referenced papers were identified through searching the ISI Web of Science (1990 onwards). Additional papers were identified through cross-referencing from retrieved papers. Only papers published in English were used. The following website addresses were used to explore ongoing and recent trials of chemopreventive agents: http://clinicaltrials.gov/; http://cancernet.nci.nih.gov/cgi-bin/srchcgi.exe; http://www.cdc.gov/cancer/index.htm; http://iarc.fr/pageroot/UNITS/CHP.HTM; and http://cancerindex.org/clinks4t.htm.

the isolated agent should be studied in parallel. One or the other should then be selected for further development, on the basis of efficacy and toxicity data. Finally, the selected intervention should be tested in pilot clinical studies that precede formal phase I–III evaluation in suitable populations. The challenge is considerable, but the potential rewards are enormous.

Acknowledgements

The authors thank Sue Spriggs and Rebekah Jukes for their help with the preparation of this manuscript and one of the referees for most helpful and constructive comments.

References

1 Singh DK, Lippman SM. Cancer chemoprevention. Part 1: Retinoids and carotenoids and other classic antoxidants. Part 2: Hormones, nonclassic antioxidant natural agents, NSAIDs, and other agents. *Oncology (Huntington)* 1998; 12: 1643–60, 1787–800.
2 Kelloff GJ. Perspectives on cancer chemoprevention research and drug development. *Adv Cancer Res* 2000; 78: 199–334.
3 Wattenberg LW. Chemoprophylaxis of carcinogenesis: a review. *Cancer Res* 1966; 26: 1520–26.
4 Manson MM, Gescher A, Hudson EA, *et al.* Blocking and suppressing mechanisms of chemoprevention by dietary constituents. *Toxicol Lett* 2000; 112: 499–505.
5 Liu JY, Lin SJ, Lin JK. Inhibitory effects of curcumin on protein kinase C activity induced by 12-O-tetradecanoylphorbol-13-acetate in NIH 3T3 cells. *Carcinogenesis* 1993; 14: 857–61.
6 Korutla L, Kumar R. Inhibitory effect of curcumin on epidermal growth factor receptor kinase activity in A431 cells. *Biochim Biophys Acta* 1994; 1224: 597-600.
7 Hong RL, Spohn WH, Hung MC. Curcumin inhibits tyrosine kinase activity of p185(neu) and also depletes p185(neu1). *Clin Cancer Res* 1999; 5: 1884–91.
8 Squires MS, McCann E, Dickens M, *et al.* Differential effect on MAP kinase signalling pathways in breast cancer cells treated with the dietary chemopreventive agents curcumin or epigallocatechin-3-gallate. Manuscript submitted.
9 Chen YR, Tan TH. Inhibition of the c-jun N-terminal kinase (JNK) signaling pathway by curcumin. *Oncogene* 1998; 17: 173–8.
10 Irani K, Xia Y, Zweier JL, *et al.* Mitogenic signaling mediated by oxidants in *ras*-transformed fibroblasts. *Science* 1997; 275: 1649–52.
11 Hanif R, Qioa L, Shiff SJ, Rigas B. Curcumin, a natural plant phenolic food additive, inhibits cell proliferation and induces cell cycle changes in colon adenocarcinoma cell lines by a prostaglandin-independent pathway. *J Lab Clin Med* 1997; 130: 576–84.
12 Liang YC, LinShiau SY, Chen CF, Lin JK. Inhibition of cyclin-dependent kinases 2 and 4 activities as well as induction of cdk inhibitors p21 and p27 during growth arrest of human breast carcinoma cells by (-)-epigallocatechin-3- gallate. *J Cell Biochem* 1999; 75: 1–12.
13 Chan MM, Huang HI, Fenton MR, Fong D. *In vivo* inhibition of nitric oxide synthase gene expression by curcumin, a cancer preventive natural product with anti-inflammatory properties. *Biochem Pharmacol* 1998; 55: 1555–62.
14 Prescott SM, Fitzpatrick FA. Cyclooxygenase-2 and carcinogenesis. *Biochim Biophys Acta* 2000; 1470: M69–78.
15 Steinbach G, Lynch PM, Phillips RKS, *et al.* The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. *N Engl J Med* 2000; 342: 1946–52.
16 Giardiello FM, Hamilton SR, Krush AJ, *et al.* Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. *N Engl J Med* 1993; 328: 1313–16.
17 Plummer SM, Holloway KA, Manson MM, *et al.* Inhibition of cyclo-oxygenase 2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF-kappa B activation via the NIK/IKK signalling complex. *Oncogene* 1999; 18: 6013–20
18 Sharma RA, Ireson CR, Verschoyle RD *et al.* Effects of dietary curcumin on glutathione S-transferase and malondialdehyde-DNA adducts in rat liver and colon mucosa: Relationship with drug levels. Manuscript submitted.
19 Sambaiah K, Ratankumar S, Kamanna VS, *et al.* Influence of turmeric and curcumin on growth, blood constituents and serum enzymes in rats. *J Food Sci Technol* 1982, 19: 187–90.
20 Yin M-J, Yamamoto Y, Gaynor RB. The anti-inflammatory agents aspirin and salicylate inhibit the activity of IκB kinase-β. *Nature* 1998; 396: 77–80.
21 Marnett LJ. Aspirin and the potential role of prostaglandins in colon cancer. *Cancer Res* 1992; 52: 5575–89.
22 Huang TS, Lee SC, Lin JK. Suppression of c-jun/AP-1 activation by an inhibitor of tumor promotion in mouse fibroblast cells. *Proc Natl Acad Sci USA* 1991; 88: 5292–96.
23 Singh S, Aggarwal BB. Activation of transcription factor NF-kappaB is suppressed by curcumin (diferuloylmethane). *J Biol Chem* 1995; 270: 24995–5000.
24 Yamamoto K, Arakawa T, Ueda N, Yamamoto S. Transcription roles of nuclear factor kappa B and nuclear factor interleukin-6 in the tumor necrosis factor alpha-dependent induction of cyclooxygenase-2 in MC3T3-E1 cells. *J Biol Chem* 1995; 270: 31315–20.
25 Barnes S, Peterson TG, Coward L. Rationale for the use of genistein-containing soy matrices in chemoprevention trials for breast and prostate cancer. *J Cell Biochem* 1995; 22: 181–87.
26 Gail MH, Brinton LA, Byar DP, *et al.* Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. *J Natl Cancer Inst* 1989; 81: 1879–86.
27 Naasani I, Seimiya H, Tsuruo T. Telomerase inhibition, telomerase shortening, and senescence of cancer cells by tea catechins. *Biochem Biophys Res Comm* 1998; 249: 391–96.
28 Cao Y, Cao R. Angiogenesis inhibited by drinking tea. *Nature* 1999; 398: 381–82.
29 Arbiser JL, Klauber N, Rohan R, *et al.* Curcumin is an *in vivo* inhibitor of angiogenesis. *Mol Med* 1998; 4: 376–83.
30 Mohan R, Sivak J, Ashton P, *et al.* Curcuminoids inhibit the angiogenic response stimulated by fibroblast growth factor-2, including expression of matrix metalloproteinase gelatinase B. *J Biol Chem* 2000; 275: 10405–12.
31 Jiang, MC, Yang-Yen HF, Yen JJ, Lin JK. Curcumin induces apoptosis in immortalized NIH 3T3 and malignant cancer cell lines. *Nutr Cancer* 1996; 26: 111–20.
32 Ahmad N, Feyes DK, Nieminen AL, *et al.* Green tea constituent epigallocatechin-3-gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. *J Natl Cancer Inst* 1997; 89: 1881–86.
33 Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000; 100: 57–70.
34 Kelloff GJ, Crowell JA, Steele VE, *et al.* Progress in cancer chemoprevention: Development of diet-derived chemopreventive agents. *J Nutr* 2000; 130: S467–71.
35 Pezzuto JM. Plant-derived anticancer agents. *Biochem Pharmacol* 1997; 53: 121–33.
36 Hudson EA, Dinh PA, Kokubun T, *et al.* Characterization of potentially chemopreventive phenols in extracts of brown rice which inhibit the growth of human breast and colon cancer cells. *Cancer Epidemiol Biomarkers Prev* 2000; 9: 1163–70.
37 Goss KH, Groden J. Biology of the adenomatous polyposis coli tumor suppressor. *J Clin Oncol* 2000, 18: 1967–79.
38 Rustgi AK. Hereditary gastrointestinal polyposis and nonpolyposis syndromes. *N Engl J Med* 1994, 331: 1694–702.
39 Potter JD. Colorectal cancer: Molecules and populations. *J Natl Cancer Inst* 1999, 91: 916–32.
40 Rao CV, Rivenson A, Simi B, Reddy BS. Chemoprevention of colon carcinogenesis by dietary curcumin, a naturally occurring plant phenolic compound. *Cancer Res* 1995; 55: 259–66.
41 Kawamori T, Lubet R, Steele VE, *et al.* Chemopreventive effect of curcumin, a naturally occurring anti-inflammatory agent, during

the promotion/progression stages of colon cancer. *Cancer Res* 1999; 59: 597–601.
42 Singh SV, Hu X, Srivastava SK, *et al.* Mechanism of inhibition of benzo[a]pyrene-induced forestomach cancer in mice by dietary curcumin. *Carcinogenesis* 1998; 19: 1357–60.
43 Mahmoud NN, Carothers AM, Grunberger D, *et al.* Plant phenolics decrease intestinal tumors in an animal model of familial adenomatous polyposis. *Carcinogenesis* 2000; 21: 921–27.
44 Yang CS, Wang ZY. Tea and cancer. *J Natl Cancer Inst* 1993; 85: 1038–49.
45 Song J, Sohn KJ, Medline A, *et al.* Chemopreventive effects of dietary folate on intestinal polyps in Apc+/-Msh2-/- mice. *Cancer Res* 2000; 60: 3191–99.
46 Lamartiniere CA, Moore JB, Brown NM, *et al.* Genistein suppresses mammary cancer in rats. *Carcinogenesis* 1995; 16: 2833–40.
47 Rao CV, Wang CX, Simi B, *et al.* Enhancement of experimental colon cancer by genistein. *Cancer Res* 1997; 57: 3717-22.
48 Torrance CJ, Jackson PE, Montgomery E, *et al.*. Combinatorial chemoprevention of intestinal neoplasia. *Nature Med* 2000; 6: 1024–28.
49 Rao CV, Tokumo K, Rigotty J, *et al.* Chemoprevention of colon carcinogenesis by dietary administration of piroxicam, α-difluoromethylornithine, 16 α-fluoro-5-androsten-17-one, and ellagic acid individually and in combination. *Cancer Res* 1991, 51: 4528–35.
50 Suganuma M, Okabe S, Kai N, *et al.* Synergistic effects of (-)epigallocatechin gallate with (-) epicatechin, sulindac, or tamoxifen on cancer-preventive activity in the human lung cancer cell line PC-9. *Cancer Res* 1999, 59: 44–47.
51 Khafif A, Schantz SP, Chou TC, *et al.* Quantitation of chemopreventive synergism between (-)-epigallocatechin-3-gallate and curcumin in normal, premalignant and malignant human oral epithelial cells. *Carcinogenesis* 1998; 19: 419–24.
52 Eberhardt MV, Lee CY, Liu RH. Antioxidant activity of fresh apples. *Nature* 2000; 405: 903–04.
53 Barnett A. Soya linked to cancer and brain damage. *The Observer* Sunday 13th August, 2000.
54 Kelloff GJ, Johnson JR, Crowell JA, *et al.* Approaches to the development and marketing approval of drugs that prevent cancer. *Cancer Epidemiol Biomarkers Prev* 1995, 4: 1–10.
55 Omenn GS. Chemoprevention of lung cancer: the rise and demise of β-carotene. *Ann Rev Public Health* 1998, 19: 73–99.
56 Sharoni Y, Danilenko M, Levy J. Molecular mechanisms for the anticancer activity of the carotenoid lycopene. *Drug Develop Res* 2000, 50: 448–56.
57 Giovannucci E, Clinton SK. Tomatoes, lycopene, and prostate cancer. *Proc Soc Exp Biol Med* 1998, 218: 129–39.
58 Rao AV, Agarwal S. Role of lycopene as antioxidant carotinoid in the prevention of chronic diseases: a review. *Nutr Res* 1999, 19: 305–23.
59 World Cancer Research Fund and American Institute for Cancer Research. Food, Nutrition and the Prevention of Cancer: a Global Perspective. World Cancer Research Fund/American Institute for Cancer Research. Washington, DC 1997.
60 Alberts DS, Martinez ME, Roe DJ, *et al.* Lack of effect of a high-fiber cereal supplement on the recurrence of colorectal adenomas. *N Engl J Med* 2000; 342: 1156–62.
61 Schatzkin A, Lanza E, Corle D, *et al.* Lack of effect of a low-fat, high-fiber diet on the recurrence of colorectal adenomas. *N Engl J Med* 2000; 342: 1149–55.
62 Janne PA, Mayer RJ. Chemoprevention of colorectal cancer. *N Engl J Med* 2000, 342: 1960–68.
63 Giovannucci E, Stampfer MJ, Colditz GA, *et al.* Multivitamin use, folate, and colon cancer in women in the Nurses' Health Study. *Ann Intern Med* 1998; 129: 517–24.
64 Chen J, Giovannucci E, Hankinson SE, *et al.* A prospective study of methylenetetrahydrofolate reductase and methionine synthase gene polymorphisms, and risk of colorectal adenoma. *Carcinogenesis* 1998, 19: 2129–32.
65 Cravo ML, Pinto AG, Chaves P, *et al.* Effect of folate supplementation on DNA methylation of rectal mucosa in patients with colonic adenomas: Correlation with nutrient intake. *Clin Nutr* 1998, 17: 45–49.
66 Kono S, Ikeda M, Tokudome S, Kutratsune M. A case-control study of gastric cancer and diet in northern Kyushu. *Jap J Cancer Res* 1988, 79: 1067–74.
67 Gao YT, McLaughlin JK, Blot WJ, *et al.* Reduced risk of esophageal cancer associated with green tea consumption. *J Natl Cancer Inst* 1994; 86: 855–58.
68 Ravindranath V, Chandrasekhara N. Absorption and tissue distribution of curcumin in rats. *Toxicol* 1980; 16: 259–65.
69 Shoba G, Joy D, Joseph T, *et al.* Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers. *Planta Medica* 1998; 64: 353–56.
70 Nakagawa K, Miyazawa T. Absorption and distribution of tea catechin, (-)-epigallocatechin-3- gallate, in the rat. *J Nutr Sci Vitaminol* 1997; 43: 679–84.
71 Yang CS, Chen LS, Lee MJ, *et al.* Blood and urine levels of tea catechins after ingestion of different amounts of green tea by human volunteers. *Cancer Epidemiol Biomarkers Prev* 1998; 7: 351–54.
72 Wattenberg LW, Wiedmann TS, Estensen RD, *et al.* Chemoprevention of pulmonary carcinogenesis by aerosolized budesonide in female A/J mice. *Cancer Res* 1997; 57: 5489–92.
73 Raleigh SM, Verschoyle RD, Bowskill C, *et al.* Pulmonary availability of isotretinoin in rats after inhalation of a powder aerosol compared to oral isotretinoin. *Brit J Cancer* 2000; 83: 935–40.
74 Ahsan H, Parveen N, Khan NU, Hadi SM. Pro-oxidant, anti-oxidant and cleavage activities on DNA of curcumin and its derivatives demethoxycurcumin and bisdemethoxycurcumin. *Chem Biol Interact* 1999; 121: 161–75.
75 Podmore ID, Griffiths HR, Herbert KE, *et al.* Vitamin C exhibits pro-oxidant properties. *Nature* 1998; 392: 559.
76 Shureiqi I, Brenner DE. Chemoprevention of epithelial cancers. *Current Opinion Oncol* 1999; 11: 408–13.
77 August DA, Landau J, Caputo D, *et al.* Ingestion of green tea rapidly decreases prostaglandin E-2 levels in rectal mucosa in humans. *Cancer Epidemiol Biomarkers Prev* 1999; 8: 709–13.
78 De Flora S, Balansky R, Scatolini L, *et al.* Adducts to nuclear DNA and mitochondrial DNA as biomarkers in chemoprevention. In: *Principles of Chemoprevention.* Stewart BW, McGregor D, Kleihues P, eds. IARC Scientific Publication No.139, Lyon, 1996, 291–301.

APPENDIX 2D: Sharma RA, Manson MM, Gescher A, Steward WP. Colorectal cancer chemoprevention: Biochemical targets and clinical development of promising agents. *Eur J Cancer* 2001, **37**: 12-22.

PERGAMON

European Journal of Cancer 37 (2001) 12–22

European Journal of Cancer

www.ejconline.com

Review

Colorectal cancer chemoprevention: biochemical targets and clinical development of promising agents

R.A. Sharma [a,*], M.M. Manson [b], A. Gescher [b], W.P. Steward [a]

[a] *University Department of Oncology, Leicester Royal Infirmary, Leicester LE1 5WW, UK*
[b] *MRC Toxicology Unit, Lancaster Road, Leicester LE1 9HN, UK*

Received 27 April 2000; received in revised form 17 July 2000; accepted 17 August 2000

Abstract

Colorectal cancer (CRC) remains a cause of significant mortality in developed countries despite extensive knowledge of its epidemiology and molecular basis. Since multiple molecular steps that collectively bring about this disease are known, its chemoprevention is a realistic proposition. Biochemical targets of CRC chemopreventive agents include carcinogen metabolising enzymes, arachidonic acid metabolism, the transcription factor nuclear factor-kappa beta (NF-κB), enzymes responsible for polyamine metabolism, and events associated with proliferation and apoptosis of preneoplastic cells. Aspirin, celecoxib, calcium and α-difluoromethylornithine are examples of drugs that have undergone clinical testing. Critical evaluation of these trials allows optimisation of methodologies for clinical advancement of novel chemopreventive agents. Cancer patients can be a suitable cohort of subjects for pilot studies of certain new agents. Such studies and larger trials in high-risk healthy individuals require the stringent use of carefully validated 'preneoplastic' biomarkers which are intrinsically related to defined stages of colorectal carcinogenesis and/or to mechanisms of action of the agent under investigation.

Keywords: Biomarker: Carcinogenesis: Clinical trial: Colorectal cancer: Chemoprevention

1. Introduction

The concept of chemoprevention is not new. For many decades, chemicals have been used to prevent dental caries, heart attacks and stroke. Similarly, many individuals modify their diets and lifestyles in an attempt to prevent or delay the onset of disease. Although the history of cancer chemoprevention is relatively brief, many studies have been reported and a few efficacious agents have been identified. The most obvious example is tamoxifen, which can now be considered an established agent in the prevention of breast cancer [1]. Furthermore the vitamin A analogues isotretinoin and retinol palmitate have been demonstrated to prevent second primary cancers in patients with malignancies of the lung and head/neck [2,3]. There is little doubt that the identification of novel chemopreventive agents with efficacy against the major human cancers could have an enormously beneficial impact on the public health of Western societies.

This review, which is targeted at a readership concerned primarily with treatment rather than prevention of cancer, will initially attempt to define the parameters which determine whether a chemoprevention study will be useful or not. Therewith, biochemical events associated with colorectal carcinogenesis will be described as potential targets for chemopreventive agents. The final aim is to summarise recent clinical chemoprevention trials of novel agents.

Colorectal cancer (CRC) is the second most common malignancy in developed countries, accounting for approximately 20–30 deaths per 100 000 standard population each year [4]. Although recent years have seen marginal improvements in mortality for white US citizens [4], the prognosis remains poor; similar to that described in 1932 by Dukes [5] and in 1954 by Astler and Coller [6]. The 5-year survival rate for a patient presenting with lymph node involvement (Dukes' C) is approximately 39% following surgery [7]. Although 80% of patients undergoing surgical resection have complete macroscopic clearance of their disease, 50%

* Corresponding author. Tel.: +44-116-258-7597; fax: +44-116-258-7599.
E-mail address: ras20@le.ac.uk (R.A. Sharma).

0959-8049/01/$ - see front matter
PII: S0959-8049(00)00326-9

suffer recurrence [8]. Moreover, approximately 25% of patients have advanced disease at presentation [4], when palliative management strategies remain the only option.

The epidemiology and molecular biology underlying CRC have been studied more thoroughly and are better understood than for most other neoplasias. Clinical and molecular evidence suggests that there are several somatic pathways to CRC, and that two of these pathways appear to parallel certain well-defined processes described for inherited forms of the disease [9]. The incidence of CRC has increased rapidly in the latter part of the twentieth century in many countries, including Italy, Japan and urban China [10]. Such changes have occurred within one generation and suggest an important role for environmental influences in the aetiology of this disease. Epidemiological studies have also pinpointed environmental factors, especially dietary, which appear to affect individuals' chances of developing CRC [11]. Diets low in vegetables and folate, and high in fat, red meat and alcohol appear to increase relative risk. Lack of exercise and cigarette smoking are also risk factors. However, the relative risk ratio for each individual factor appears small when compared with factors linked to other cancers, such as smoking which increases the risk of lung cancer almost 20-fold. Red meat intake, for example, may at most double the risk for CRC [11]. Prospective cohort studies have confirmed certain links, such as the protective role of high-dose folate [12], but have failed to substantiate others such as the benefits of dietary fibre [13]. Large prospective studies of dietary risk factors are ongoing, such as the European Prospective Investigation of Cancer, in which data from over 400 000 individuals are being evaluated [14].

Intense research efforts are aimed at integration of our knowledge of molecular pathways with that of the epidemiology of CRC. Integration may ultimately allow the generation of 'risk profiles' for individuals, based on knowledge of family history, lifestyle, dietary factors, genetic analysis and risk/preneoplastic biomarkers (see below). Such profiles may play a significant role in the selection of subjects for clinical chemoprevention trials. Improved genetic screening for inherited disease traits and an increasing number of pilot screening programmes for sporadic cancer in normal populations [15] are likely to identify increasing numbers of individuals with early stages of colon carcinogenesis. Thus the need for intervention strategies in high-risk individuals and those with preneoplastic lesions will undoubtedly increase.

Cancer chemoprevention can be defined as the inhibition, retardation or reversal of carcinogenic processes by chemical means [16]. Most clinical trials focus on the prevention of carcinogenesis at a premalignant stage or an early phase of malignancy, which is termed 'primary' or 'secondary' chemoprevention, depending on whether it involves normal or high-risk individuals. The treatment with chemopreventive agents of patients who have undergone successful therapy of a primary malignancy but are at increased risk of a second malignancy, is referred to as 'tertiary' chemoprevention [17]. It is notable that the original definition of the term chemoprevention also embraces inhibition of growth and delay of progression of cancers [18].

2. Features of clinical trials

Clinical trials of cancer chemotherapeutic agents have traditionally been divided into three phases; phase I studies to assess potential toxicity and pharmacokinetics; phase II studies to assess potential benefit; and phase III studies to compare the efficacy and toxicity of the new treatment with current therapeutic regimes. The clinical evaluation of agents for chemopreventive properties can also be separated into three phases, albeit with objectives which differ significantly from those in chemotherapy trials. Table 1 summarises salient features of the design of contemporary chemoprevention trials. Unique challenges posed by these studies include trial duration, agent safety and number of subjects enrolled. Although the initiation phase of carcinogenesis may last only hours or days, promotion and progression

Table 1
Basic generalised design for chemoprevention trials (modified from [19])

Phase	Number of subjects	Design	Primary endpoint
I	15–30	Small, short-term pilot study in cancer patients with rapid dose escalation if no toxicity observed	1. Pharmacokinetics
			2. Preliminary data on preneoplastic biomarkers
IIa	30–60	Small, short-term dose de-escalation study	Sensitivity and response of preneoplastic biomarkers
IIb	500–1500	Larger, medium-term randomised study in high-risk individuals	Response in definitive preneoplastic biomarkers
III	Several thousand	Large, long-term randomised study with low- and high-risk levels	1. Response in definitive preneoplastic biomarkers
			2. Cancer incidence and mortality

of tumours probably take years, if not decades. Potentially efficacious cancer chemopreventive agents are therefore likely to be administered regularly to healthy individuals who will gain no visible benefit for many years. It is, therefore, important that there be a minimal risk of toxicity. Any adverse effect must be carefully considered in the risk/benefit estimation for the subject population chosen. Moreover, the number of individuals in chemoprevention efficacy trials is generally substantially greater than the number of patients in most chemotherapeutic trials in order to allow observations to reach significance levels.

3. Choice of subjects

Thousands of years ago, Hippocrates and Galen cautioned against the treatment of hidden cancers, arguing that treatment more often than not hastened death [20]. The ethical dilemmas of treating healthy or 'at risk' individuals were highlighted recently by two major lung cancer intervention trials, the α-tocopherol, β-carotene study [21], and the β-carotene and retinol efficacy study [22]. The rationale for the studies was the finding that eating fruit and vegetables is associated with consistently elevated levels of β-carotene and a lower incidence of cancer. However, the results of the trials suggested that β-carotene not only failed to protect against lung cancer, in high-risk groups of smokers and/or workers occupationally exposed to asbestos, it even increased the risk of developing the disease. Several criticisms have been levelled at the study [23], and consideration of these criticisms may be useful in optimising the design of future CRC chemoprevention studies. Especially noteworthy among the criticisms are the lack of a rigorous scientifically based mechanistic rationale for the trial and the absence of adequate pharmacokinetic/dynamic information to decide proper dose and scheduling. The importance of detailed mechanistic studies, as a prelude to clinical evaluation, in order to minimise the occurrence of unexpected adverse effects was highlighted recently by the suggestion that β-carotene boosts the detrimental effects of cigarette smoke carcinogens by upregulating carcinogen-metabolising enzyme activities [24]. Furthermore, it is conceivable that the fruit or vegetable constituent which is responsible for chemopreventive efficacy is not β-carotene, but a different agent such as its isomer α-carotene [23].

It is also clearly desirable to obtain information on pharmacokinetics and potential toxicity in humans before administering chemopreventive agents over long periods of time to healthy volunteers. Some of the agents under consideration in cancer chemoprevention inhibit steps in the late stages of carcinogenesis and have chemotherapeutic activity in clinical trials. This notion is certainly true for tamoxifen in breast cancer prevention and treatment, and there is evidence in preclinical models and case reports to suggest similar activity for putative CRC chemopreventive agents such as curcumin, an active constituent of the spice turmeric [25,26]. Therefore it may be justifiable to conduct small pilot studies of certain chemopreventive agents in patients with cancer, with measurement of radiological/chemical tumour markers as well as preneoplastic biomarkers (see below) and pharmacokinetics.

4. Preneoplastic biomarkers

Biomarkers are increasingly used in programmes of screening, chemoprevention and chemotherapy, and there is some degree of confusion in the literature regarding their classification. 'Surrogate endpoint biomarkers' (SEBs) have been identified for several different stages in the carcinogenic process, and represent a means of monitoring disease progression without having to wait for true neoplasia and metaplasia to develop. SEBs can be termed 'preneoplastic biomarkers', and should be distinguished from 'risk biomarkers' and 'tumour markers'. In Table 2 these three basic marker types are defined and their uses outlined. Of these three types, preneoplastic biomarkers are the most useful for assessing the efficacy of cancer chemopreventive agents [27,28].

Carcinoma incidence is the ultimate endpoint of cancer chemopreventive intervention. However, if this acts

Table 2
Types of biomarkers (modified from [27])

Type of biomarker	Stage of disease	Description	Application
Risk biomarker	No disease detectable	Genetic predisposition, past medical history, lifestyle factors, exposure	Useful to molecular epidemiologists to assess risk of developing cancer
Preneoplastic biomarker	Initiation, promotion, progression	Biological alterations representing the early and intermediate stages of carcinogenesis, which may show alterations by chemopreventive agents	Identification of carcinogenesis and chemoprevention
Tumour markers	Established neoplasia, adhesion–migration	Elevated levels associated with particular cancers	Diagnosis and treatment

as the only endpoint, clinical trials are beset by practical difficulties, most notably the prolonged time periods necessary to obtain results. For example, the delay before preventive effects are seen may be 9 years for aspirin [29] and as long as 15 years for high-dose folate [12]. Therefore, sensitive and specific biomarkers are required that accurately reflect the development of malignancy, and may be differentially expressed during the multiple stages of carcinogenesis. In Table 3 some preneoplastic biomarkers of potential use in CRC chemoprevention studies are shown. The frequency of measurement of biomarkers depends on factors such as the accessibility of the biological material required and the potential risk involved in making the measurement. In cardiovascular chemoprevention for example, clinical measurement of arterial blood pressure and laboratory measurement of blood cholesterol are well established as valid predictive markers upon which intervention can be based. Fortunately, in the case of CRC the target organ is anatomically accessible with a relatively small risk from biopsy collection. But it could be desirable to correlate preneoplastic biomarker levels in the target organ with those in peripheral blood. An example of such a correlation has been provided recently in a pilot study [31] preceding a chemoprevention study of broccoli supplements [32]. Although glutathione S-transferase (GST) activity of colon mucosa correlated with that of blood lymphocytes in 29 subjects at increased risk of colorectal cancer, neither was raised significantly by the supplements. Apart from colonic biopsies and blood, patient samples that may be collected for preneoplastic biomarker measurement are urine, stool and colonic lavage fluid.

Preneoplastic biomarkers have not only to be identified, but also painstakingly validated. This task involves the judicious use of the most suitable animal models which reflect the multifactorial nature of human colorectal cancer [33]. Furthermore, the relationship between biomarker and risk of cancer has to be established in prospective studies in high-risk individuals. Such studies constitute a significant undertaking, but are necessary to bridge the gap between preclinical model systems and clinical trials.

5. Biochemical targets and chemopreventive mechanisms

5.1. Features of colorectal cancer

Most CRC arises within pre-existing adenomatous polyps or adenomas, which are common lesions, particularly in the elderly [34]. However, it is relatively rare for individual adenomas to progress to malignancy, despite the high rate of cell division [35], and it is, therefore, important to identify 'high-risk' polyps. In order to assess this risk, molecular biological criteria are increasingly used in addition to histological ones. A cell accumulates a combination of genetic defects, including activation of oncogenes and inactivation of tumour suppressor genes, to undergo full malignant transformation [36]. This concept, known as the multistep model of carcinogenesis, has been characterised in some detail for CRC (Fig. 1). Although in reality the progression of events may be less linear and less temporal than this simplified scheme suggests, the model has provided the basis for understanding the interaction between genetic predisposition and environmental factors as outlined in several contemporary reviews [8,9]. Irreversible mutations are known to occur in the initiation phase, followed by continued mutations, uncontrolled cellular proliferation and clonal expansion in the promotion phase. Progression involves genotypically altered cells developing the histological changes associated with CRC.

Identification of high-risk polyps should not only be based on histological criteria and assessment of genetic defects, but might also benefit from consideration of cell signalling pathways, downstream from isolated gene

Table 3
Examples of preneoplastic biomarkers identified for colorectal cancer (modified from [30])

Type of biomarker	Variable measured	Biomarker
Pathological	Histology	Adenoma, aberrant crypt foci
Cellular	Proliferation	BrDU, PCNA, Ki67, lectin labelling
	Differentiation	Lectin labelling
	Apoptosis	TUNEL assay
Biochemical	Arachidonate metabolism	Prostaglandins, COX-2, arachidonic acid, lipooxygenase, leucotrienes
	Polyamine metabolism	Polyamines, ornithine decarboxylase
	Detoxification enzymes	GST, DT-diaphorase
Molecular	DNA methylation, DNA adducts	Methyl groups, MTHFR, MDA–DNA adducts
	Cell cycle	Cyclin D1, TGFα
Genetic	Gene/product	K-ras, APC, DCC

BrDU, bromodeoxyuridine; PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxyribonucleotidyl transferase mediated nick-end labelling; COX-2, cyclooxygenase-2; GST, glutathione S-transferase; MTHFR, methylenetetrahydrofolate reductase; MDA, malondialdehyde; TGF-α, transforming growth factor-α. APC, adenomatous polyposis coli; DCC, deleted in colorectal cancer.

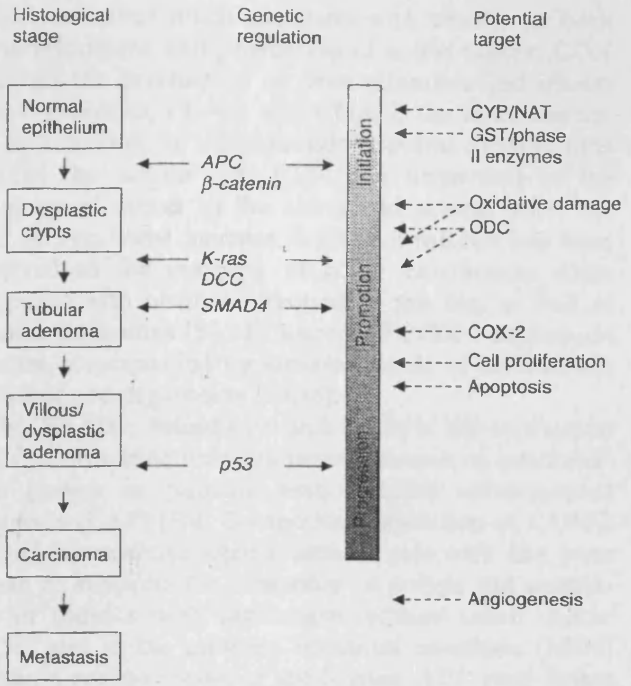


Fig. 1. Multistep model of carcinogenesis with targets for chemopreventive agents (modified from [36]). APC, adenomatous polyposis coli; COX-2, cyclooxygenase-2; CYP, cytochromes P450; *DCC*, deleted in colorectal cancer; GST, glutathione S-transferase; NAT, N-acetyltransferases; ODC, ornithine decarboxylase.

defects which give rise to an abnormal phenotype. It is now recognised that multiple, sequential mutations in genes critical to the control of cellular signal transduction, transcription and thus proliferation and apoptosis are important throughout carcinogenesis [37]. An example is provided by the *APC* gene which is commonly mutated in polyps and early cancer [38]. Loss of wild-type *APC* leads to transcriptional activation of *c-myc* expression through the β-catenin Tcf-4 complex [39], which may affect a number of downstream targets including the initiation factor eIF-4E [40]. Elucidation of the signalling pathway downstream of the wild-type *APC* gene has pinpointed a crucial role for β-catenin and E-cadherin, the expression of which is frequently abnormal in colorectal cancer [41]. Although *APC* function may be normal, mutations in *β-catenin* can result in the abnormalities of adhesion–migration and proliferative signalling found in neoplasia [42].

5.2. Chemopreventive agents

Historically, chemopreventive agents have been categorised as either 'blocking' or 'suppressing' according to the stage of the carcinogenic process with which they interfere [43]. Blocking agents exert their effect at the initiation stage, for example by altering carcinogen-metabolising activities in target tissues or by antioxidant effects. Suppressing agents act at more advanced stages in the carcinogenic process during promotion and/or progression, by inhibiting cell proliferation and/or inducing apoptosis. Although it is now known that single chemopreventive agents may exert effects at multiple stages (see below), this broad and simple classification remains useful.

It is not yet clear whether highly specific inhibitors, e.g. selective inhibitors of the enzyme cyclooxygenase-2 (COX-2), or agents with more 'pleiotropic' effects, e.g. naturally occurring polyphenols, offer the greatest potential in the chemoprevention of human cancers. In order to effectively counteract processes leading to a disease as multifactorial as cancer, a satisfactory chemopreventive agent may have to possess a variety of mechanistically distinct, but complementary, anticarcinogenic properties. Some important biochemical targets for cancer chemopreventive agents are outlined below.

5.3. Antioxidants and agents which affect xenobiotic metabolism

Reactive oxygen species, such as superoxide anions and hydroxyl radicals, are thought to be involved in carcinogenesis [44]. Consequently, mopping up activated oxygen species is a chemopreventive mechanism displayed by some blocking agents such as flavonoids, vitamin E and isothiocyanates. Phase I drug metabolising enzymes, particularly cytochromes P450, activate many carcinogens, and phase II enzymes, including GST, detoxify carcinogenic metabolites. The balance between carcinogen activation and detoxification is probably a critical arbiter of an individual's risk of developing cancer [45]. This balance is influenced by many blocking agents. One example is indole-3-carbinol which, despite inducing both cytochromes P450 and phase II enzymes, prevents aflatoxin B1-induced hepatocarcinogenesis in rodents [46]. Another example is the isothiocyanate sulphoraphane contained in broccoli, which not only induces xenobiotic conjugating enzymes [47], but also inhibits some cytochrome P450 isoenzymes, prominent among them CYP2E1 [48].

Preclinical and clinical studies have established an association between decreased GST enzyme activity and increased risk for CRC [31]. In subjects at risk of colorectal cancer, the putative chemopreventive agent oltipraz significantly increased GST expression in colon mucosa and in blood lymphocytes [49]. Similarly, administration of non-steroidal anti-inflammatory drugs (NSAIDs) and curcumin has been shown to increase levels of GST isoenzymes in the gastrointestinal tract in preclinical models [50,51].

5.4. Modulators of arachidonic acid metabolism

The arachidonic acid pathway and one of its key enzymes, COX, or prostaglandin H synthase, have

recently received much attention with respect to both the development and prevention of colon cancer. COX catalyses the production of prostaglandins and occurs as two isozymes, COX-1 and COX-2, the latter inducible by infection or inflammation. Several observations support the notion that COX-2 is important in the aetiology of cancer of the colon and several other tissues. A significant increase in COX-2 mRNA has been observed in the majority of colon carcinomas when compared with normal surrounding mucosa, as well as in some adenomas [52,53]. Increased COX-2 expression is often accompanied by elevated levels of eicosanoids including prostaglandins [54,55].

The NSAID, sulindac, which inhibits the expression of COX non-selectively, causes regression of adenomatous polyps in patients with familial adenomatous polyposis (FAP) [56]. Competitive inhibition of COX-2 activity by selective agents such as celecoxib has been shown to suppress the formation of polyps and neoplasias in rodents with carcinogen-induced colon cancer [57,58] and in the minimal intestinal neoplasia (MIN) mouse, a rodent model of the human *APC* gene defect [59]. When $APC^{\Delta 716}$ knockout mice which possess a phenotype similar to that of the MIN mouse, were crossed with COX-2 knockout mice, the resultant offspring were significantly protected from tumour development [60].

5.5. Inhibitors of NF-κB activation

Nuclear factor-kappa beta (NF-κB) is a transcription factor pivotal for the expression of many genes regulating proliferation, immunity, inflammatory response and cellular adhesion. It is sequestered in an inactive state in the cytoplasm by an inhibitory protein, IκB [61]. Upon stimulation of the cell by tumour promoters, cytokines or the products of oxidative stress, IκB is phosphorylated by upstream kinases and degraded to release NF-κB. NF-κB undergoes translocation to the nucleus where it initiates upregulation of genes containing suitable binding sites. Activation of NF-κB has been shown to inhibit apoptosis [62]. This finding suggests that its inhibition could downregulate genes involved in the promotion and progression of carcinogenesis by restoration of the sensitivity of cells towards apoptotic stimuli [63]. Both aspirin and its hydrolysis product salicylate [64], as well as curcumin [65], interfere with NF-κB activation by inhibiting the IκB kinase (IKK) complex involving a heterodimeric kinase IKK-α and -β.

5.6. Inhibitors of polyamine biosynthesis

Polyamines are short-chain aliphatic molecules required for normal cellular growth. Their concentration in tissues has been shown to correlate with cellular proliferation [66]. Polyamines appear to be involved in the activation of the proto-oncogenes *c-myc* and *c-fos* [67]. Proliferating tissues and tumours differ from normal non-proliferating tissues not only because they produce more polyamines, but also in that they contain individual polyamines at different concentrations. For example, N1-acetylspermidine is generally undetectable in normal mammalian tissues, but is present in high levels in human CRC [68]. The rate-limiting step in polyamine biosynthesis is the enzyme ornithine decarboxylase (ODC), which is constitutively overexpressed in colorectal dysplasia and neoplasia [69,70]. Levels of ODC and polyamines, especially putrescine, are significantly elevated in the colorectal mucosa of individuals with the *APC* germ line mutation before they develop polyposis [71]. ODC has been suggested to be critical for cell transformation [72], and it might thus act as a preneoplastic biomarker of colorectal carcinogenesis [73]. α-Difluoromethylornithine (DFMO) is an irreversible inhibitor of ODC that is currently under clinical evaluation (see below). Many diet-derived chemopreventive agents, such as genistein, curcumin, indole-3-carbinol and green tea polyphenols, have also been shown to inhibit ODC activity [74–77].

5.7. Modulators of cell proliferation and apoptosis

The evidence for antiproliferative and pro-apoptotic properties as important mechanistic determinants of chemopreventive activity is particularly robust in the case of the NSAIDs. Aspirin has been shown to retard proliferation of human colorectal tumour cells by inducing arrest in the G0/G1 phase of the cell cycle and programmed cell death [78]. Salicylate increased the susceptibility of cells at a late stage of neoplastic progression towards induction of apoptosis. Intriguingly, aspirin suppressed the mutator phenotype associated with hereditary non-polyposis CRC by genetic selection for a subset of cells that do not express microsatellite instability [79]. Other chemopreventive agents like curcumin and indole-3 carbinol have also been shown to induce cell cycle arrest and/or apoptosis in cancer cell lines [80,81].

In general, cancer chemopreventive agents have cytostatic rather than cytotoxic properties. A case in point is the specific COX-2 inhibitor SC-58125, which arrested the growth of COX-2-expressing human colon adenocarcinoma cells both *in vitro* and when implanted into nude mice, but it did not affect adenocarcinoma cells lacking COX-2 [82]. This degree of selective activity of a chemopreventive agent is reminiscent of the current emphasis in modern anticancer drug development on compounds which target cellular signalling events selectively [83]. Many of these 'antisignalling molecules', in contrast to their classical anticancer drug precursors, also cause cytostasis rather than cytotoxicity. Such similarities between chemopreventive and antisignalling

chemotherapeutic agents intimate a convergence in the philosophies underpinning new agent development in both areas. This notion is supported by the fact that many cellular signal transduction targets such as *ras*, the mitogen-activated protein kinase pathway and cell cycle regulating molecules, are equally attractive to both areas.

6. Clinical trials

Agents that have entered clinical chemoprevention trials have been chosen on the basis of epidemiological research and activity in preclinical models. The relevance of published clinical trials is discussed in the following paragraphs. Relevant ongoing trials of the same agents, posted on commonly accessed international cancer websites, are shown in Table 4 to allow the reader to monitor future progress.

6.1. Celecoxib

Celecoxib is a highly selective COX-2 inhibitor (see above), which was recently approved in the USA as an adjunct to standard care for patients with FAP based on the results of a double-blind, placebo-controlled, multicentre trial in 77 patients [84]. Of the two dose levels studied for six months, 400 mg of celecoxib twice daily was found to cause a significant reduction in the number of colorectal polyps on endoscopy. Further NCI-sponsored phase II/III trials in patients with sporadic polyps and hereditary non-polyposis coli are ongoing. Preclinical studies of such agents [82] and clinical experience of NSAIDs such as sulindac [56] would suggest that the chemopreventive effects of highly selective COX-2 inhibitors are likely to be transient, and that polyps may increase in size and number once treatment is curtailed. Long-term safety is being elucidated (see Table 4).

6.2. Aspirin

A number of retrospective epidemiological studies suggest a decreased incidence of colorectal cancer of up to 50% in regular aspirin users [85–87]. One case–control study of 5815 cases of CRC over a 14-year period has suggested that there may be a 9-year delay before any preventive effects are seen from daily aspirin use, and that the reduction in risk may be dose-depen-

Table 4
A selection of clinical trials in progress measuring preneoplastic colorectal biomarkers (obtained from commonly accessed cancer websites, June 2000)

Agent	Trial design[a]	Subject population (recruitment status)	Variables measured[b]	Principal investigator/institution
Celecoxib	Phase I/II	Patients with HNPCC (R)	Pharmacokinetics Histology Others N/D	National Cancer Institute (Bethesda, MD, USA)
Celecoxib	Phase III	Patients with polyps resected (R)	Histology	Bertagnolli M (Boston, MA, USA)
Aspirin	Phase III	Patients with sporadic polyps (R)	Histology	Baron J. (Dartmouth Uni., NH, USA)
DFMO + Sulindac	Phase IIb	Patients with polyps resected previously (N)	Histology Gene/product Apoptosis Arachidonate metabolism Polyamine metabolism	Meyskens F.L. (California Irvine University, CA, USA)
Sulindac versus curcumin/rutin/quercetin	Phase I/II	Normal volunteers (R)	N/D	Shiff S. (Rockefeller University, New York, USA)
Curcumin	Phase I	Patients with advanced CRC (R)	Pharmacokinetics DNA adducts Detoxification enzymes Arachidonate metabolism	Sharma R.A. (Leicester University, UK)
Calcium	Phase III	Patients with sporadic polyps (C)	Histology, others N/D	Baron J. (Dartmouth Uni., NH, USA)
Calcium/Fibre	Phase III	Patients with polyps (R)	Histology, others N/D	Biasco G. (Bologna, Italy)
Folate	Phase II	Patients with polyps resected (R)	Histology, DNA methylation	Mason J. (Boston, MA, USA)

R, recruiting; N/D, not defined; N, not yet recruiting; C, recruitment completed; DFMO, α-difluoromethyl ornithine; HNPCC, hereditary non-polyposis colorectal cancer; CRC, colorectal cancer.

[a] See Table 1.

[b] See Table 3.

dent [29]. However, the interpretation of retrospective studies is fraught with potential pitfalls, such as individual selection, compliance and confounding variables.

Unfortunately, prospective studies of the incidence of colorectal cancer among users of aspirin or other NSAIDs are still lacking. The only large study published so far has been the Physicians' Health Study [88]. This randomised trial assessed the effects of 325 mg of aspirin taken on alternate days in 22 071 male physicians in the USA. The trial was closed prematurely because of a significant benefit with respect to cardiovascular mortality in the aspirin group. Analysis of 12-year follow-up of the aspirin group has not shown any significant effect on CRC incidence [89]. Questions have been raised about the suitability of the subject population, the dose regime adopted, and the fact that the actual period of continual dosing lasted only five years and subsequent data were collected post-trial when follow-up of subjects may have been less stringent.

More recently, strategies to investigate the chemopreventive potential of aspirin have changed. In a pilot phase I de-escalating dose study involving 65 healthy human subjects taking the drug daily for two weeks, preneoplastic biomarkers were measured in colonic tissue in addition to plasma levels of aspirin and salicylic acid [90]. The results of this study suggest that the optimal dose for aspirin as a chemopreventive agent may be 81 mg/day, which is lower than the dosage previously considered necessary for efficacy. Colonic prostaglandin levels continued to be suppressed long after aspirin and salicylic acid had been cleared from the plasma. Subsequent phase II results in patients with colon adenomas have proved encouraging [91], and phase III studies of aspirin and other NSAIDs are ongoing.

6.3. Calcium

Calcium ingested orally forms salts with bile acids and fatty acids once it reaches the colon, and it may thus have a direct anticarcinogenic effect on colonic epithelial cells [92]. Although a few case–control epidemiological studies suggest an inverse association between calcium intake and incidence of colorectal cancer [93], the results are by no means consistent. Similarly, chemoprevention trials have studied histological endpoints in the colon, with mixed results. In certain studies, calcium supplementation decreased epithelial cell hyperproliferation [94,95] and reduced the incidence of metachronous colorectal adenomas [96]. However, two large studies failed to show an effect of calcium on colonic mucosal proliferation [97,98]. Although two recent studies of patients with a previous history of dysplasia or neoplasia have suggested that calcium supplementation may decrease adenoma recurrence [99,100], the optimal dosing regime and the role of confounding dietary constituents remain unclear.

6.4. DFMO

Another agent which has been administered to individuals at risk for CRC is DFMO. Unlike chemoprevention trials in which only histological endpoints were measured, contemporary chemoprevention trials of DFMO have focused on potential toxicity and the tissue levels of polyamines and ODC as preneoplastic biomarkers. In two studies, significant changes in biomarker levels were observed in the target organ, but reversible ototoxicity was experienced at high-dose levels [101,102]. Depending on the subject population being targeted, safety may prove to be the main obstacle in the development of DFMO as a cancer chemopreventive.

6.5. Selenium

Often the secondary analyses of results of negative chemoprevention trials provide surprising results, as exemplified by the selenium skin cancer study [103]. Selenium (200 μg/day) was administered as a constituent of brewer's yeast to 1312 patients with a history of skin cancers, living in low selenium-intake regions of America. Secondary subset analysis found statistically significant reductions in total cancer mortality, and specifically in the incidence of prostate, lung and colon malignancies.

6.6. Other potential agents

Agents which are currently under consideration for colorectal cancer chemoprevention trials are the dietary substances curcumin, isothiocyanates, vitamin E, vitamin D3, folate, perillyl alcohol and polyphenols from tea [104,105]. On account of their long history of use in food, these compounds are considered harmless, at least at the dose levels consumed in certain diets. Whether this assumption is reasonable for the compounds in their pure forms remains to be demonstrated. Non-dietary compounds under consideration include oestrogens, oltipraz and N-acetylcysteine.

7. Conclusions

With improved molecular and epidemiological knowledge of colorectal carcinogenesis and risk of developing CRC, effective chemoprevention of this common disease is a realistic proposition. Reactive oxygen species, metabolising enzymes, COX-2, NF-κB, polyamines, and key molecules in cell proliferation and apoptosis are potential targets for chemopreventive agents. Integration of knowledge of the molecular biology of CRC with known mechanisms of action of such agents should lead to the discovery of new efficacious

agents and optimisation of clinical trials. CRC chemoprevention trials of aspirin, celecoxib, calcium and DFMO have provided valuable insights into choice of subject cohort, dose levels and the use of biomarkers. The trial of β-carotene in lung cancer patients has highlighted the need for new approaches to subject selection and trial design, especially the desirability of incorporating preclinical and early clinical mechanistic data. There is a need for small pilot studies of putative agents in healthy subjects or cancer patients to obtain information on safety, pharmacokinetics and pharmacodynamics. On account of the prolonged time periods necessary for chemoprevention trials, optimisation will depend on the selection and validation of preneoplastic biomarkers. Adherence to these principles may facilitate the discovery and development of effective chemopreventive agents. The old adage 'to name is to know, a disease known is half cured' may not currently hold for the chemotherapy of CRC. Nevertheless, the optimised understanding of this disease may ultimately contribute to its prevention.

Acknowledgements

We thank Jo Arden, Clare Winfield and Sue Spriggs for their help with the references.

References

1. Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J Natl Cancer Inst* 1998, **90**, 1371–1388.
2. Hong WK, Lippman SM, Itri LM, Karp DD, Lee JS, Byers RM. Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck. *N Engl J Med* 1990, **323**, 795–801.
3. Pastorino U, Infante M, Maioli M, *et al.* Adjuvant treatment of stage I lung cancer with high-dose vitamin A. *J Clin Oncol* 1993, **11**, 1216–1222.
4. Ries LAG, Eisner MP, Kosary CL, *et al. SEER Cancer Statistics Review 1973–1997.* Bethesda, MD, USA, National Cancer Institute, 2000.
5. Dukes CE. The classification of cancer of the rectum. *J Pathol Bacteriol* 1932, **35**, 323–333.
6. Astler VB, Coller FA. The prognostic significance of direct extension of carcinoma of the colon and rectum. *Ann Surg* 1954, **192**, 846–850.
7. Arnaud JP, Buyse M, Nordlinger B. Adjuvant therapy of poor prognosis colon cancer with levamisole: results of an EORTC double-blind randomized clinical trial. *Br J Surg* 1989, **76**, 284–289.
8. Midgley R, Kerr D. Colorectal cancer. *Lancet* 1999, **353**, 391–399.
9. Potter JD. Colorectal cancer: molecules and populations. *J Natl Cancer Inst* 1999, **91**, 916–932.
10. Muir C, Waterhouse J, Mack T, Powell J, Whelan S, Smans M, eds. *Cancer Incidence in Five Continents.* Lyon, France, International Agency for Research on Cancer, 1987.
11. Cummings JH, Bingham SA. Diet and the prevention of cancer. *Br Med J* 1998, **317**, 1636–1640.
12. Giovannucci E, Stampfer MJ, Colditz GA, *et al.* Multivitamin use, folate and colon cancer in women in the Nurses' Health Study. *Ann Intern Med* 1998, **129**, 517–524.
13. Fuchs CS, Giovannucci EL, Colditz GA, Willett WC. Dietary fiber and the risk of colorectal cancer and adenoma in women. *N Engl J Med* 1999, **340**, 169–176.
14. Riboli E. Nutrition and cancer: background and rationale of EPIC. *Ann Oncol* 1992, **3**, 783–791.
15. Kronberg O, Fenger C, Olsen J. Randomised study of screening for colorectal cancer with fecal occult blood test. *Lancet* 1996, **348**, 1467–1471.
16. Lippman SM, Benner SE, Hong WK. Cancer chemoprevention. *J Clin Oncol* 1994, **12**, 851–873.
17. De Flora S, Balansky R, Scatolini L, *et al.* Adducts to nuclearDNA and mitochondrial DNA as biomarkers in chemoprevention. In Stewart BW, McGregor D, Kleihues P, eds. *Principles of Chemoprevention.* Lyon, France, IARC Scientific Publication No. 139, 1996, 291–301.
18. Sporn MB, Newton DL. Chemoprevention of cancer with retinoids. *Fed Proc* 1979, **38**, 2528–2534.
19. Alberts DS. Advanced clinical trial design: the use of surrogate end point biomarkers in cancer chemoprevention trials. In Perry MC, ed. *American Society of Clinical Oncology Educational Book.* Baltimore, USA, Lippincott, 1999, 76–80.
20. Cantor D. Cancer. In Bynum WF, Porter R, eds. *Companion Encyclopedia of the History of Medicine Vol. 1.* London, UK, Routledge, 1993, 552.
21. The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group. The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. *N Engl J Med* 1994, **330**, 1029–1035.
22. Omenn GS, Goodman GE, Thornquist MD, Balmes J, Cullen MR, Glass A. Risk factors for lung cancer and for intervention effects in CARET, the beta-carotene and retinol efficacy trial. *J Natl Cancer Inst* 1996, **88**, 1550–1559.
23. Knekt P, Järvinen R, Teppo L, Aromaa A, Seppänen R. Role of various carotenoids in lung cancer prevention. *J Natl Cancer Inst* 1999, **91**, 182–184.
24. Paolini M, Cantelii-Forti G, Perocco P, Pedulli GF, Abdel-Rahman SZ, Legator MS. Co-carcinogenic effect of beta-carotene. *Nature* 1999, **398**, 760–761.
25. Jiang M-C, Yang-Yen H-F, Yen JJ-Y, Lin J-K. Curcumin induces apoptosis in immortalized NIH 3T3 and malignant cancer cell lines. *Nutr Cancer* 1996, **26**, 111–120.
26. Arbiser JL, Klauber N, Rohan R, *et al.* Curcumin is an in vivo inhibitor of angiogenesis. *Mol Medicine* 1998, **4**, 376–383.
27. Sharma RA. Cancer chemoprevention: a clinical reality. *J Roy Soc Med* 2000, in press.
28. Einspahr JG, Alberts DA, Gapstur SM, Bostick RM, Emerson SS, Gerner EW. Surrogate end-point biomarkers as measures of colon cancer risk and their use in cancer chemoprevention trials. *Cancer Epid Biomarkers Prev* 1997, **6**, 37–48.
29. Collet J-P, Sharpe C, Belzile E, Boivin J-F, Hanley J, Abenhaim L. Colorectal cancer prevention by non-steroidal anti-inflammatory drugs: effects of dosage and timing. *Br J Cancer* 1999, **81**, 62–68.
30. Krishnan K, Ruffin MT, Brenner DE. Clinical models of chemoprevention for colon cancer. *Hematol Oncol Clinics N America* 1998, **12**, 1079–1113.
31. Szarka CE, Pfeiffer GR, Hum ST, Everley LC, Balshem AM, Moore DF. Glutathione *S*-transferase activity and glutathione *S*-transferase μ expression in subjects at risk for colorectal cancer. *Cancer Res* 1995, **55**, 2789–2793.
32. Clapper ML, Szarka CE, Pfeiffer GR, *et al.* Preclinical and clinical evaluation of broccoli supplements as inducers of glutathione S-transferase activity. *Clin Cancer Res* 1997, **3**, 25–30.

33. Shureiqi I, Reddy P, Brenner DE. Chemoprevention: general perspective. *Crit Rev Oncol/Hematol* 2000, **33**, 157–167.
34. Vogelstein B, Fearon ER, Hamilton SR. Genetic alterations during colorectal-tumour development. *N Engl J Med* 1988, **319**, 525–532.
35. Bird JP. Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings. *Cancer Lett* 1987, **37**, 147–151.
36. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990, **61**, 759–767.
37. Loeb L. Cancer cells exhibit a mutator phenotype. *Adv Cancer Res* 1998, **72**, 25–56.
38. Powell SM, Zilz N, Beazer-Barclay Y, Bryan TM, Hamilton SR, Thibodeau SN. APC mutations occur early during colectroal tumorigenesis. *Nature* 1992, **359**, 235–237.
39. He TC, Sparks AB, Rago C, *et al.* Identification of c-MYC as a target of the APC pathway. *Science* 1998, **281**, 1509–1512.
40. Rosenwald IB, Chen J-J, Wang S, Savas L, London IM, Pullman J. Upregulation of protein synthesis initiation factor eIF-4E is an early event during colon carcinogenesis. *Oncogene* 1999, **18**, 2507–2517.
41. Sparks AB, Morin PJ, Vogelstein B, Kinzler KW. Mutational analysis of the APC/β-catenin/Tcf pathway in colorectal cancer. *Cancer Res* 1998, **58**, 1130–1134.
42. Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B. Activation of β-catenin-Tcf signaling in colon cancer by mutations in β-catenin or APC. *Science* 1997, **275**, 1787–1790.
43. Wattenberg LW. Chemoprevention of cancer. *Cancer Res* 1985, **45**, 1–8.
44. Kensler TW, Trush MA, Guyton KZ. Free radicals as targets for cancer chemoprevention: prospects and problems. In Steel VE, Stoner GDM, Boon CW, Kelloff GJ, eds. *Cellular and Molecular Targets for Chemoprevention.* Boca Raton, USA, CRC Press, 1992, 173–191.
45. Clapper ML, Szarka CE. Glutathione *S*-transferasesv — biomarkers of cancer risk and chemopreventive response. *Chem Biol Interact* 1998, **112-112**, 377–388.
46. Manson MM, Hudson EA, Ball HWL, *et al.* Chemoprevention of aflatoxin B_1-induced carcinogenesis by indole-3-carbinol in rat liver — predicting the outcome using early biomarkers. *Carcinogenesis* 1998, **18**, 1729–1738.
47. Zhang Y, Talalay P, Cho C-G, Posner GH. A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. *Proc Natl Acad Sci USA* 1992, **89**, 2399–2403.
48. Barcelo S, Gardiner JM, Gescher A, Chipman JK. CYP2E1-mediated mechanism of anti-genotoxicity of the broccoli constituent sulforaphane. *Carcinogenesis* 1996, **17**, 277–282.
49. O'Dwyer PJ, Szarka CE, Yao K-S, *et al.* Modulation of gene expression in subjects at risk for colorectal cancer by the chemopreventive dithiolethione oltipraz. *J Clin Invest* 1996, **98**, 1210–1217.
50. Van Lieshout EMM, Tiemessen DM, Roelofs HMJ, Peters WHM. Nonsteroidal anti-inflammatory drugs enhance glutathione *S*-transferase theta levels in rat colon. *Biochim Biophys Acta* 1998, **1381**, 305–311.
51. Nijhoff WA, Groen GM, Peters WHM. Induction of rat hepatic and intestinal glutathione S-transferases and glutathione by dietary naturally occurring anticarcinogens. *Int J Oncol* 1993, **3**, 1131–1139.
52. Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology* 1994, **107**, 1183–1188.
53. Kargmann SL, O'Neill GP, Vickers PJ, Evans JF, Mancini JA, Jothy S. Expression of prostaglandin G/H synthase-1 and -2 protein in human colon cancer. *Cancer Res* 1995, **55**, 2556–2559.
54. Bennett A, Tacca MD, Stamford IF, Zebro T. Prostaglandins from tumours of human large bowel. *Br J Cancer* 1977, **35**, 881–884.
55. Brock TG, McNish RW, Peters-Golden M. Arachidonic acid is preferentially metabolized by cyclooxygenase-2 to prostacyclin and prostaglandin E2. *J Biol Chem* 1999, **274**, 11660–11666.
56. Giardiello FM, Hamilton SR, Krush AJ, Piantadosi S, Hylind LM, Celano P. Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. *N Engl J Med* 1993, **328**, 1313–1316.
57. Kawamori T, Rao CV, Seibert K, Reddy BS. Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. *Cancer Res* 1998, **58**, 409–412.
58. Reddy BS, Hirose Y, Lubet R, *et al.* Chemoprevention of colon cancer by specific cyclooxygenase-2 inhibitor, celecoxib, administered during different stages of carcinogenesis. *Cancer Res* 2000, **60**, 293–297.
59. Roy HK, Lu K. Nabumetone inhibition of intestinal tumorigenesis in MIN mice: modulation of Bcl-2. *Gastroenterology* 1997, **112**, A647.
60. Oshima M, Dinchuk JE, Kargman SL, *et al.* Suppression of intestinal polyposis in $Apc^{\Delta 716}$ knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell* 1996, **87**, 803–809.
61. Simeonidis S, Stauber D, Chen GY, Hendrickson WA, Tahnos D. Mechanisms by which IκB proteins control NF-κB activity. *Proc Natl Acad Sci USA* 1999, **96**, 49–54.
62. Beg AA, Baltimore D. An essential role for NF-κB in preventing TNF-α-induced death. *Science* 1996, **274**, 782–784.
63. Waddick KG, Uckun FM. Innovative treatment programs against cancer: NF-κB as a molecular target. *Biochem Pharmacol* 1999, **57**, 9–17.
64. Yin M-J, Yamamoto Y, Gaynor RB. The anti-inflammatory agents aspirin and salicylate inhibit the activity of IkB kinase-β. *Nature* 1998, **396**, 77–80.
65. Plummer SM, Holloway KA, Manson MM, *et al.* Inhibition of cyclooxygenase-2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF-kB activation via the NIK/IKK signalling complex. *Oncogene* 1999, **18**, 6013–6020.
66. Pegg AE. Recent advances in the biochemistry of polyamines in eukaryotes. *Biochem J* 1988, **234**, 249–262.
67. Tabib A, Bachrach A. Activation of the proto-oncogene c-*myc* and c-*fos* by c-*ras*. Involvement of polyamines. *Biochem Biophys Res Commun* 1994, **202**, 720–727.
68. Takenoshita S, Matsuzaki S, Nakano G, *et al.* Selective elevation of the N^1-acetylspermidine level in human colorectal adenocarcinomas. *Cancer Res* 1984, **44**, 845–847.
69. Luk GD, Baylin SB. Ornithine decarboxylase as a biologic marker in familial colonic polyposis. *N Engl J Med* 1984, **311**, 80–83.
70. Narisawa T, Takahashi M, Niwa M, *et al.* Increased mucosal ornithine decarboxylase activity in large bowel with multiple tumors, adenocarcinoma and adenoma. *Cancer* 1989, **63**, 1572–1576.
71. Giardiello FM, Hamilton SR, Hylind LM, Yang VW, Tamez P, Casero Jr RA. Ornithine decarboxylase and polyamines in familial adenomatous polyposis. *Cancer Res* 1997, **57**, 199–201.
72. Auvinen M, Paasinen A, Andersson LC, Hölttä E. Ornithine decarboxylase activity is critical for cell transformation. *Nature* 1992, **360**, 355–358.
73. Wang W, Liu LQ, Higuchi CM. Mucosal polyamine measurements and colorectal cancer risk. *J Cell Biochem* 1996, **63**, 252–257.
74. Tseng C-P, Verma AK. Inhibition of 12-O-Tetradecanoylphorbol-13-acetate-induced ornithine decarboxylase activity by genistein, a tyrosine kinase inhibitor. *Mol Pharmacol* 1996, **50**, 249–257.
75. Rao CV, Simi B, Reddy BS. Inhibition by dietary curcumin of azoxymethane-induced ornithine decarboxylase, tyrosine protein kinase, arachidonic acid metabolism and aberrant crypt foci formation in the rat colon. *Carcinogenesis* 1993, **14**, 2219–2225.

76. Hudson EA, Howells L, Ball HWL, Pfeiifer AMA, Manson MM. Mechanisms of action of indole-3-carbinol as a chemopreventive agent. *Biochem Soc Trans* 1998, **26**, S370.
77. Gupta S, Ahmad N, Mohan RR, Husain MM, Mukhtar H. Prostate cancer chemoprevention by green tea: *in vitro* and *in vivo* inhibition of testosterone-mediated induction of ornithine decarboxylase. *Cancer Res* 1999, **39**, 2115–2120.
78. Elder DJ, Hague A, Hicks DJ, Paraskeva C. Differential growth inhibition by the aspirin metabolite salicylate in human colorectal tumor cell lines: enhanced apoptosis in carcinoma and in vitro-transformed adenoma relative to adenoma cell lines. *Cancer Res* 1996, **56**, 2273–2276.
79. Ruschoff J, Wallinger S, Dietmaier W, Bocker T, Brockhoff G, Hofstadter F. Aspirin suppresses the mutator phenotype associated with hereditary nonpolyposis colorectal cancer by genetic selection. *Proc Natl Acad Sci USA* 1998, **95**, 11301–11306.
80. Samaha HS, Kelloff GJ, Steele V, Rao CV, Reddy BS. Modulation of apoptosis by sulindac, curcumin, phenylethyl-3-methylcaffeate, and 6-phenylhexyl isothiocyanate: apoptotic index as a biomarker in colon cancer chemoprevention and promotion. *Cancer Res* 1997, **57**, 1301–1305.
81. Cover CM, Hsieh SJ, Tran SH, *et al.* Indole-3-carbinol inhibits the expression of cyclin-dependent kinase-6 and induces a G1 cell cycle arrest of human breast cancer cells independent of estrogen receptor signaling. *J Biol Chem* 1998, **273**, 3838–3847.
82. Sheng H, Shao J, Kirkland SC, *et al.* Inhibition of human colon cancer cell growth by selective inhibition of cyclooxygenase-2. *J Clin Invest* 1998, **273**, 2254–2259.
83. Gelman KA, Eisenhauer EA, Harris AL, Ratain MJ, Workman P. Anticancer agents targeting signaling molecules and cancer cell environment: challenges for drug development? *J Natl Cancer Inst* 1999, **91**, 1281–1287.
84. Steinbach G, Lynch PM, Phillips RKS, Wallace MH, Hawk E, Levin B. The effect of celecoxib, a cyclooxygenase-2 inhibitor in familial adenomatous polyposis. *N Engl J Med* 2000, **342**, 1946–1952.
85. Thun MJ, Namboodiri MM, Heath Jr CW. Aspirin use and reduced risk of fatal colon cancer. *N Engl J Med* 1991, **325**, 1593–1596.
86. Giovannucci E, Rimm EB, Stampfer MJ, Colditz GA, Ascherio A, Willett WC. Aspirin use and the risk for colorectal cancer and adenoma in male health professionals. *Ann Intern Med* 1994, **121**, 241–246.
87. Kune GA, Kune S, Watson LF. Colorectal cancer risk, chronic illnesses, operations and medications: case–control results from the Melbourne Colorectal Cancer Study. *Cancer Res* 1988, **48**, 4399–4404.
88. Gann PH, Manson JE, Glynn RJ. Low-dose aspirin and the incidence of colorectal tumors in a randomized trial. *J Natl Cancer Inst* 1993, **85**, 1220–1224.
89. Sturmer T, Glynn RJ, Lee IM. Aspirin use and colorectal cancer: post-trial follow-up data from the Physicians' Health Study. *Ann Intern Med* 1998, **128**, 713–720.
90. Krishnan K, Ruffin MT, Brenner DE. Colon cancer chemoprevention: clinical development of aspirin as a chemopreventive agent. *J Cell Biochem Suppl* 1997, **28/29**, 148–158.
91. Barnes CJ, Hamby-Mason RL, Hardman WE, *et al.* Effect of aspirin on prostaglandin E2 formation and transforming growth factor alpha expression in human rectal mucosa from individuals with a history of adenomatous polyps of the colon. *Cancer Epidemiol Biomarkers Prevention* 1999, **8**, 311–315.
92. Scalmati A, Lipkin M, Newmark H. Calcium, vitamin D and colon cancer. *Clin Appl Nutr* 1992, **2**, 67–74.
93. Potter JD, Slattery ML, Bostick RM, Gapstur SM. Colon cancer: a review of the epidemiology. *Epidemiol Rev* 1993, **15**, 499–545.
94. Wargovich MJ, Isbell G, Shabot M. Calcium supplementation decreases rectal epithelial cell proliferation in subjects with sporadic adenomas. *Gastroenterology* 1992, **103**, 92–97.
95. Bostick RM, Fosdick L, Wood JR, Grambsch P, Grandits GA, Lillemoe TJ. Calcium and colorectal epithelial cell proliferation in sporadic adenoma patients: a randomized double-blinded, placebo-controlled clinical trial. *J Natl Cancer Inst* 1995, **87**, 1307–1315.
96. Baron JA, Beach M, Mandel JS, van Stolk RU, Haile RW, Sandler RS. Calcium supplements for the prevention of colorectal adenomas. *N Engl J Med* 1999, **340**, 101–107.
97. Gregoire PC, Stern HS, Yeung KS. Effect of calcium supplementation on mucosal cell proliferation in high risk patients for colon cancer. *Gut* 1989, **30**, 376–382.
98. Baron JA, Toteson TD, Wargovich MJ. Calcium supplementation and rectal mucosal proliferation: a randomized controlled trial. *J Natl Cancer Inst* 1995, **87**, 1275–1277.
99. Whelan RL, Hovath KD, Gleason NR, *et al.* Vitamin and calcium supplement use difluoromethylornithine on rectal mucosal levels of polyamines in a randomized, double-blinded trial for colon cancer prevention. *J Natl Cancer Inst* 1998, **90**, 1212–1218.
100. Baron JA (for the Calcium Polyp Prevention Study Group). Calcium supplements for the prevention of colorectal adenomas. *N Engl J Med* 1999, **340**, 101–107.
101. Love RR, Jacoby R, Newton MA, *et al.* A randomized, placebo-controlled trial of low-dose α-difluoromethylornithine in individuals at risk for colorectal cancer. *Cancer Epidemiol Biomarkers Prev* 1998, **7**, 989–992.
102. Meyskens FL, Gerner EW, Emerson S, *et al.* Effect of α-difluoromethylornithine on rectal mucosal levels of polyamines in a randomized, double-blinded trial for colon cancer prevention. *J Natl Cancer Inst* 1998, **90**, 1212–1218.
103. Clark LC, Combs GF Jr, Turnbull BW, Slate EH, Chalker DK, Chow J. Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. *J Am Med Assoc* 1997, **277**, 1520.
104. Kellof GJ, Sigman CC, Greenwald P. Cancer chemoprevention: progress and promise. *Eur J Cancer* 1999, **35**, 1755–1762.
105. Kakizoe T. Asian studies of cancer chemoprevention: latest clinical results. *Eur J Cancer* 2000, **277**, 1303–1309.

APPENDIX 2E: Sharma RA, Ireson CR, Verschoyle RD, Hill KA, Williams ML, Leuratti C, Manson MM, Marnett LJ, Steward WP, Gescher A. Effects of Dietary Curcumin on Glutathione S-Transferase and Malondialdehyde-DNA Adducts in Rat Liver and Colon Mucosa: Relationship with Drug Levels. *Clin Cancer Res* 2001, **7**: 1452-1458.

Effects of Dietary Curcumin on Glutathione *S*-Transferase and Malondialdehyde-DNA Adducts in Rat Liver and Colon Mucosa: Relationship with Drug Levels[1]

Ricky A. Sharma,[2] Christopher R. Ireson, Richard D. Verschoyle, Kirsti A. Hill, Marion L. Williams, Chiara Leuratti, Margaret M. Manson, Lawrence J. Marnett, William P. Steward, and Andreas Gescher

Department of Oncology [R. A. S., K. A. H., M. L. W., W. P. S.] and MRC Centre for Mechanisms of Human Toxicity [C. R. I., R. D. V., C. L., M. M. M., A. G.], University of Leicester, Leicester LE1 9HN, United Kingdom, and Vanderbilt Cancer Center, Nashville, Tennessee 37232 [L. J. M.]

ABSTRACT

Curcumin prevents colon cancer in rodent models. It inhibits lipid peroxidation and cyclooxygenase-2 (COX-2) expression and induces glutathione *S*-transferase (GST) enzymes. We tested the hypothesis that 14 days of dietary curcumin (2%) affects biomarkers relevant to cancer chemoprevention in the rat. Levels of inducible COX-2, as reflected by prostaglandin E_2 production by blood leukocytes, were measured *ex vivo*. Total GST activity and adducts of malondialdehyde with DNA (M_1G), which reflect endogenous lipid peroxidation, were measured in colon mucosa, liver, and blood leukocytes. Curcumin and its metabolites were analyzed by high-performance liquid chromatography in plasma, and its pharmacokinetics were compared following a diet containing 2% curcumin *versus* intragastric (i.g.) administration of curcumin suspended in an amphiphilic solvent. The curcumin diet did not alter any of the markers in the blood but increased hepatic GST by 16% and decreased colon M_1G levels by 36% when compared with controls. Administration of carbon tetrachloride during the treatment period increased colon M_1G levels, and this increase was prevented by dietary curcumin. Dietary curcumin yielded low drug levels in the plasma, between 0 and 12 nM, whereas tissue concentrations of curcumin in liver and colon mucosa were 0.1–0.9 nmol/g and 0.2–1.8 μmol/g, respectively. In comparison with dietary administration, suspended curcumin given i.g. resulted in more curcumin in the plasma but much less in the colon mucosa. The results show that curcumin mixed with the diet achieves drug levels in the colon and liver sufficient to explain the pharmacological activities observed and suggest that this mode of administration may be preferable for the chemoprevention of colon cancer.

INTRODUCTION

Cancer mortality rates in the developed world have risen throughout most of this century, and it is already the leading cause of death in some Western countries (1, 2). This observation has engendered much research activity aimed at the identification of cancer chemopreventive agents, especially substances derived from the diet (3). Turmeric, the dried ground rhizome of the perennial herb *Curcuma longa*, is an example of one such agent. This spice is consumed in the diet in quantities up to 4 g/adult/day in some countries (4), which also appear to have low incidence rates of colorectal cancer (1). Turmeric contains curcuminoids and essential oils. Curcumin (diferuloylmethane), a low molecular weight polyphenol and the major curcuminoid in the plant, is regarded as the constituent with the highest biological activity. Curcumin has been shown to inhibit tumor formation in the skin, forestomach, duodenum, and colon of mice and in the tongue, colon, mammary glands, and sebaceous glands of rats (5). Mechanisms by which curcumin causes cancer chemoprevention are thought to involve antioxidation (6), inhibition of kinases (7), interference with the activity of transcription factors such as nuclear factor-κB and activator protein-1 (8), and suppression of expression of the enzyme COX-2[3] (9).

In a program of preclinical work designed to help optimize the clinical evaluation of curcumin as a colon cancer chemopreventive agent, we tested the hypothesis that curcumin in the diet alters biomarkers of its chemopreventive efficacy. Three biomarkers were selected to represent possible mechanisms of the chemopreventive activity of curcumin *in vivo*. These were total GST activity, DNA adducts formed by MDA, and inducible COX-2 expression as reflected by PGE_2 production. Induction of GSTs, phase II enzymes that detoxify certain carcinogens, is regarded as a potential mechanism of blockade of the early stages of carcinogenesis (3). Such induction is a property

Received 12/8/00; revised 1/31/01; accepted 2/5/01.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Supported in part by a Leicester Royal Infirmary Research Fellowship (to R. A. S.), United Kingdom Ministry for Agriculture, Fisheries, and Food Contract FS1735 (to C. L.), and NIH Grant CA-77839 (to L. J. M.).
[2] To whom requests for reprints should be addressed, at Oncology Department, Hodgkin Building, University of Leicester, Leicester LE1 9HN, United Kingdom. Phone: 44-116-252-5541; Fax: 44-116-252-5616; E-mail: ras20@le.ac.uk.

[3] The abbreviations used are: COX, cyclooxygenase; GST, glutathione *S*-transferase; MDA, malondialdehyde; PG, prostaglandin; M_1G, malondialdehyde-deoxyguanosine adduct; HPLC, high-performance liquid chromatography; LPS, lipopolysaccharide; CDNB, 1-chloro-2,4-dinitrobenzene as substrate; i.g., intragastric.

of several agents with chemopreventive activity in preclinical models, including curcumin (10) and the broccoli constituent sulforaphane (11). MDA is a naturally occurring product of lipid peroxidation (12), which is also formed during prostaglandin biosynthesis via COX (13), two enzymatic processes that have been implicated in the pathogenesis of a number of cancers, especially colon cancer (14–16). PGE_2 is a product of COX-2, the isoenzyme of COX induced during infection, inflammation, and malignant transformation. Inhibition of COX-2 is thought to be an important mechanism of chemoprevention, exhibited by nonsteroidal anti-inflammatory drugs (14) and dietary agents such as curcumin (9). MDA reacts with DNA under physiological conditions to form adducts, predominantly with deoxyguanosine (M_1G). Accumulated DNA damage may be important in the etiology of many cancers, and such damage may be reflected by exocyclic DNA adducts such as M_1G (17). M_1G has been implicated in the induction of G→T transversions by MDA and is considered a potentially useful "dosimeter" of MDA-induced DNA modification (18). M_1G levels have been described in malignant and nonmalignant human colon cells *in vitro*[4] and in leukocytes, pancreas, breast, and liver in healthy volunteers (12); indeed they are comparable with levels of DNA adducts formed by exogenous carcinogens such as polycyclic hydrocarbons.

The ability of curcumin to prevent malignancies in the colorectal tract in rodents has been particularly well documented (7, 19, 20). It has also been shown to inhibit lipid peroxidation (21) and carbon tetrachloride-mediated hepatotoxicity (22, 23). We therefore aimed to test the hypothesis that dietary curcumin affects GST and M_1G levels in the intestinal mucosa and prevents increases in M_1G levels when lipid peroxidation is induced in this tissue by CCl_4. For comparison, levels of GST and M_1G were also investigated in liver and blood leukocytes. Because COX-2 is not expressed significantly in normal tissues without provocation, an *ex vivo* model was used to study its inducibility in peripheral blood indirectly. To be able to rationalize potential pharmacodynamic changes and interpret them in terms of efficacious drug levels, curcumin and its glucuronide or sulfate conjugation products were determined in intestinal mucosa, plasma, and liver. Studies in rodents suggest that curcumin has poor systemic bioavailability when given p.o. and is cleared rapidly from the plasma when administered i.v. (24), but overall the information on its pharmacokinetic behavior is scarce. Whereas in cancer intervention studies using curcumin it has generally been administered admixed in the diet, studies of its pharmacokinetics and metabolism have used i.g. gavage or non-oral routes of administration. In the light of the difficult pharmaceutical properties of curcumin, *i.e.*, its lipophilicity and susceptibility to rapid metabolism, we also tested the hypothesis that drug and metabolite levels after i.g. gavage are representative of those following curcumin ingestion in the diet.

[4] R. A. Sharma, A. Gescher, J. P. Plastaras, C. Leuratti, R. Singh, L. J. Marnett, W. P. Steward, and S. M. Plummer. Role of cyclooxygenase-2 in modulation of malondialdehyde levels and its deoxyguanosine adduct in human colon cells. Carcinogenesis (Lond.), submitted for publication.

MATERIALS AND METHODS

Reagents. Curcumin was purchased from Apin Chemicals Ltd. (Abingdon, United Kingdom). Analysis by HPLC/mass spectrometry established that the material contained 91% curcumin and 9% desmethoxycurcumin. CCl_4, corn oil, glutathione, CDNB, glycerol formal (consisting of 60% 5-hydroxy-1,3-dioxane and 40% 4-hydroxymethyl-1,3-dioxalone) and Cremophore were purchased from Sigma Chemical Co. (Poole, United Kingdom). Murine M_1G monoclonal antibody D10A1 was prepared as described previously (25). Antimurine horseradish peroxidase antibody was purchased from Dako (Ely, United Kingdom). M_1G standards were synthesized and characterized as described previously (26).

Treatments. Female F344 rats (6-weeks of age; 160–180 g), obtained from Charles River UK Ltd. (Margate, United Kingdom) had access to water and a standard RMI diet (Special Diet Services) *ad libitum*. Rats were kept in groups of four per cage at 20°C on a 12-h light/dark cycle. For dietary administration, curcumin and corn oil were mixed with the diet to furnish 2% for each. This level of curcumin in the diet, which rats received for 14 days, has been shown previously to prevent gastrointestinal tract tumors in several rodent models of chemically induced carcinogenesis (5, 10). Control animals received a diet containing corn oil. CCl_4 was dissolved in corn oil (2 ml/kg) and administered by i.g. gavage at 0.5 ml/kg on day 10 after commencement of dietary curcumin intake. This dose of CCl_4 has been shown previously to raise liver M_1G levels (18). Control animals received the equivalent dose of corn oil.

In the pharmacokinetic study, administration of dietary curcumin was compared with that of an i.g. bolus. For administration by gavage, curcumin was suspended and partially dissolved in a mixture of glycerol formal:cremophore:water (5:2:2), which in preliminary experiments was found to yield a suitable formulation in terms of acceptable viscosity and stability of suspension. This formulation was administered at 500 mg curcumin/kg by i.g. gavage once only or daily for 7 consecutive days. Control animals received the excipient mixture only. Tissue samples were collected 30 min postdose, a time point at which in preliminary experiments curcumin levels had been found to be maximal. For an optimal comparison of this administration mode with dietary curcumin, we exploited the fact that feeding habits of rats are subject to a diurnal cycle with two eating peaks, one of which occurs at around 8:00 p.m. (27). Rats were deprived of food for 6 h, commencing at 1:00 p.m. They then received the curcumin-containing diet for 3 h, coinciding with the eating peak. Subsequently, food was withdrawn for ~30 min, after which blood and tissue samples were collected. This "starvation-refeeding" protocol was performed either in unpretreated animals or in rats that had received the curcumin diet continually for 7 days.

At the end of the feeding period, or subsequent to i.g. administration, rats were subjected to terminal anesthesia (halothane/nitrous oxide), and blood was removed by cardiac puncture. Blood was placed in heparinized tubes and plasma obtained by centrifugation (1100 × *g* at 4°C for 25 min). The large intestine was flushed with PBS and dissected out. Cytosol was prepared by standard procedures (28), and blood leukocytes were isolated using Ficoll-Paque Plus (Amersham Pharmacia

Biotech, Buckinghamshire, United Kingdom). Histological examination ensured accurate scraping of colonic mucosa only. Blood and tissue samples were protected from light, frozen in liquid nitrogen, and stored at −80°C until pharmacodynamic and pharmacokinetic analyses were performed.

Pharmacodynamic Analyses. Total GST activity of cytosol samples was measured spectrophotometrically using glutathione and CDNB (29). Results were corrected for protein levels using the Bio-Rad protein assay (Bio-Rad, Hemel Hempstead, United Kingdom). Extraction of genomic DNA and analysis of M_1G adduct levels by immunoslot blot was performed as described previously (30). Discrepancies in the amount of DNA in each slot were corrected by staining the nitrocellulose filter with propidium iodide and performing UV light densitometry. The detection limit for M_1G was five adducts per 10^8 nucleotides. The model for assessing inducibility of COX-2 *ex vivo* was based on published methods and has been well established in clinical studies as well as preclinical models (31, 32). Aliquots (0.5 ml) of fresh blood were incubated with acetylsalicylic acid (200 μM) for 30 min at 37°C to inactivate platelet COX-1 irreversibly. LPS (10 μg/ml) was added to half the samples and mixed well, and samples were reincubated for 24 h. Plasma was separated by centrifugation and stored at −80°C. PGE_2 in these plasma samples was measured by competitive enzyme immunoassay (Cayman Chemicals, Ann Arbor, MI), with a detection limit of ~30 pg/ml plasma.

Analysis of Curcumin and Its Conjugates. Curcumin and its metabolites were measured as described before (24). Plasma and tissues were extracted with twice the volume of ethyl acetate, and the organic layer was evaporated under nitrogen. Extraction efficiencies from plasma for curcumin, curcumin glucuronide, and curcumin sulfate determined by HPLC (see below) at 0.1 μg/ml were 92 ± 7, 45 ± 10, and 49 ± 9% (mean ± SD, $n = 6$), respectively; the extraction efficiencies at 40 μg/ml were very similar to these values. Extraction efficiencies from liver and colon mucosal scrapings were not significantly different from those obtained in plasma. The reversed-phase HPLC method for detection and quantitation of curcumin and its conjugates used a Symmetry Shield RP 18 column (150 × 3.9 mm; particle size, 5 μm; Waters) and a Varian Prostar (230 model) solvent delivery system coupled to a UV-visible detector (310 model) and autosampler (model 410). Detection of curcumin, curcumin sulfate, 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. A linear gradient of 5–45% acetonitrile in 0.01% ammonium acetate (pH 4.5) was used for 30 min, followed by an increase over 20 min to 95% acetonitrile (flow rate, 1 ml/min). The limits of detection for curcumin, curcumin glucuronide, and curcumin sulfate were 5 pmol/ml plasma or 25 pmol/g tissue, and the limits of quantitation were near 10 pmol/ml plasma and 50 pmol/g tissue. Quantitation of the conjugates was based on calibration curves obtained for curcumin, because we had established previously that the specific absorption at 420 nm was almost identical for the three compounds (24). The quantitative method for curcumin was validated using a 0.1 and 40 μg/ml solution yielding intra-day and inter-day coefficients of variation of 17 and 13%, and 16 and 19%, respectively ($n = 4$).

Table 1 Effect of dietary curcumin[a] on GST levels in rat liver, colon mucosa, and blood lymphocytes

Tissue	Control	Curcumin-fed
Liver	634 ± 66[b]	860 ± 73[c]
Colon mucosa	84 ± 13	69 ± 16
Blood lymphocytes	20 ± 4	16 ± 4

[a] Animals were exposed to curcumin (2%) in the diet for 14 days.
[b] Values, expressed as nmol/min/mg protein using CDNB as substrate, are the mean ± SD of eight separate animals. For experimental details see "Materials and Methods."
[c] The difference between control and treated animals is significant ($P < 0.01$, by ANOVA).

Statistical Evaluation. Results were subjected to ANOVA and Spearman's test of rank correlation, using Excel and Minitab (both Windows 1997) software packages. *Post hoc* Fisher's Least Significant Difference Test was performed.

RESULTS

Pharmacodynamic Effects of Dietary Curcumin. Rats were fed a diet supplemented with 2% curcumin for 2 weeks. On the basis of food consumption, the approximate daily dose ingested was 1.2 g curcumin/kg. Dietary curcumin at this dose did not affect the animals' body weight gain. Levels of GST activity in the liver of curcumin-fed rats were elevated by 36% over those in control animals (Table 1). GST levels in colon mucosa and lymphocytes were marginally decreased by curcumin; however, this difference was not significant. Levels of M_1G adducts in colon mucosa of rats which received curcumin were moderately, but significantly, lower than those in control rats (Fig. 1). M_1G adduct levels in leukocytes and liver of curcumin-fed rats were also slightly decreased as compared with controls, but these differences were not significant. Treatment of rats with CCl_4 increased levels of M_1G adducts in liver and colon mucosa by 49 and 25%, respectively, over control values (Fig. 1). Dietary curcumin attenuated this increase in the liver and completely prevented it in the colon.

To study whether dietary curcumin led to altered COX-2 expression in blood cells, the concentration of PGE_2 in plasma was determined after induction with LPS added *in vitro*. LPS increased blood PGE_2 levels ~3-fold, but curcumin feeding failed to interfere with this increase (result not shown).

Levels of Curcumin after Dietary Administration. Plasma, colon mucosa, and liver from rats that had received curcumin in their diet as described above for 2 weeks were analyzed for the presence of curcuminoids. Curcumin and its metabolites, curcumin glucuronide and curcumin sulfate, could not be detected in the plasma obtained by cardiac puncture or from the hepatic portal vein. Levels of curcumin were 1.8 ± 0.8 μmol/g tissue in the colon mucosa and 0.8 ± 0.3 nmol/g in the liver. Curcumin was also present in the feces (8.6 ± 0.6 μmol/g dried feces). Curcumin glucuronide or curcumin sulfate was not detected in either tissues or feces.

Pharmacokinetic Comparison of Modes of Administration. To compare the availability of curcumin admixed with the diet or suspended in an amphiphilic solvent and adminis-

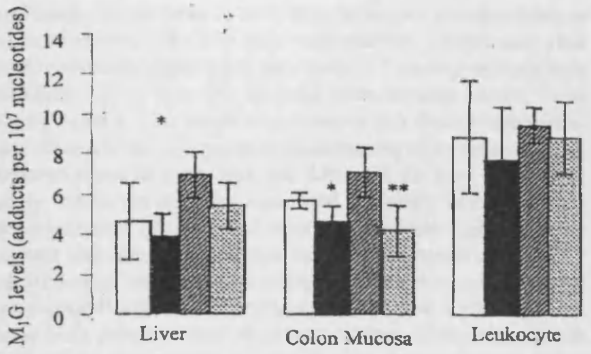


Fig. 1 Effect of dietary curcumin on M_1G adduct levels in liver, colon mucosa, and blood leukocytes. □, control animals; ■ and ▩, animals that received dietary curcumin (2%) for 14 days; ▨ and ▩, animals that received carbon tetrachloride (0.5 ml/kg) via the i.g. route on the 10th day of the study diet. Values are means of eight animals; *bars*, SD. * and **, the difference between control and curcumin-treated animals (*, $P < 0.01$, by ANOVA) or between animals that received carbon tetrachloride either with or without curcumin (**, $P < 0.005$, by ANOVA) is significant. For experimental details, see "Materials and Methods."

Table 2 Curcumin and curcumin conjugates in the plasma of rats that received curcumin in the diet or by the i.g. route

		Plasma levels (pmol/ml)	
Dosing schedule[a]	Curcumin	Curcumin glucuronide	Curcumin sulfate
Short feeding	<10[b]	ND[c]	ND
Single i.g.	30 ± 9	56 ± 36	134 ± 100
7-day feeding	12 ± 5	ND	ND
Repeated i.g.	65 ± 28	638 ± 146	360 ± 160

[a] Rats received curcumin as an i.g. bolus (500 mg/kg) once ("Single i.g.") or daily on 7 consecutive days ("Repeated i.g."), or with the diet (2%) for 3 h after a period of starvation (6 h) once ("Short feeding") or after unlimited access to curcumin containing diet for 7 days ("7-day feeding"). The dose, as calculated by weighing food removal, was between 200 and 400 mg/kg. For details of curcumin administration and measurement of curcumin and metabolites, see "Materials and Methods."

[b] Values are mean ± SD from three to four animals.

[c] ND, not detectable.

tered by i.g. bolus, we studied drug levels after short-term feeding (2% in the diet for 3 h) or single i.g. bolus (500 mg/kg) and after continual feeding or repeated bolus administration for 7 days. To render the dietary dose ingested and the sample timing comparable with those associated with the i.g. bolus, we exploited the "starvation-refeeding" protocol described in "Materials and Methods." The dose ingested with the diet during the refeeding period, as adjudged by the amount of food removed from the hoppers, was between 200 and 400 mg/kg, *i.e.*, between 40 and 80% of, and thus comparable with, the i.g. dose. Tables 2 and 3 show that the levels of curcumin and its conjugates differed substantially, depending on the mode of administration. Plasma levels of curcumin were approximately three to five times higher after the i.g. bolus than after dietary ingestion, and curcumin conjugates were observed in the plasma only when the agent was given via the i.g. route (Table 2). Repeated

Table 3 Curcumin levels in colon mucosa of rats that received curcumin in the diet or by the i.g. route

Dosing schedule[a]	Curcumin levels (nmol/g tissue)
Short feeding	279 ± 295
Single i.g.	1.7 ± 0.9[b]
7-day feeding	482 ± 412
Repeated i.g.	18 ± 24

[a] Rats received curcumin as an i.g. bolus (500 mg/kg) once ("Single i.g.") or daily for 7 consecutive days ("Repeated i.g."), or with the diet (2%) for 3 h after a period (6 h) of starvation ("Short feeding"), or after a starvation-refeeding regimen after unlimited access to a curcumin-containing diet for 7 days ("7-day feeding"). The dose of curcumin when given with the diet, as calculated by weighing food removal, was between 200 and 400 mg/kg. For details of curcumin administration and its measurement, see "Materials and Methods."

[b] Values are mean ± SD from three to four animals.

i.g. administration for 7 days led to an increase in plasma levels of curcumin and its conjugates as compared with single i.g. administration. Curcumin levels were doubled, and levels of curcumin sulfate and curcumin glucuronide were 2.7 and 11 times higher than those seen after single i.g. administration. In contrast, plasma levels of curcumin after its consumption in the diet for a week, at the end of which rats were subjected to the starvation-refeeding regime, did not differ substantially from those measured after short-term dietary consumption. The variation between animals in colon mucosal curcumin levels (Table 3) after dietary consumption was very high, probably reflecting considerable differences in food intake between individual animals during the 3-h period of refeeding. Curcumin levels in the colon mucosa after short-term dietary consumption were 164 times higher than those seen after single i.g. bolus; after dietary consumption for 1 week, they were 27 times higher than after the last of seven daily i.g. bolus doses (Table 3). Liver levels of curcumin were ~0.1 nmol/g, irrespective of route of administration, and there was little difference in liver levels between single or repeated administration via the diet or gavage.

DISCUSSION

The results of this study allow two conclusions that may help to optimize clinical trials of curcumin as a cancer chemopreventive agent: (*a*) the bioavailability of curcumin in blood and tissues is dramatically affected by the way in which it is p.o. administered; and (*b*) colon mucosa and liver are pharmacological targets of dietary curcumin.

The first conclusion is based on the observed differences in levels of curcumin and its conjugates in plasma and tissues after the two administration modes. Dietary curcumin elicited concentrations of the drug in the colon mucosa of between 0.3 and 1.8 μmol/g, whereas plasma levels were around the limit of detection. Curcumin suspended in a solvent mixture and given by i.g. bolus furnished levels of drug in the plasma that were 3–6-fold higher than those seen after dietary administration. Curcumin metabolites were detectable in the plasma only after i.g. administration. Conversely, colon mucosal levels of curcumin after i.g. bolus were only a fraction of those observed after dietary administration.

Curcumin exerts its effects on growth, COX-2 expression,

and transcription factor activity in cells *in vitro* at concentrations of 5 μM or above (9). The colon mucosal level of curcumin after 14 days feeding observed in this study, 1.8 μmol/g, is more than 300-fold higher than the minimal concentration shown to be active *in vitro*. This result demonstrates that dietary administration of curcumin can produce pharmacologically relevant drug concentrations in colon mucosa. Although the dose used in this study, 2% in the diet, has been used frequently in rodent intervention studies (5, 10, 33), it is at least 10 times higher than the highest estimated daily human intake of curcumin as a dietary constituent (4) and does not reflect normal dietary use. In recent intervention studies, dietary levels of 0.2 and 0.1% curcumin have been demonstrated to protect rodents from colon cancer induced by azoxymethane (7, 19, 20) and to reduce polyp numbers in the Min mouse (34), a model of the genetic defect found in human familial adenomatous polyposis. Our results therefore intimate that dietary curcumin at doses considerably lower than those used here might yield pharmacologically efficacious levels in the colon mucosa and perhaps also in the liver.

Intriguingly, curcumin glucuronide and curcumin sulfate were not detected in plasma or tissues after its administration in the diet, whereas they were the major drug-derived species present in the plasma after i.g. administration. In a recent report, a small amount of a curcumin reduction product, tetrahydrocurcumin, was found in plasma samples from rats fed a curcumin-containing diet (1%) subsequent to treatment of plasma with enzymes that hydrolyze xenobiotic glucuronides and sulfates (35). In contrast, products of curcumin reduction were not unequivocally detected in plasma after dietary curcumin given for 14 days in the experiments described here, although hexahydrocurcumin and hexahydrocurcuminol were identified by mass spectrometry in the bile.[5] Overall, this part of the study suggests that the pharmacokinetic behavior of curcumin after administration of an i.g. bolus of curcumin in suspension is clearly unrepresentative of that of curcumin mixed into the diet. This finding is important because, although in intervention studies curcumin is generally given as a constituent of the diet, studies of its pharmacokinetics and metabolism have been performed mostly with the drug formulated in suspension, using DMSO, aqueous carboxymethyl cellulose solution, or arachis oil as solvents, and administered as an i.g. bolus or via the i.p. route (36–39). The results presented here suggest that should oral curcumin be advocated in the chemoprevention of malignancies remote from the liver or gastrointestinal tract, improvement of its oral bioavailability might be necessary, perhaps by formulating it as a solution.

Our second conclusion is borne out by the findings that dietary curcumin elevated hepatic GST levels, reduced colon mucosal M_1G adduct levels, and decreased the elevation of M_1G adduct levels elicited by a powerful lipid peroxidative stimulus in the liver and colon mucosa. These potentially beneficial effects correlated with curcumin levels of 1.8 μmol/g in the colon mucosa and 0.8 nmol/g in liver. The high concentrations of curcumin in the colon mucosa described here are consistent with the outcome of intervention studies in preclinical rodent models of colon cancer (7, 19, 20, 34). Although the decrease in M_1G levels in colon mucosa was modest, it underlines in principle that curcumin supplementation of the diet can achieve drug concentrations sufficient to decrease levels of DNA adducts formed as a corollary of lipid peroxidation. Curcumin glucuronide and curcumin sulfate were not found in liver or colon mucosa after dietary administration of curcumin, strongly suggesting that parent curcumin rather than either conjugate affects GST activities and M_1G adduct levels *in vivo*.

The levels of M_1G adducts in rat liver measured here were approximately three times higher than those described previously in Sprague Dawley rats and are more analogous to background levels found in normal human liver (18). Differences may be related to the age, sex, and strain of the animals used, or fat composition of the diets used. M_1G adduct levels in rat leukocytes and colon mucosa have not been documented previously. CCl_4 has been shown to increase MDA and M_1G adduct levels in rat liver (18), and increases in hepatic MDA levels have been attenuated by feeding rats 100 mg/kg curcumin for 4 weeks (23). The results described here extend these findings, because they show that CCl_4 also engenders M_1G adducts in the colon mucosa, and that this increase can be prevented by dietary curcumin. These findings are pertinent in the light of a recent study in which oxidative DNA damage linked to lipid peroxidation was detected in biopsies from normal human colon (40). The M_1G-lowering effect of curcumin provides a tentative rationale for the regular use of dietary curcumin in the protection of the colon mucosa against oxidative damage, perhaps in premalignant conditions such as ulcerative colitis.

Liver GST activity was raised over controls after curcumin ingestion in the diet. The effects of curcumin on GST and its expression are complex and may involve competitive enzyme inhibition (41) as well as indirect enzyme induction (42). Our finding that 14 days of dietary curcumin induces GST activity in the liver is consistent with an earlier study of the same dose of curcumin in diet and water fed to 8-week-old mice (10). Similarly, mice that received curcumin dissolved in aqueous sodium carboxymethylcellulose via i.g. gavage for 15 days at a dose approximately one-fifth of that used here were found to have significantly higher GST levels in liver compared with controls (41). In contrast, in a more recent study of 8-week-old Sprague Dawley rats, curcumin dissolved in corn oil given daily for 14 days via the i.g. route at various doses failed to induce hepatic GST at doses >6% of the dietary dose used here (43). This discrepancy may relate to differences between the studies in rodent species and strain, age of the animals, dose of curcumin, and its route of administration. Compatible with earlier rodent studies of dietary curcumin (10, 44), we failed to detect any significant alteration in GST activity in the colonic mucosa.

In cells *in vitro*, we have shown previously that curcumin inhibits COX-2 expression by a mechanism involving interference with nuclear factor-κB activation and inhibition of the IκB kinase complex (9). In the study described here, PGE_2 production induced *ex vivo* as an indicator of leukocyte COX-2 activity was not affected by dietary curcumin. The fact that curcumin administered in the diet did not affect COX-2 inducibility, GST activity, or M_1G levels in rat blood leukocytes is consistent with the finding that this mode of administration furnished extremely low levels of parent curcumin in the plasma.

[5] Unpublished result.

In conclusion, the results presented above show that dietary administration of curcumin to rats produces pharmacologically active levels of unmetabolized curcumin in the colon mucosa and liver, capable of decreasing M_1G levels and elevating GST activity. These effects may contribute to cancer chemoprevention. The results also suggest that dietary admixture may be the preferable mode of administration for curcumin in the chemoprevention of colon cancer. The chemopreventive efficacy of oral curcumin in the colorectum and the liver merits clinical evaluation.

ACKNOWLEDGMENTS

We thank Samuel Oustric, Simon Plummer, Raj Singh, Eliot Deag, and Don Jones for technical assistance and Clare Winfield and Sue Spriggs for helping to prepare the manuscript.

REFERENCES

1. Greenlee, R. T., Murray, R., Bolden, S., and Wingo, P. A. Cancer statistics, 2000. CA Cancer J. Clin., *50:* 7–33, 2000.

2. Tattersall, M. H. N., and Thomas, H. Recent advances: oncology. Br. Med. J., *318:* 445–448, 1999.

3. Sharma, R. A., Manson, M. M., Gescher, A., and Steward, W. P. Colorectal cancer chemoprevention: biochemical targets and clinical development of promising agents. Eur. J. Cancer, *37:* 12–22, 2001.

4. Sambaiah, K., Ratankumar, S., Kamanna, V. S., Satyanarayana, M. N., and Rao, M. V. L. Influence of turmeric and curcumin on growth, blood constituents, and serum enzymes in rats. J. Fed. Sci. Technol. (Mysore), *19:* 187–190, 1982.

5. Kelloff, G. J., Crowell, J. A., Hawk, E. T., Steel, V. E., Lubet, R. A., Boone, C. W., Covey, J. M., Doody, L. A., Omenn, G. S., Greenwald, P., Hong, W. K., Parkinson, D. R., Bagheri, D., Baxter, G. T., Blunden, M., Doeltz, M. K., Eisenhauer, K. M., Johnson, K., Knapp, G. G., Longfellow, D. G., Malone, W. F., Nayfield, S. G., Seifried, H. E., Swall, L. M., and Sigman, C. C. Strategy and planning for chemopreventive drug development: Clinical Development Plans II. J. Cell. Biochem., *26S:* 54–71, 1996.

6. Jovanovic, S. V., Steenken, S., Boone, C. W., and Simic, M. G. H-atom transfer is a preferred antioxidant mechanism of curcumin. J. Am. Chem. Soc., *121:* 9677–9681, 1999.

7. Rao, C. V., Rivenson, A., Simi, B., and Reddy, B. S. Chemoprevention of colon carcinogenesis by dietary curcumin, a naturally occurring plant phenolic compound. Cancer Res., *55:* 259–266, 1995.

8. Huang, T. S., Lee, S. C., and Lin, J. K. Suppression of c-jun/AP-1 activation by an inhibitor of tumor promotion in mouse fibroblast cells. Proc. Natl. Acad. Sci. USA, *88:* 5292–5296, 1991.

9. Plummer, S. M., Holloway, K. A., Manson, M. M., Munks, R. J. L., Kaptein, A., Farrow, S., and Howells, L. Inhibition of cyclo-oxygenase 2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF-κB activation via the NIK/IKK signaling complex. Oncogene, *18:* 6013–6020, 1999.

10. Singh, S. V., Hu, X., Srivastava, S. K., Singh, M., Xia, H., Orchard, J. L., and Zaren, H. A. Mechanism of inhibition of benzo[*a*]pyrene-induced forestomach cancer in mice by dietary curcumin. Carcinogenesis (Lond.), *19:* 1357–1360, 1998.

11. Zhang, Y., Kensler, T. W., Cho, C. G., Posner, G. H., and Talalay, P. Anticarcinogenic activities of sulforaphane and structurally related synthetic norbonyl isothiocyanates. Proc. Natl. Acad. Sci. USA, *91:* 3147–3150, 1994.

12. Marnett, L. J. Lipid peroxidation–DNA damage by malondialdehyde. Mutat. Res., *424:* 83–95, 1999.

13. Diczfalusy, U., Falardeau, P., and Hammarstrom, S. Conversion of prostaglandin endoperoxides to C17-hydroxyacids catalysed by human platelet thromboxane synthase. FEBS Lett., *84:* 271–274, 1977.

14. Taketo, M. N. Cyclooxygenase-2 inhibitors in tumorigenesis (part II). J. Natl. Cancer Inst., *90:* 1609–1620, 1998.

15. Hendrickse, C. W., Kelly, R. W., Radley, S., Donovan, I. A., Keighley, M. R. B., and Neoptolemos, J. P. Lipid peroxidation and prostaglandins in colorectal cancer. Br. J. Surg., *81:* 1219–1223, 1994.

16. Wang, M. Y., Dhingra, K., Hittelman, W. N., Liehr, J. G., de Andrade, M., and Li, D. H. Lipid peroxidation-induced putative malondialdehyde-DNA adducts in human breast tissues. Cancer Epidemiol. Biomark. Prev., *5:* 705–710, 1996.

17. Marnett, L. J. Oxyradicals and DNA damage. Carcinogenesis (Lond.), *21:* 361–370, 2000.

18. Chaudhary, A. K., Nokubo, M., Reddy, G. R., Yeola, S. N., Morrow, J. D., Blair, I. A., and Marnett, L. J. Detection of endogenous malondialdehyde-deoxyguanosine adducts in human liver. Science (Wash. DC), *265:* 1580–1582, 1994.

19. Samaha, H. S., Kelloff, G. J., Steele, V., Rao, C. V., and Reddy, B. S. Modulation of apoptosis by sulindac, curcumin, phenylethyl-3-methylcaffeate, and 6-phenylhexyl isothiocyanate: apoptotic index as a biomarker in colon cancer chemoprevention and promotion. Cancer Res., *57:* 1301–1305, 1997.

20. Kawamori, T., Lubet, R., Steele, V. E., Kellof, G. J., Kaskey, R. B., Rao, C. V., and Reddy, B. S. Chemopreventative effect of curcumin, a naturally occurring anti-inflammatory agent, during the promotion/progression stages of colon cancer. Cancer Res., *59:* 597–601, 1999.

21. Ramirez Bosca, A., Soler, A., Gutierrez, M. A. C., Alvarez, J. L., and Almagro, E. Q. Antioxidant *Curcuma* extracts decrease the blood lipid levels of human subjects. Age, *18:* 167–169, 1995.

22. Subramanian, L., and Selvam, R. Prevention of CCl_4-induced hepatotoxicity by aqueous extract of turmeric. Nutr. Res., *19:* 429–441, 1999.

23. Park, E. J., Jeon, C. H., Ko, G., Kim, J., and Sohn, D. H. Protective effect of curcumin in rat liver injury induced by carbon tetrachloride. J. Pharm. Pharmacol., *52:* 437–440, 2000.

24. Ireson, C. R., Orr, S., Jones, D. L., Verschoyle, R., Lim, C. K., Luo, J. L., Howells, L., Plummer, S. P., Jukes, R., Williams, M., Steward, W. P., and Gescher, A. Characterization of metabolites of the chemopreventive agent curcumin in humans and rat hepatocytes and in rat plasma and evaluation of their ability to inhibit cyclooxygenase-2 expression. Cancer Res., *61:* 1058–1064, 2001.

25. Sevilla, C. L., Mahle, N. H., Eliezer, N., Uzieblo, A., O'Hara, S. M., Nokubo, M., Miller, R., Rouzer, C. A., and Marnett, L. J. Development of monoclonal antibodies to the malondialdehyde-deoxyguanosine adduct, pyrimidopurinone. Chem. Res. Toxicol., *10:* 172–180, 1997.

26. Chapeau, M. C., and Marnett, L. J. Enzymatic synthesis of purine deoxynucleoside adducts. Chem. Res. Toxicol., *4:* 636–638, 1991.

27. Vermeulen, J. K., De Vries, A., Schlingmann, F., and Remie, R. Food deprivation: common sense or nonsense? Anim. Technol., *48:* 45–54, 1997.

28. Manson, M. M., Ball, H. W. L., Judah, D. J., Williamson, G., and Neal, G. E. Mechanism of action of dietary chemoprotective agents in rat liver: induction of phase I and II drug-metabolizing enzymes and aflatoxin B1 metabolism. Carcinogenesis (Lond.), *18:* 1729–1738, 1997.

29. Habig, W. H., Pabst, M. J., and Jakoby, W. B. Glutathione *S*-transferases. J. Biol. Chem., *249:* 7130–7139, 1974.

30. Leuratti, C., Singh, R., Lagneau, C., Farmer, P. B., Marnett, L. J., and Shuker, D. E. G. Determination of malondialdehyde-induced DNA damage in human tissues using an immunoslot blot assay. Carcinogenesis (Lond.), *19:* 1919–1924, 1998.

31. Patrignani, P., Panara, M. R., Greco, A., Fusco, O., Natoli, C., Iacobelli, S., Cipollone, F., Ganci, A., Créminion, C., Maclouf, J., and Patrono, C. Biochemical and pharmacological characterization of the cyclooxygenase activity of human blood prostaglandin endoperoxide synthases. J. Pharmacol. Exp. Ther., *271:* 1705–1712, 1994.

32. Warner, T. D., Giuliano, F., Vojnovic, I., Bukasa, A., Mitchell, J. A., and Vane, J. R. Nonsteroidal drug selectivities for cyclooxygenase-1 rather than cyclooxygenase-2 are associated with human gastrointestinal toxicity: a full *in vitro* analysis. Proc. Natl. Acad. Sci. USA, *96:* 7563–7568, 1999.

33. Huang, M. T., Newmark, H. L., and Frenkel, K. Inhibitory effects of curcumin on tumorigenesis in mice. J. Cell. Biochem., *67:* 26–34, 1994.

34. Mahmoud, N. N., Carothers, A. M., Grunberger, D., Bilinski, R. T., Churchill, M. R., Martucci, C., Newmark, H. L., and Bertagnolli M. M. Plant phenolics decrease intestinal tumors in an animal model of familial adenomatous polyposis. Carcinogenesis (Lond.), *21:* 921–927, 2000.

35. Inano, H., Onoda, M., Inafuku, N., Kubota, M., Kamada, Y., Osawa, T., Kobayashi, H., and Wakabayashi, K. Potent preventive action of curcumin on radiation-induced initiation of mammary tumorigenesis in rats. Carcinogenesis (Lond.), *21:* 1835–1841, 2000.

36. Holder, G. M., Plummer, J. L., and Ryan, A. J. The metabolism and excretion of curcumin [1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] in the rat. Xenobiotica, *8:* 761–768, 1978.

37. Ravindranath, V., and Chandrasekhara, N. Absorption and tissue distribution of curcumin in rats. Toxicology, *16:* 259–265, 1980.

38. Shoba, G., Joy, D., Joseph, T., Majeed, M., Rajendran, R., and Srinivas, P. S .S. R. Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers. Planta Med., *64:* 353–356, 1998.

39. Pan, M. H., Huang, T. M., and Lin, J. K. Biotransformation of curcumin through reduction and glucuronidation in mice. Drug Metab. Dispos., *27:* 486–494, 1999.

40. Pool-Zobel, B. L., Abrahamse, S. L., Collins, A. R., Kark, W., Gugler, R., Oberreuther, D., Siegel, E. G., Treptow van Lishaut, S., and Rechkemmer, G. Analysis of DNA strand breaks, oxidized bases, and glutathione *S*-transferase P1 in human colon cells from biopsies. Cancer Epidemiol. Biomark. Prev., *8:* 609–614, 1999.

41. Oetari, S., Sudibyo, M., Commandeur, J. N. M., Samhoedi, R., and Vermeulen, N. P. E. Effect of curcumin on cytochrome P450 and glutathione *S*-transferase activities in rat liver. Biochem. Pharmacol., *51:* 39–45, 1996.

42. Piper, T., Singhal, S. S., Salameh M., Torman, R. T., Awasthi, Y. C., and Awasthi, S. Mechanisms of anticarcinogenic properties of curcumin: the effect of curcumin on glutathione linked detoxification enzymes in rat liver. Int. J. Biochem. Cell Biol., *30:* 445–456, 1998.

43. Susan, M., and Rao, M. N. A. Induction of glutathione *S*-transferase activity by curcumin in mice. Drug Res., *42:* 962–964, 1992.

44. Nijhoff, W. A., Groen, G. M., and Peters, W. H. M. Induction of rat hepatic and intestinal glutathione *S*-transferases and glutathione by dietary naturally occurring anticarcinogens. Int. J. Oncol., *3:* 1131–1139, 1993.

APPENDIX 2F: Sharma RA, Gescher AJ, McLelland HR, Ireson CR, Hill KA, Euden SA, Manson MM, Pirmohamed M, Marnett LJ, Steward WP. Pharmacodynamic and pharmacokinetic study of oral *Curcuma* extract in patients with colorectal cancer. *Clin Cancer Res* 2001, in press.

Pharmacodynamic and Pharmacokinetic Study of Oral *Curcuma* Extract in Patients with Colorectal Cancer[1]

Ricky A. Sharma,[2] Heather R. McLelland, Kirsti A. Hill, Christopher R. Ireson, Stephanie A. Euden, Margaret M. Manson, Munir Pirmohamed, Lawrence J. Marnett, Andreas J. Gescher, and William P. Steward

Oncology Department [R. A. S., H. R. M., K. A. H., S. A. E., W. P. S.] and Medical Research Council Toxicology Unit [A. J. G., C. R. I., M. M. M.], University of Leicester, Leicester LE1 9HN, United Kingdom; Department of Pharmacology and Therapeutics, University of Liverpool, Liverpool L69 3GE, United Kingdom [M. P.]; and Vanderbilt Cancer Center, Nashville, Tennessee 37232 [L. J. M.]

ABSTRACT

***Curcuma* spp. extracts, particularly the dietary polyphenol curcumin, prevent colon cancer in rodents. In view of the sparse information on the pharmacodynamics and pharmacokinetics of curcumin in humans, a dose-escalation pilot study of a novel standardized *Curcuma* extract in proprietary capsule form was performed at doses between 440 and 2200 mg/day, containing 36–180 mg of curcumin. Fifteen patients with advanced colorectal cancer refractory to standard chemotherapies received *Curcuma* extract daily for up to 4 months. Activity of glutathione *S*-transferase and levels of a DNA adduct (M_1G) formed by malondialdehyde, a product of lipid peroxidation and prostaglandin biosynthesis, were measured in patients' blood cells. Oral *Curcuma* extract was well tolerated, and dose-limiting toxicity was not observed. Neither curcumin nor its metabolites were detected in blood or urine, but curcumin was recovered from feces. Curcumin sulfate was identified in the feces of one patient. Ingestion of 440 mg of *Curcuma* extract for 29 days was accompanied by a 59% decrease in lymphocytic glutathione *S*-transferase activity. At higher dose levels, this effect was not observed. Leukocytic M_1G levels were constant within each patient and unaffected by treatment. Radiologically stable disease was demonstrated in five patients for 2–4 months of treatment. The results suggest that (*a*) *Curcuma* extract can be administered safely to patients at doses of up to 2.2 g daily, equivalent to 180 mg of curcumin; (*b*) curcumin has low oral bioavailability in humans and may undergo intestinal metabolism; and (*c*) larger clinical trials of *Curcuma* extract are merited.**

INTRODUCTION

Curcumin (diferuloylmethane), a low molecular weight polyphenol derived from the rhizomes of *Curcuma* spp., has been shown to prevent cancer in the skin, forestomach, duodenum, and colon of mice and in the tongue, colon, mammary glands, and sebaceous glands of rats (1). Of particular interest is the ability of dietary curcumin to interfere with colon carcinogenesis in chemical and genetic rodent models (2–4). Curcumin has also been associated with regression of established malignancy in humans (5). Curcumin is the major constituent of the spice turmeric, which is abundantly used in the diet on the Indian subcontinent, an area that has a low incidence of colorectal cancer (6). Mechanisms by which curcumin prevents cancer are thought to involve up-regulation of carcinogen-detoxifying enzymes, such as GSTs[3] (7–9), antioxidation (10–16), and suppression of expression of the enzyme cyclooxygenase-2 (17, 18). The pharmacokinetic properties of curcumin in humans remain unexplored. In rodents, curcumin undergoes avid metabolism by conjugation and reduction, and its disposition after oral dosing is characterized by poor systemic bioavailability (9, 17, 19, 20).

In view of the paucity of pharmacodynamic and pharmacokinetic information regarding curcumin in humans, we conducted a dose-escalation pilot study of a standardized *Curcuma* extract in patients with advanced colorectal cancer refractory to standard chemotherapy. The aims of the study were threefold. Firstly, we wished to evaluate the safety of curcumin administered p.o. as *Curcuma* extracts. Secondly, we wanted to investigate the suitability of two potential biomarkers of the pharmacological efficacy of curcumin in patients' blood leukocytes: GST activity and the levels of the adduct (M_1G) formed by the reaction of malondialdehyde with deoxyguanosine in DNA. GST enzyme activity has been shown to be up- or down-regulated in rat tissues after oral curcumin treatment, depending on the dose and route of administration (7–9). To aid the interpretation of GST activity data, patients were genotyped for *GSTM1*, *GSTT1*, and *GSTP1*. These represent the three major GST subclasses found in human lymphocytes, at least one of which is relevant to colorectal cancer and resistance to chemo-

Received 2/1/01; revised 4/10/01; accepted 4/13/01.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Supported by program grants from the United Kingdom Medical Research Council (to A. J. G. and M. M. M.), funds from Phytopharm plc. (to W. P. S.), NIH Grant CA77839 (to L. J. M.), and a fellowship from the University Hospitals of Leicester (to R. A. S.).
[2] To whom requests for reprints should be addressed, at Chemoprevention Research Group, Oncology Department, University of Leicester, Leicester LE1 9HN, United Kingdom. Phone: 44-116-252-5541; Fax: 44-116-252-5616; E-mail: ras20@le.ac.uk.

[3] The abbreviations used are: GST, glutathione *S*-transferase; NSAID, nonsteroidal anti-inflammatory drug; HPLC, high-performance liquid chromatography; CEA, carcinoembryonic antigen; CT, computed tomography; CDNB, 1-chloro-2,4-dinitrobenzene; AUC, area under curve.

therapy (21–23). Malondialdehyde is a naturally occurring product of lipid peroxidation and prostaglandin biosynthesis via cyclooxygenase (24, 25). These two cellular processes implicated in the pathogenesis of colorectal cancer (26, 27) are inhibited by curcumin in preclinical models (17, 28). In a recent study in rats, dietary curcumin was shown to up-regulate GST activity in the liver and diminish M_1G levels in colon mucosa, and these effects were accompanied by measurable tissue levels of curcumin (9). The third aim of the study described here was therefore to test the hypothesis that curcumin or products of its metabolism can be detected in blood or excreta of humans. Overall, the study was designed to define parameters that might help to optimize the clinical evaluation of curcumin in Phase I/II trials.

PATIENTS AND METHODS

Patients. The trial was approved by the local ethics committee and the United Kingdom Medicines Control Agency. Fifteen patients enrolled between September 1999 and September 2000 at the Leicester Royal Infirmary met the following eligibility criteria: (*a*) histologically proven adenocarcinoma of the colon or rectum for which no further conventional therapies were available; (*b*) measurable or evaluable disease; (*c*) age > 18 years; (*d*) WHO performance status of 0–2 and life expectancy greater than 12 weeks; (*e*) absolute neutrophil count $\geq 1.5 \times 10^9$/liter; (*f*) hemoglobin $\geq$ 10 g/dl; (*g*) platelets $\geq 100 \times 10^9$/liter; (*h*) aspartate aminotransferase and alanine aminotransferase $< 2.5 \times$ the upper limit of normal; (*i*) serum bilirubin and creatinine $< 1.5 \times$ the upper limit of normal; and (*j*) no previous investigational or chemotherapeutic drugs within 28 days prior to enrollment. Exclusion criteria included: (*a*) active chronic inflammatory or autoimmune disease; (*b*) active infection, including viral infection; (*c*) significant impairment of gastrointestinal function or absorption; (*d*) active peptic ulcer disease; (*e*) known biliary obstruction or biliary insufficiency; and (*f*) use of NSAIDs within 14 days of enrollment. Patients were asked to abstain from NSAID use and the consumption of foods containing the spice turmeric during the study period, and their general practitioners were asked not to prescribe NSAIDs. Written informed consent was obtained from each patient before enrollment. Demographic and baseline characteristics of patients are shown in Table 1. All patients were Caucasian, and all had undergone previous surgery. Three patients stopped NSAID medication 3 weeks before enrollment.

Study Design and Treatment. P54FP was provided in soft gelatin capsules by Phytopharm plc. (Godmanchester, United Kingdom). Each capsule contained 20 mg of curcuminoids (18 mg of curcumin and 2 mg of desmethoxycurcumin) suspended in 200 mg of essential oils derived from *Curcuma* spp. Typical constituents of *Curcuma* essential oil mixtures are tumerone, atlantone, and zingiberene. Chemical analysis by HPLC/mass spectrometry confirmed the content of curcuminoids. This formulation, which in the following text will be referred to as "*Curcuma* extract," was selected on account of the curcumin dose, which equates to dietary intake of turmeric (see below), the reproducibility of curcuminoid content, and the fact that the capsules contained extracts of *Curcuma* plants used in traditional Indian and Southeast Asian medicine. There were

Table 1 Patient characteristics at enrollment

	Daily dose of *Curcuma* extract (mg)				
	440	880	1320	1760	2200
Sex					
Male	3	3	2	1	3
Female	0	0	1	2	0
Age (yrs)					
Mean	67.3	61	60.3	53	63.7
Range	65–71	53–72	59–66	42–73	51–71
WHO performance					
0	2	2	2	3	2
1	1	1	1	0	1
Sites of measurable disease					
Colorectum	1	0	0	0	0
Liver	2	2	2	2	3
Lung	1	0	0	0	1
Lymph nodes	2	2	0	0	1
Other	0	0	0	1	0
Peritoneum	0	1	2	0	1
Previous chemotherapy					
5-Fluorouracil	3	3	3	3	3
Other	2	3	2	2	2
Previous radiotherapy	1	1	0	2	1

three patients per dose level. After at least a 2-h fast, patients consumed 2, 4, 6, 8, or 10 capsules once daily with water. This translates to doses of 440, 880, 1320, 1760, and 2200 mg of *Curcuma* extract per day containing 36, 72, 108, 144, and 180 mg of curcumin, respectively. Treatment was continued until disease progression was established or consent was withdrawn.

Clinical Measurements. Blood, urine, and feces were collected on days 1, 2, 8, and 29 and protected from light and stored at −80°C. Blood was collected before dose administration and at 0.5, 1, 2, 3, 6, and 8 h after dose administration. Samples were collected in tubes pretreated with lithium-heparin (Sarstedt, Loughborough, United Kingdom). Full blood cell count and urea, electrolytes, liver, and bone function were measured in venous samples, and physical examination was performed before treatment and on treatment days 1, 2, 8, and 29 and monthly thereafter. Venous blood levels of the tumor markers CEA and CA19.9 were measured before treatment and after every month of treatment. Radiological assessment of target lesions was performed every 2 months by CT or magnetic resonance imaging scan, in addition to monthly chest X-rays. Blood samples for analysis of GST activity and M_1G levels were collected 1 week before treatment and on days 1, 2, 8, and 29 of treatment, immediately before dosing for M_1G or immediately before and 1 h after each dose for GST. Lymphocytes were separated from fresh blood using Ficoll-Paque Plus (Amersham Pharmacia Biotech, Bucks, United Kingdom), resuspended in 1 ml of 10 mM Tris-HCl (pH 7.8), and stored at −80°C. Patients completed the European Organization for Research and Treatment of Cancer quality of life questionnaire GLQ-C30 (version 2.0) before treatment and monthly during treatment (29).

Chemical Analysis. Extraction of curcumin and its metabolites curcumin glucuronide, curcumin sulfate, hexahydrocurcumin, and hexahydrocurcuminol from plasma and urine was performed, and their recovery efficiency was established, as described previously (17). Curcumin and curcumin sulfate were extracted from feces with 2 parts (w/v) of acetonitrile:water (7:3). Curcuminoids were separated from other fecal constituents by C18 solid phase extraction (Varian, Walton-on-Thames, United Kingdom) and eluted from the column with acetonitrile (2 ml). The reverse-phase HPLC method with UV-visible detection used to analyze curcuminoids in extracts of plasma, urine, or feces has been reported previously (17). The limit of detection for curcumin in plasma and urine was 5 pmol/ml. The synthesis of curcumin sulfate and its identification by electrospray mass spectrometry were performed as described previously (17).

Measurement of GST Activity and M_1G Levels. Glutathione and CDNB were purchased from Sigma Chemical Co. (Poole, United Kingdom). Once thawed, lymphocyte samples were sonicated for 30 s (Fisher 550 sonicator; Fisher, Pittsburgh, PA) on ice and centrifuged at 3000 × *g* (15 min, 4°C). Total GST activity in the supernatant was measured spectrophotometrically using glutathione and CDNB as substrates, in triplicate for each sample (30). Results were corrected for protein levels using the Bio-Rad protein assay (Bio-Rad, Hemel Hempstead, United Kingdom). The GST activity values were quoted as nmol CDNB conjugated with glutathione/min/mg lymphocytic protein. The *GSTM1*, *GSTT1*, and *GSTP1* genotypes were determined by PCR methods described previously (31, 32). Murine M_1G monoclonal antibody D10A1 was prepared as described previously (33). Antirabbit and antimurine horseradish peroxidase antibodies were purchased from Dako (Ely, United Kingdom). M_1G standards were synthesized and characterized, genomic DNA was extracted from whole blood, and leukocytic M_1G adduct levels were analyzed by immunoslot blot in triplicate as described previously (34). Discrepancies in the amount of DNA/slot were corrected for by staining the nitrocellulose filter with propidium iodide and performing UV light densitometry (9). The detection limit for M_1G was 5 adducts/10^8 nucleotides. The assay had been validated previously by collaborating laboratories at the University of Leicester and the Vanderbilt Cancer Center by exchange of samples.

Statistical Evaluation. GST and M_1G values were subjected to a balanced repeated measure ANOVA and linear regression analysis using Minitab (version 10.2) software package. Plots of residuals were used to ensure that variances were homogeneous and that the residuals had a normal distribution.

RESULTS

Tolerability of Oral *Curcuma* Extract. Patients with advanced colorectal cancer ingested P54FP capsules once daily for up to 4 months at doses between 440 and 2200 mg of *Curcuma* extract containing between 36 and 180 mg of curcumin. The treatment was well tolerated at all dose levels, and there was no dose-limiting toxicity. Two types of adverse events, both gastrointestinal, were possibly related to *Curcuma* consumption. One patient on 1320 mg of *Curcuma* extract daily experienced nausea during the first month of treatment (National Cancer Institute toxicity grade 1), which resolved spontaneously despite continuation of treatment. Two patients (one each on 880 and 2200 mg of *Curcuma* extract daily) developed diarrhea (National Cancer Institute grades 2 and 1, respectively) 4 months and 1 month into treatment, respectively. Both patients withdrew from the study before the cause of the diarrhea could be investigated.

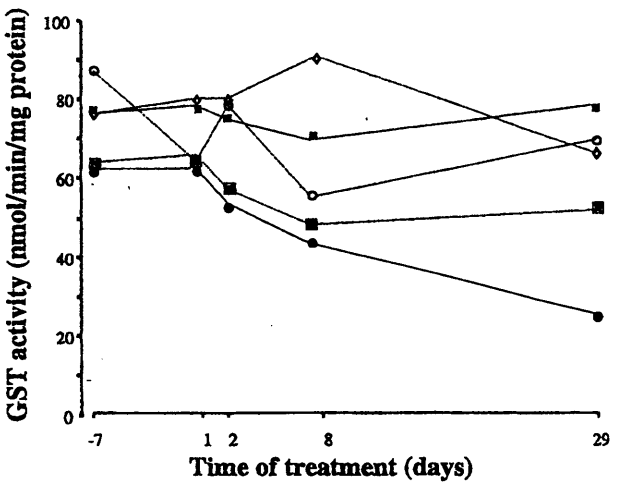


Fig. 1 Mean lymphocytic GST levels for patients on the following daily doses of *Curcuma* extract: 440 (●), 880 (*concentric squares*), 1320 (◇), 1760 (■), and 2200 mg (○), containing 36, 72, 108, 144, and 180 mg of curcumin, respectively. Each point represents means of values for three patients, calculated from triplicate readings for two samples per patient per day (immediately before dose administration and 1 h after dose administration). Time is relative to first dose on day 1. The pooled SD, including all data points, is 10.9 nmol/min/mg protein. For details of patients and measurement, see "Materials and Methods."

Pretreatment GST and M_1G Levels in Relation to GST Polymorphisms. Lymphocytic total GST activity and leukocytic M_1G levels differed substantially between patients (Figs. 1 and 2). Patients were genotyped for GST isoenzymes *GSTM1*, *GSTP1*,and *GSTT1*. Two-thirds of the patients lacked *GSTM1*, slightly more than the 40–60% proportion expected in healthy Caucasians (21), with an even distribution across the five dose levels. In patients who displayed the null genotype for *GSTM1*, pretreatment levels of leukocytic M_1G were 7.6 ± 4.3 adducts/10^7 nucleotides, 74% higher than those in patients expressing *GSTM1*, in whom adduct levels were 4.3 ± 2.6 adducts/10^7 nucleotides ($P < 0.001$ by ANOVA). Two patients were null for *GSTT1*. Their pretreatment levels of leukocytic M_1G (mean levels pooling triplicate readings from both time points, 5.8 ± 1.6 adducts/10^7 nucleotides) were marginally lower than those in patients expressing *GSTT1* (6.6 ± 4.3 adducts/10^7 nucleotides; $P = 0.02$ by ANOVA). Leukocytic levels of M_1G did not correlate with total GST activity, active smoking status ($n = 2$), vegetarianism ($n = 2$), or age.

Biological Effects of Oral *Curcuma* Extract. In patients taking 440 mg of *Curcuma* extract (36 mg of curcumin) daily, lymphocytic GST activity decreased gradually with time from a pretreatment GST value of 64 ± 19 nmol/min/mg protein

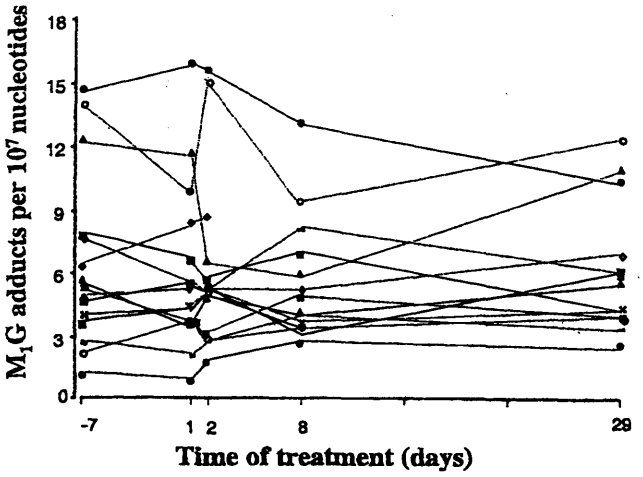


Fig. 2 Leukocytic M_1G adduct levels for patients on *Curcuma* extract over a 5-week period. Each *line* represents one patient; each *point* is the mean of three to five readings from one sample taken before dose administration on each day (shown relative to first dose on day 1). For experimental details, see "Materials and Methods." The interpatient variation is significant ($P < 0.001$ by ANOVA), but there is no significant intrapatient change with time. The pooled SD, including all data points, is 5.4 adducts/10^7 nucleotides.

(measured in triplicate at two time points for each patient) to 26 ± 13 nmol/min/mg protein on day 29 of *Curcuma* consumption ($P < 0.001$ by ANOVA; Fig. 1). This decline was not observed at the higher dose levels. Retrospective subset analysis revealed that pretreatment lymphocytic GST levels (53 ± 10 nmol/min/mg protein) in the five patients who subsequently exhibited stable disease radiologically for more than 3 months of treatment were lower ($P = 0.001$ by ANOVA) than those in the other 10 patients (84 ± 25 nmol/min/mg protein). Leukocytic M_1G levels varied considerably between patients, with no significant difference between subsets of patients as defined by GST polymorphisms or radiological criteria. Consumption of *Curcuma* extract did not affect M_1G levels (Fig. 2).

Levels of Curcuminoids in Blood and Excreta. Neither curcumin nor its metabolites curcumin glucuronide, curcumin sulfate, hexahydrocurcumin, or hexahydrocurcuminol were found in the plasma or urine at up to 29 days of daily treatment. Incubation of plasma and urine samples with glucuronidase and sulfatase enzymes, which would hydrolyze curcumin conjugates, did not generate measurable amounts of curcumin. To test the hypothesis that curcumin, a very lipophilic molecule, is sequestered in blood cells and/or total blood lipoproteins, which may confound its detection in the plasma, these fractions were also analyzed. Curcuminoids were not detected. Feces obtained from patients on days 8 and 29 of consumption of *Curcuma* extract were investigated for the presence of curcuminoids. HPLC analysis furnished a prominent peak in fecal samples from all patients, which coeluted with curcumin. The mass spectrum of this peak was characterized by the presence of the molecular ion ($m/z = 367$) and major fragments of authentic curcumin including its base peak ($m/z = 149$), confirming the identity of this species as curcumin. Levels of curcumin in day 29 fecal samples from patients consuming 1760 and 2200 mg of *Curcuma* extract daily (containing 144 and 180 mg of curcumin) were between 144 and 519 nmol/g dried feces at the lower dose and between 64 and 1054 nmol/g dried feces at the higher dose. The feces of one of the patients on the highest dose contained a species which on HPLC analysis coeluted with curcumin sulfate. Mass spectral investigation of the HPLC peak by selected ion monitoring afforded the molecular ion of $m/z = 447$, and the fragmentation pattern was compatible with that of the authentic reference compound, corroborating the identity of the peak as curcumin sulfate.

Chemotherapeutic Efficacy of Oral *Curcuma* Extract. All patients enrolled exhibited radiological evidence of progressive malignant disease before recruitment. Levels of the tumor marker CEA in venous blood were above the normal range in all patients, and those of CA19.9 were abnormal in 80% of patients. In one patient, who received 440 mg of *Curcuma* extract (equivalent to 36 mg of curcumin) daily, venous blood CEA levels decreased from a pretreatment value of 310 ± 15 to 175 ± 9 μg/liter after 2 months of treatment. This patient experienced stabilization of disease in the colon but progression in the liver, as demonstrated on CT scan. None of the other patients had measurable disease in the colon because the primary tumor had been resected previously. Levels of CA19.9 did not change with treatment. Five patients exhibited stable disease on CT scan [three (on 440, 880, and 1760 mg of *Curcuma* extract) for 3 months and two (on 880 and 1320 mg of *Curcuma* extract) for 4 months of treatment]. Significant changes in quality of life variables were not recorded.

DISCUSSION

The study presented here constitutes the first clinical evaluation of a standardized *Curcuma* extract in patients with cancer including pharmacodynamic and pharmacokinetic measurements. The results allow three conclusions, which will help to optimize the design of future clinical trials of curcumin or *Curcuma* extracts: (*a*) oral administration of *Curcuma* extract for several months at doses of up to 2.2 g daily (equivalent to 180 mg of curcumin) appears safe in the framework of this Phase I study; (*b*) the systemic bioavailability of p.o. administered curcumin is low in humans; and (*c*) *Curcuma* extract may cause clinical benefit in patients with advanced refractory colorectal cancer.

Our first conclusion regarding the apparent safety of *Curcuma* extracts is consistent with previous reports of clinical studies of curcumin and turmeric. Soni and Kuttan (35) treated 10 volunteers with 500 mg of curcumin daily for 7 days and failed to observe clinical toxicity. Two clinical trials designed to study the efficacy of curcumin as an anti-inflammatory agent in the treatment of arthritis or postoperative inflammation found that daily doses of 1.2–2.1 g of curcumin for 2–6 weeks did not cause adverse effects (36, 37). In a pilot study published in abstract form (38), tablets of turmeric extract containing 99.8% curcumin did not cause any treatment-related toxicity at doses as high as 8 g/day. Furthermore, a single dose of 50–200 mg of micronized curcumin formulated as capsules or sachets was administered to 18 volunteers without causing significant tox-

icity.[4] Clinical trials of oral curcumin incorporating larger subject populations will be required to establish the safety of chronic administration. Although certain communities in the Indian subcontinent consume up to 1.5 g of dietary turmeric per person per day, curcumin constitutes only 2–8% of most turmeric preparations (39). The acceptable daily intake of curcumin as an additive has been defined by the WHO as 0–1 mg/kg body weight (40). Thus the largest dose administered in the study presented here (2.2 g of *Curcuma* extract, containing 180 mg of curcumin) exceeds that of dietary consumption.

Our finding that curcumin was detectable only in the feces of patients and not in plasma, blood cells, or urine is in keeping with the low systemic bioavailability of p.o. administered curcumin seen in rodents (9, 17, 19, 20) and suspected in humans (38).[4] After a single oral dose of 2 g, curcumin levels were transiently detectable in the serum of healthy volunteers (41). In that study, coingestion of curcumin with the pepper constituent l-piperoylpiperidine, which is thought to inhibit xenobiotic glucuronidation, appeared to increase curcumin serum AUC by a factor of 20. The presence of curcumin sulfate in the feces of one patient at the highest dose level described here is consistent with the suspicion that curcumin can undergo metabolic conjugation in the gut (42). We are currently testing the hypothesis that sulfation is the major biotransformation route of the curcumin molecule catalyzed by intestinal tissue.

Two potential biomarkers of the systemic efficacy of curcumin were evaluated in the pilot study described here. Lymphocytic GST activity decreased with time in the three patients who received the lowest dose level of *Curcuma* extract. This decrease may have been associated with the treatment, but in light of the small number of patients studied and the fact that GST activity was not decreased in patients on higher dose levels, the interpretation of this observation has to be tentative. Rats fed dietary curcumin at approximately 250 mg/kg body weight and above were found to have decreased hepatic GST activity compared to controls, and competitive enzyme inhibition by the curcumin molecule was thought to be responsible (7). It is unlikely that this observation can be used to rationalize the decline observed in our patients because the dose used in the rats was more than 60-fold higher than that given to the patients. Lymphocytic GST activity, as measured by the CDNB assay, has been shown to be independent of age and gender (43) and constant within subjects, as borne out by measurements on at least three occasions over a 2–4-week period in normal individuals and those at increased risk of developing colorectal cancer (44). The observations made in the study reported here propose similar consistency for patients with advanced cancer. Whether or not lymphocytic GST activity correlates with colon mucosal GST levels in patients with colon cancer, as was demonstrated in individuals at risk of developing colon cancer (44), remains to be established.

This study provides the first description of leukocytic M_1G levels in patients with colorectal cancer. The lower levels shown in Fig. 2 are comparable with those reported previously in healthy volunteers, whereas the highest levels resemble those seen in humans on pro-oxidant diets (45). M_1G adduct levels were unaffected by *Curcuma* consumption. It is conceivable that higher doses of curcumin, which furnish measurable plasma curcumin concentrations, are required to elicit an antilipid peroxidative effect in the blood. Nevertheless, the intraindividual reproducibility over time of M_1G adduct levels supports the potential suitability of this adduct as a biomarker of the systemic effects of curcumin or other chemopreventive antioxidants. The putative link between the *GSTM1* null genotype and elevated leukocytic M_1G adduct levels observed in the patients in this trial is congruous with associations reported previously between *GSTM1* genotype and levels of aflatoxin B_1-induced DNA adducts (46) but is the first suggestion of such an association for an adduct formed by an endogenous product of lipid peroxidation.

One-third of the patients in this study experienced stable disease for 3 months or longer, and in one additional patient, *Curcuma* extract may have been linked to a decrease in venous tumor marker level and abatement of progression of the primary colon tumor without a cytostatic effect on liver metastases. The possibility that patients with colorectal cancer may benefit from consumption of *Curcuma* extract merits evaluation at higher dose levels and ultimately within the framework of larger studies incorporating control groups.

In conclusion, despite the lack of reproducible effects of *Curcuma* extracts on the biomarkers studied, this pilot study of *Curcuma* extract in patients with colorectal cancer provides information that might help optimize the design of the future clinical evaluation of curcumin. Doses of up to 2.2 g of *Curcuma* extract (containing 180 mg of curcumin) per day can be administered to patients with cancer for up to 4 months, and in this pilot study, such treatment was safe. Clinical trials of *Curcuma* extracts as potential cancer chemopreventive agents should focus on the effects of such doses in target tissues, particularly colon epithelium. Moreover, because consumption of *Curcuma* extract was not detrimental to patients with advanced cancer, future trials of *Curcuma* extracts as potential cancer chemotherapeutic agents should study the systemic effects of higher dose levels. Leukocytic GST activity and M_1G levels merit further exploration as potentially suitable biomarkers of pharmacological efficacy in this regard.

ACKNOWLEDGMENTS

We thank the following: the patients who participated in this study; N. Rush, C. Sanganee, A. Alfirevic, and S. Feehan (Leicester and Liverpool Universities) for help with the patients' samples; Drs. R. Singh, D. Jones, S. Plummer, M. Festing, M. Williams, and C. K. Lim and Profs. D. Shuker and P. B. Farmer (MRC Toxicology Unit) for help with experiments and useful discussions; Prof. D. Barnett and Dr. P. Woll for reviewing the case report forms; and Phytopharm plc., particularly Drs. R. Grover and A. Kelly, for advice and provision of P54FP capsules.

REFERENCES

1. Kelloff, G. J., Crowell, J. A., Hawk, E. T., Steel, V. E., Lubet, R. A., Boone, C. W., Covey, J. M., Doody, L. A., Omenn, G. S., Greenwald,

[4] D. E. Brenner, I. Shureiqi, J. Bailey, D. Normolle, G. Kelloff, J. Crowell, C. Ireson, A. Gescher, C. L. Rock, and M. T. Ruffin. A Phase I study of curcumin: bioavailability and safety profile for low doses, submitted for publication.

P., Hong, W. K., Parkinson, D. R., Bagheri, D., Baxter, G. T., Blunden, M., Doeltz, M. K., Eisenhauer, K. M., Johnson, K., Knapp, G. G., Longfellow, D. G., Malone, W. F., Nayfield, S. G., Seifried, H. E., Swall, L. M., and Sigman, C. C. Strategy and planning for chemopreventive drug development: clinical development plans II. J. Cell. Biochem., *26S:* 54–71, 1996.

2. Rao, C. V., Rivenson, A., Simi, B., and Reddy, B. S. Chemoprevention of colon carcinogenesis by dietary curcumin, a naturally occurring plant phenolic compound. Cancer Res., *55:* 259–266, 1995.

3. Kawamori, T., Lubet, R., Steele, V. E., Kelloff, G. J., Kaskey, R. B., Rao, C. V., and Reddy, B. S. Chemopreventative effect of curcumin, a naturally occurring anti-inflammatory agent, during the promotion/progression stages of colon cancer. Cancer Res., *59:* 597–601, 1999.

4. Mahmoud, N. N., Carothers, A. M., Grunberger, D., Bilinski, R. T., Churchill, M. R., Martucci, C., Newmark, H. L., and Bertagnolli, M. M. Plant phenolics decrease intestinal tumors in an animal model of familial adenomatous polyposis. Carcinogenesis (Lond.), *21:* 921–927, 2000.

5. Kuttan, R., Sudheeran, P. C., and Josph, C. D. Turmeric and curcumin as topical agents in cancer therapy. Tumori, *73:* 29–31, 1987.

6. Greenlee, R. T., Murray, R., Bolden, S., and Wingo, P. A. Cancer statistics, 2000. CA Cancer J. Clin., *50:* 7–33, 2000.

7. Piper, J. T., Singhal, S. S., Salameh, M., Torman, R. T., Awasthi, Y. C., and Awasthi, S. Mechanisms of anticarcinogenic properties of curcumin: the effect of curcumin on glutathione linked detoxification enzymes in rat liver. Int. J. Biochem. Cell. Biol., *30:* 445–456, 1998.

8. Susan, M., and Rao, M. N. A. Induction of glutathione *S*-transferase activity by curcumin in mice. Arzneim. Forsch., *42:* 962–964, 1992.

9. Sharma, R. A., Ireson, C. R., Verschoyle, R. D., Hill, K. A., Williams, M. L., Leuratti, C., Manson, M. M., Marnett, L. J., Steward, W. P., and Gescher, A. Effects of dietary curcumin on glutathione *S*-transferase and malondialdehyde-DNA adducts in rat liver and colon mucosa: relationship with drug levels. Clin. Cancer Res., *7:* 1542–1548, 2001.

10. Subramanian, M., Sreejayan, M., Rao, M. N., Devasagayam, T. P., and Singh, B. B. Diminution of singlet oxygen induced DNA damage by curcumin and related antioxidants. Mutat. Res., *311:* 249–255, 1994.

11. Tonnesen, H. H., and Greenhill, J. V. Studies on curcumin and curcuminoids. XXII. Curcumin as a reducing agent and as a radical scavenger. Int. J. Pharm. (Amst.), *87:* 79–87, 1992.

12. Kunchandy, E., and Rao, M. N. A. Oxygen radical scavenging activity of curcumin. Int. J. Pharm. (Amst.), *58:* 237–240, 1990.

13. Reddy, A. C. H. P., and Lokesh, B. R. Studies on the inhibitory effects of curcumin and eugenol on the formation of reactive oxygen species and the oxidation of ferrous iron. Mol. Cell. Biochem., *137:* 1–8, 1994.

14. Donatus, I. A., Sardjoko, X. X., and Vermeulen, N. P. E. Cytotoxic and cytoprotective activities of curcumin. Biochem. Pharmacol., *39:* 1869–1875, 1990.

15. Mukhopadhary, A., Basu, N., Ghatak, N., and Gujral, P. K. Anti-inflammatory and irritant activities of curcumin analogues in rats. Agents Actions, *12:* 508–515, 1982.

16. Sharma, S. C., Mukhtar, H., Sharma, S. K., and Krishna Murt, C. R. Lipid peroxide formation in experimental inflammation. Biochem. Pharmacol., *21:* 1210–1214, 1972.

17. Ireson, C. R., Orr, S., Jones, D. L., Verschoyle, R., Lim, C. K., Luo, J. L., Howells, L., Plummer, S. M., Jukes, R., Williams, M., Steward, W. P., and Gescher, A. Characterization of metabolites of the chemopreventive agent curcumin in humans and rat hepatocytes and in rat plasma and evaluation of their ability to inhibit cyclooxygenase-2 expression. Cancer Res., *61:* 1058–1064, 2001.

18. Plummer, S. M., Holloway, K. A., Manson, M. M., Munks, R. J., Kaptein, A., Farrow, S., and Howells, L. Inhibition of cyclo-oxygenase 2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF-κB activation via the NIK/IKK signalling complex. Oncogene, *18:* 6013–6020, 1999.

19. Holder, G. M., Plummer, J. L., and Ryan, A. J. The metabolism and excretion of curcumin (1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) in the rat. Xenobiotica, *8:* 761–768, 1978.

20. Wahlstrom, B., and Blennow, G. A study on the fate of curcumin in the rat. Acta Pharmacol. Toxicol., *43:* 86–92, 1978.

21. Hayes, J. D., and Pulford, D. J. The glutathione *S*-transferase supergene family: regulation of GST and contribution of the isoenzymes to cancer chemoprevention and drug resistance. Crit. Rev. Biochem. Mol. Biol., *30:* 445–600, 1995.

22. Mulder, T. P. J., Verspaget, H. W., Sier, C. F. M., Roelofs, H. M., Ganesh, S., Griffioen, G., and Peters, W. H. Glutathione *S*-transferase π in colorectal tumors is predictive for overall survival. Cancer Res., *55:* 2696–2702, 1995.

23. Tew, K. D. Gluathione-associated enzymes in anticancer drug resistance. Cancer Res., *54:* 4313–4320, 1994.

24. Marnett, L. J. Lipid peroxidation: DNA damage by malondialdehyde. Mutat. Res., *424:* 83–95, 1999.

25. Diczfalusy, U., Falardeau, P., and Hammarstrom, S. Conversion of prostaglandin endoperoxides to C17-hydroxy acids catalyzed by human platelet thromboxane synthase. FEBS Lett., *84:* 271–274, 1977.

26. Taketo, M. M. Cyclooxygenase-2 inhibitors in tumorigenesis (part II). J. Natl. Cancer Inst. (Bethesda), *90:* 1609–1620, 1998.

27. Hendrickse, C. W., Kelly, R. W., Radley, S., Donovan, I. A., Keighley, M. R. B., and Neoptolemos, J. P. Lipid peroxidation and prostaglandins in colorectal cancer. Br. J. Surg., *81:* 1219–1223, 1994.

28. Venkatesan, P., and Rao, M. N. A. Structure-activity relationships for the inhibition of lipid peroxidation and the scavenging of free radicals by synthetic symmetrical curcumin analogues. J. Pharm. Pharmacol., *52:* 1123–1128, 2000.

29. Aaronson, N. K., Ahmedzai, S., Bergman, B., Bullinger, M., Cull, A., Duez, N. J., Filiberti, A., Flechtner, H., Fleishman, S. B., and de Haes, J. C. The European Organization for Research and Treatment of Cancer QLQ-C30: a quality of life instrument for use in international clinical trials in oncology. J. Natl. Cancer Inst. (Bethesda), *85:* 365–376, 1993.

30. Habig, W. H., Pabst, M. J., and Jakoby W.B. Glutathione *S*-transferases. J. Biol. Chem., *249:* 7130–7139, 1974.

31. DeSousa, M., Pirmohamed, M., Kitteringham, N. R., Woolf, T., and Park, B. K. No association between tacrine transaminitis and the glutathione transferase θ genotype in patients with Alzheimer's disease. Pharmacogenetics, *8:* 353–355, 1998.

32. Harries, L. W., Stubbins, M. J., Forman, D., Howard, G. C., and Wolf, C. R. Identification of genetic polymorphisms at the glutathione *S*-transferase π locus and association with susceptibility to bladder, testicular and prostate cancer. Carcinogenesis (Lond.), *18:* 641–644, 1997.

33. Sevilla, C. L., Mahle, N. H., Eliezer, N., Uzieblo, A., O'Hara, S. M., Nokubo, M., Miller, R., Rouzer, C. A., and Marnett, L. J. Development of monoclonal antibodies to the malondialdehyde-deoxyguanosine adduct, pyrimidopurinone. Chem. Res. Toxicol., *10:* 172–180, 1997.

34. Leuratti, C., Singh, R., Lagneau, C., Farmer, P. B., Marnett, L. J., and Shuker, D. E. G. Determination of malondialdehyde-induced DNA damage in human tissues using an immunoslot blot assay. Carcinogenesis (Lond.), *19:* 1919–1924, 1998.

35. Soni, K. B., and Kuttan, R. Effect of oral curcumin administration on serum peroxides and cholesterol levels in human volunteers. Indian J. Physiol. Pharmacol., *36:* 273–275, 1992.

36. Deodhar, S. D., Sethi, R., and Srimal, R. C. Preliminary study on antirheumatic activity of curcumin (diferuloyl methane). Indian J. Med. Res., *71:* 632–634, 1980.

37. Satoskar, R. R., Shah, S. J., and Shenoy, S. G. Evaluation of anti-inflammatory property of curcumin (diferuloyl methane) in patients with post-operative inflammation. Int. J. Clin. Pharmacol. Ther. Toxicol., *24:* 651–654, 1986.

38. Cheng, A. L., Lin, J. K., Hsu, M. M., Shen, T. S., Ko, J. Y., Lin, J. T., Wu, M. S., Yu, H. S., Jee, S. H., Chen, G. S., Chen, T. M., Chen, C. A., Lai, M. K., Pu, Y. S., Pan, M. H., Wang, U. J., Tsai, C. C., and

Hsieh, C. Y. Phase I chemoprevention clinical trial of curcumin. Proc. Am. Soc. Clin. Oncol., *17:* 558a, 1998.

39. Sambaiah, K., Ratankumar, S., Kamanna, V. S., Satyanarayana, M. N., and Rao, M. V. L. Influence of turmeric and curcumin on growth, blood constituents and serum enzymes in rats. J. Food Sci. Technol., *19:* 187–190, 1982.

40. Evaluation of Certain Food Additives: 51st Report of the Joint FAO/WHO Expert Committee on Food Additives, WHO Technical Report Series 891. Geneva: WHO, 2000.

41. Shoba, G., Joy, D., Joseph, T., Majeed, M., Rajendran, R., and Srinivas, P. S. S. R. Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers. Planta Med., *64:* 353–356, 1998.

42. Ravrindanath, V., and Chandrasekhara, N. *In vitro* studies on the intestinal absorption of curcumin in rats. Toxicology, *20:* 251–257, 1981.

43. van Lieshout, E. M. M., and Peters, W. H. M. Age and gender dependent levels of glutathione and glutathione *S*-transferases in human lymphocytes. Carcinogenesis (Lond.), *19:* 1873–1875, 1998.

44. Szarka, C. E., Pfeiffer, G. R., Hum, S. T., Everley, L. C., Balshem, A. M., Moore, D. F., Litwin, S., Goosenberg, E. B., Frucht, H., and Engstrom, P. F. Glutathione *S*-transferase activity and glutathione *S*-transferase μ expression in subjects at risk for colorectal cancer. Cancer Res., *55:* 2789–2793, 1995.

45. Fang, J. L., Vaca, C. E., Valsta, L. M., and Mutanen, M. Determination of DNA adducts of malondialdehyde in humans: effects of dietary fatty acid composition. Carcinogenesis (Lond.), *17:* 1035–1040, 1996.

46. Liu, Y. H., Taylor, J., Linko, P., Lucier, G. W., and Thompson, C. L. Gluathione *S*-transferase mu in human lymphocyte and liver: role in modulating formation of carcinogen-derived DNA adducts. Carcinogenesis (Lond.), *12:* 2269–2275, 1991.

APPENDIX 2G: Sharma RA, Gescher A, Plastaras JP, Leuratti C, Singh R, Gallacher-Horley B, Offord E, Marnett LJ, Steward WP, Plummer SM. Cyclooxygenase-2, malondialdehyde and pyrimidopurinone adducts of deoxyguanosine in human colon cells. *Carcinogenesis* 2001, in press.

Carcinogenesis vol.22 no.9 pp.1557–1560, 2001

SHORT COMMUNICATION

Cyclooxygenase-2, malondialdehyde and pyrimidopurinone adducts of deoxyguanosine in human colon cells

Ricky A.Sharma[4], Andreas Gescher[1], John P.Plastaras[2], Chiara Leuratti[1], Rajinder Singh[1], Barbara Gallacher-Horley[1], Elizabeth Offord[3], Lawrence J.Marnett[2], William P.Steward and Simon M.Plummer[1]

Oncology Department and [1]MRC Toxicology Unit, University of Leicester, Leicester LE1 9HN, UK, [2]Vanderbilt–Ingram Cancer Center, Nashville, TN 37232, USA and [3]Nestlé Research Center, Lausanne PO Box 44, CH-1000 Lausanne 26, Switzerland

[4]To whom correspondence should be addressed
Email: ras20@le.ac.uk

Cyclooxygenases (COX) catalyse the oxygenation of arachidonic acid to prostaglandin (PG) endoperoxides. Activity of one of the COX isoforms, COX-2, results in production of prostaglandin E_2 (PGE_2) via the endoperoxide PGH_2. COX-2 has been implicated in the pathogenesis of colorectal cancer. Malondialdehyde (MDA) is a mutagen produced by spontaneous and enzymatic breakdown of PGH_2. MDA reacts with DNA to form adducts, predominantly the pyrimidopurinone adduct of deoxyguanosine (M_1G). Here the hypothesis was tested that COX-2 activity in human colon cells results in formation of MDA and generation of M_1G adducts. M_1G was detected in basal cultures of human non-malignant colon epithelial (HCEC) and malignant SW48, SW480, HT29 and HCA-7 colon cells, at levels from 77 to 148 adducts/10^8 nucleotides. Only HCA-7 and HT29 cells expressed COX-2 protein. Levels of M_1G correlated significantly ($r = 0.98$, $P < 0.001$) with those of intracellular MDA determined colorimetrically in the four malignant cell types, but neither parameter correlated with expression of COX-2 or PG biosynthesis. Induction of COX-2 expression by phorbol 12-myristate 13-acetate in HCEC cells increased PGE_2 production 20-fold and MDA concentration 3-fold. Selective inhibition of COX-2 activity in HCA-7 cells by NS-398 significantly inhibited PGE_2 production, but altered neither MDA nor M_1G levels. Malondialdehyde treatment of HCEC cells resulted in a doubling of M_1G levels. These results show for the first time in human colon cells that COX-2 activity is associated with formation of the endogenous mutagen, MDA. Moreover, they demonstrate the correlation between MDA concentration and M_1G adduct levels in malignant cells.

Colorectal cancer is the second commonest malignancy in developed countries and a significant cause of mortality. The epidemiological and molecular basis of colorectal cancer has been characterized in some detail and intense efforts are currently focused on applying this knowledge to improve risk assessment, screening and intervention for early stages of colorectal carcinogenesis (1). Accumulated DNA damage appears to contribute substantially to the aetiology of colorectal cancer (2). Many types of DNA damage have been observed in human tissues with several of these being related to normal cellular biochemistry, such as oxidative stress. The genotoxicity of exogenous and endogenous oxidizing agents arises either from direct damage to DNA or from reactions with other biomolecules that lead to formation of DNA-reactive electrophilic species (3). The oxidation of lipids, giving rise to products such as MDA and certain characteristic DNA adducts, provides an example of the indirect pathway. Since 'control' DNA from tissue unexposed to endogenous mutagens does not exist *in vivo*, correlative studies are necessary to relate changes in DNA adduct levels to carcinogenic events or intervention with chemopreventive agents (4).

Cyclooxygenase (COX) catalyses the conversion of arachidonic acid to PGG_2 and PGH_2 (Figure 1, step 1) and occurs as two isozymes, COX-1 and COX-2. COX-2 can be induced by tumour promoting stimuli *in vitro* (5) and by infection and inflammation *in vivo* (6). COX-2 mRNA and protein levels are markedly increased in many human colon adenomas and carcinomas in comparison to normal colon tissue (7,8). Inhibition of COX-2 causes cytostasis of colon cancer cells *in vitro* and impairs the growth of intestinal adenocarcinoma xenografts in mice (9). Therefore COX-2 inhibitors are currently under evaluation as potential colorectal cancer chemopreventive agents (1).

Malondialdehyde (MDA) can be generated during COX catalysis in human platelets and liver cells (10,11) by breakdown of PGH_2 via at least three routes (Figure 1). Consistent with this notion is the finding that high levels of MDA in colorectal cancer tissue appear to correlate with levels of prostaglandin E_2 (PGE_2) (12), a principal product of COX-2 (13). Malondialdehyde is mutagenic in bacterial and mammalian cells, and carcinogenic in rats (4). Malondialdehyde reacts with DNA under physiological conditions to form adducts, predominantly with deoxyguanosine to generate pyrimidopurinone-deoxyguanosine adducts (M_1G) (14). M_1G has also been associated with mutagenesis and has been detected in a variety of human tissues in the range of 3–150 adducts/10^8 nucleotides (4). Whilst oxidative DNA damage is known to occur in the human colon and has been linked with lipid peroxidation (15), M_1G in human colon tissue has not yet been described. M_1G levels in rat colon mucosa have recently been measured (16).

In the light of these results, we postulated that COX-2 activity may contribute to the development of the malignant phenotype in human colon cells via generation of mutagenic M_1G adducts. In order to test this hypothesis, basal levels of COX-2 and PGE_2 were compared with those of MDA and M_1G in five human colon cell lines: human non-malignant colon epithelial cells (HCEC) and adenocarcinoma SW48, SW480, HT29 and HCA-7 cell lines. Furthermore, cellular COX-2 activity was pharmacologically manipulated to study possible consequences for cellular MDA and M_1G levels.

Abbreviations: COX, cyclooxygenase; HCEC, human colon epithelial cells; MDA, malondialdehyde; M_1G, pyrimidopurinone–deoxyguanosine adduct; PG, prostaglandin; PMA, phorbol 12-myristate 13-acetate.

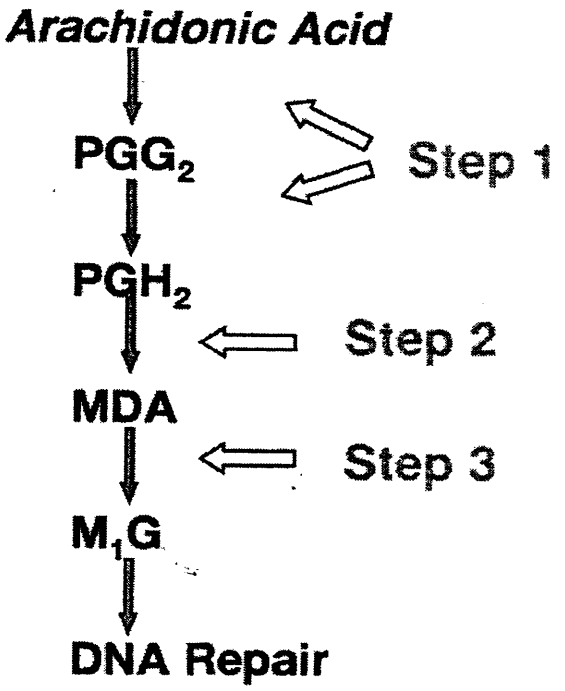


Fig. 1. Conversion of arachidonic acid to prostaglandin endoperoxides resulting in formation of MDA and potentially M_1G adducts. Step 1 incorporates both oxygenase and peroxidase activities of the COX enzyme. Step 2 represents breakdown of PGH_2 to MDA and hydroxyheptadecatrieonic acid, which can occur spontaneously or via catalysis by thromboxane synthases or other cytochromes P450 (11). Step 3 is one possible mechanism of formation of the M_1G adduct.

Non-malignant colonic epithelial HCEC cells (5) were passaged in B50 medium (Biofluids, Rockville, MD) containing bovine serum albumin, bovine pituitary extracts, retinoic acid, vitamin C and dexamethasone. Malignant colorectal carcinoma cell lines SW48, SW480, HT29 and HCA-7, which were obtained from Dr S.MacKay (University of Florida, Gainesville, FL), Prof. C.Paraskeva (Bristol University, Bristol, UK) and Dr S.Kirkland (Imperial College, London, UK), were cultured routinely in DMEM with Glutamax-1 (Life Technologies, Paisley, UK) containing 10% fetal calf serum. Experiments with all five cell types were conducted in this medium and repeated in serum-free medium to demonstrate that the presence of serum did not affect the parameters measured (data not shown). COX-2 polyclonal antibody and COX-2 protein standard were purchased from Oxford Biomedical (Oxford, UK). Anti-α-tubulin monoclonal antibody was obtained from Amersham Pharmacia Biotech (Little Chalfont, Bucks, UK). Murine M_1G monoclonal antibody D10A1, M_1G standards and NaMDA, the sodium salt of monomeric MDA, were synthesized and characterized as previously described (17,18). Anti-rabbit and anti-murine horseradish peroxidase antibodies were purchased from Dako (Ely, UK). Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma (Poole, UK) and the highly selective COX-2 inhibitor, NS-398, from Cayman (Ann Arbor, MI). Both agents were dissolved in dimethylsulphoxide before dilution in cell culture medium.

Experiments were conducted with cells grown to ~70% confluence. In order to stimulate COX-2 expression, HCEC cells were incubated with PMA (75 nM) for up to 72 h. For chemical generation of M_1G adducts, HCEC cells were treated with NaMDA (1 mM) for 24 h (19). In order to inhibit COX-2 activity, cells were incubated for up to 72 h with NS-398 at 17.7 μM, which is 10-fold higher than the reported IC_{50} value for enzyme inhibition *in vitro* (20). An aliquot (1 ml) of the culture medium was removed for measurement of PGE_2. Cells were counted after staining with trypan blue using a haemocytometer. Antioxidants that prevent lipid peroxidation were not added during workup, since previous studies have found no evidence for the generation of M_1G artifactually in hepatic and testicular tissues, with a limit of detection of 3 adducts/10^9 nucleotides (21). Malondialdehyde levels of cell lysates were assessed immediately by the colorimetric Lipid Peroxidation Assay kit (Calbiochem, San Diego, CA), which has been validated as a measure of intracellular MDA concentration in microsomes *in vitro* (22). The detection limit was ~0.1 nmol/mg protein. PGE_2 levels were determined by competitive enzyme immunoassay (Cayman); the detection limit was 10 pg/10^6 cells or 30 pg/ml culture medium. Levels were normalized with respect to cell number. COX-2 protein levels were assessed by western blotting as previously described (5). Blots were stripped and re-analysed for α-tubulin to control for protein loading and transfer. Extraction of genomic DNA and analysis of M_1G adduct levels by immunoslot blot was performed as previously described (23). The detection limit for M_1G was 5 adducts/10^8 nucleotides. M_1G results were confirmed by exchange of samples and repeat analyses at the participating laboratories in Leicester and Vanderbilt. Variation for repeat analyses of the same sample was <10%. The immunoslot blot assays were validated using M_1G standards and DNA from RKO human colorectal carcinoma cells by gas chromatography–electron capture negative chemical ionization mass spectrometry, as previously described (19,23). In case PMA treatment resulted in *de novo* DNA synthesis, the M_1G data presented in Table II were normalized to the number of viable cells used for DNA extraction. Results were subjected to analyses of variance (ANOVA) and covariance with a 5% significance level using Minitab software (Minitab Inc., State College, PA). Plots of residuals were used to ensure that variances were homogeneous and that the residuals had a normal distribution.

Expression of COX protein and PGE_2 production were compared with basal levels of intracellular MDA and M_1G adducts in the five human-derived colon cell types. All the cells contained low but similar levels of COX-1 protein when corrected for protein loading (Figure 2). Detectable COX-2 protein was found only in HCA-7 cells and, to a slight extent, in HT29 cells under basal culture conditions (Figure 2). Measurable levels of M_1G adducts were detected in all five cell lines (Table I) and the differences observed between SW480, HT29, HCA-7 and SW48 cells were statistically significant ($P < 0.005$ by ANOVA). In the malignant cell types, levels of MDA correlated significantly with those of M_1G, whereas the non-malignant HCEC cells did not fit this correlation (Figure 3). There was no correlation of M_1G levels with COX-2 protein or PGE_2 production (Table I). Incubation of HCEC cells with 1 mM NaMDA for 24 h caused an increase in M_1G levels from 79 ± 25 adducts/10^8 nucleotides in control cells to 140 ± 34 adducts/10^8 nucleotides (mean ± SD; $n = 3$).

Incubation of HCEC cells with the phorbol ester PMA induced COX-2 expression (Figure 2) but did not affect COX-1 levels. It also elicited a concomitant increase in PGE_2 production, which after 4 h was ~20-fold higher than control

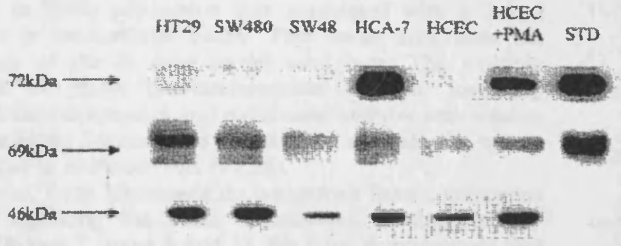


Fig. 2. COX expression in human-derived colon cell lines in basal culture and after treatment of human colon epithelial cells (HCEC) with PMA. SDS–PAGE western blots show constitutive levels of COX-2 (72 kDa) and COX-1 (69 kDa) protein in HT29, SW480, SW48 and HCA-7 colorectal carcinoma cells and non-malignant HCEC colon cells in basal culture; and in HCEC cells after incubation with 75 nM PMA for 4 h. The blots were re-probed for α-tubulin (46 kDa) to control for equal protein loading and transfer. The results shown are typical of three separate experiments. For details of western analysis see Materials and methods.

Table I. Basal cellular levels of COX-2, PGE_2, MDA and M_1G adducts

Cell type	COX-2[a]	PGE_2 (pg/10^6 cells)[b]	MDA (nmol/mg protein)[b]
HCEC	ND[c]	31 ± 20	0.38 ± 0.12
SW48	ND	122 ± 30	0.42 ± 0.18
SW480	ND	78 ± 26	0.12 ± 0.10
HT29	+[d]	410 ± 220	0.19 ± 0.10
HCA-7	+++	10 700 ± 2100	0.24 ± 0.10

[a]By Western analysis.
[b]For experimental details see Materials and methods. Values are the mean ± SD of three to five separate experiments.
[c]ND, not detectable.
[d]+, Detectable; +++, very strong band.

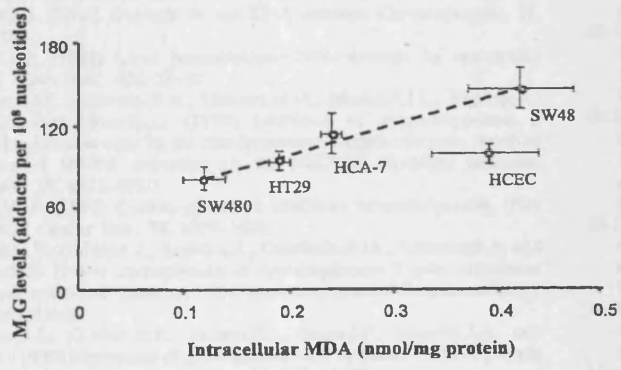


Fig. 3. Relationship between intracellular MDA and M_1G adduct levels in unstimulated culture of colon cell lines. The line of best fit ($r = 0.98$, $P < 0.001$) links values for the malignant cell types and excludes non-malignant HCEC cells. Values are the mean ± SD from three separate experiments. For experimental details see Materials and methods.

levels (Table II). COX-2 induction was also accompanied by an increase in intracellular MDA, which after 4 h was ~3-fold over control values (Table II). In contrast, M_1G adduct levels were unaffected by PMA treatment. PMA treatment of SW480 and HT29 cells also resulted in increases in COX-2, PGE_2 and MDA levels similar to those seen in HCEC cells, without significant changes in M_1G levels (data not shown). Incubation of the COX-2 expressing HCA-7 cells with the selective inhibitor NS-398 reduced PGE_2 levels from 10.7 ± 2.1 ng/

Table II. Effect of treatment of HCEC cells with PMA on levels of cellular COX-2 protein, PGE_2 in the medium, intracellular MDA and M_1G adducts

	Incubation time (h)		
	0	4	24
COX-2[a,b]	ND[c]	+++[d]	+
PGE_2 (pg/10^6 cells)[b]	30 ± 16	745 ± 210	408 ± 170
MDA (nmol/mg protein)[b]	0.38 ± 0.12	1.07 ± 0.32	0.8 ± 0.36
M_1G (adducts per cell)[b]	4600 ± 670	5300 ± 820	4950 ± 1050

[a]Based on Western blot analysis.
[b]For experimental details see Materials and methods. Values are the mean ± SD of three separate experiments.
[c]ND, not detectable.
[d]+, Detectable; +++, very strong band.

10^6 cells in control cells to 1.3 ± 0.6 ng/10^6 cells after incubation for 24 h. Incubation of cells with NS-398 for up to 72 h did not alter levels of intracellular MDA or M_1G adducts.

These results describe the relationship in colon cells between MDA, M_1G adducts and COX activity, and thus contribute to our understanding of the role of COX-2 in colon cancer. We demonstrate for the first time that levels of M_1G adducts in colon cancer cells reflect constitutive intracellular levels of MDA. Similar to COX-2 protein expression, there was considerable heterogeneity in constitutive M_1G adduct levels in colorectal cancer cells, ranging from <5 adducts/10^8 nucleotides in RKO cells to 148 ± 22 adducts/10^8 nucleotides in SW48 cells. However, there appeared to be no correlation between constitutive COX-2 expression in cultured cells and levels of M_1G adducts. Similarly, inhibition and induction of COX-2 activity did not alter M_1G levels.

There are a number of possible explanations for this lack of association. The addition of exogenous MDA in high concentrations to non-malignant HCEC cells almost doubled M_1G adduct levels, to reach values similar to those observed constitutively in malignant SW48 and HCA-7 cells. The relative insensitivity of M_1G levels in cells in culture towards challenge by MDA is consistent with the possibility that routes other than intracellular production of MDA may be the major sources of M_1G formation *in vivo*, particularly since MDA is generated predominantly at the cell membrane, some distance from cellular DNA. Evidence in favour of this hypothesis was provided recently in a study of human gastric mucosal biopsies (24), in which regression analysis of samples from 39 individuals with normal gastric histology and no evidence of *Helicobacter pylori* infection demonstrated that 200% increase in MDA concentration was reflected by an average increase in M_1G of only 23%. Little is known about alternative routes of M_1G formation, but oxidation of DNA by bleomycin has been shown to give rise to M_1G adducts via base propenal formation in the absence of lipid peroxidation and MDA (25). The apparent insensitivity of M_1G adduct levels to changes in MDA concentration may also be related to rapid repair by nucleotide excision repair pathways (26).

Alternatively, the products of COX catalysis may be less efficiently converted to MDA in cultured cells than *in vivo*. We have shown previously (11) that PGH_2 can be converted to MDA by certain cytochromes P450 (Figure 1, step 2), which may be overexpressed in colon cancer tissue but poorly preserved in cells in culture. However, breakdown of PGH_2 can also occur spontaneously, resulting in modest changes in MDA concentration. In the study described here, a 20-fold

increase in PGE_2 production was associated with a 3-fold elevation in intracellular MDA. This result underlines the limitations of the *in vitro* model used here. The multiple pathways of MDA biotransformation *in vivo*, involving aldehyde dehydrogenases and reductases, cellular antioxidants and glutathione *S*-transferase isoenzymes, may also be poorly represented in cultured cells (27,28).

However, these limitations do not detract from conclusions reached regarding the other components of the pathway studied (Figure 1, steps 1 and 3). We have demonstrated that increased COX-2 activity in non-malignant and malignant human colon cells is associated with production of MDA. Moreover, basal levels of MDA correlate with those of M_1G adduct levels in malignant colon cells, and exposure of non-malignant colon cells to MDA can increase M_1G levels. In view of the convincing role of COX-2 in the pathogenesis of colorectal cancer (6) and of M_1G adduct levels as indicators of oxidative DNA damage (4), both merit investigation *in vivo* as biomarkers of colorectal carcinogenesis.

Acknowledgements

We thank Dr S.MacKay (University of Florida, Gainesville, FL), Prof. C.Paraskeva (Bristol University, Bristol, UK) and Dr S.Kirkland (Imperial College, London, UK) for provision of cell lines; D.Wakelin, P.Shepherd, E.Griech, S.Daniels and E.Deag for technical support; and Dr M.Manson and Prof. D.Shuker for their comments on the manuscript. This study was supported in part by contract FS1735 from the UK Ministry of Agriculture, Fisheries and Food (to C.L. and R.S.), grant CA77839 from the NIH (to L.J.M.) and a Research Fellowship from the University Hospitals of Leicester (to R.A.S.).

References

1. Sharma,R.A., Manson,M.M., Gescher,A. and Steward,W.P. (2001) Colorectal cancer chemoprevention: Biochemical targets and clinical development of promising agents. *Eur. J. Cancer*, **37**, 12–22.
2. Fearon,E.R. and Jones,P.A. (1992) Progressing towards a molecular description of colorectal cancer development. *FASEB J.*, **6**, 2783–2790.
3. Marnett,L.J. (2000) Oxyradicals and DNA damage. *Carcinogenesis*, **21**, 361–370.
4. Marnett,L.J. (1999) Lipid peroxidation—DNA damage by malondialdehyde. *Mutat. Res.*, **424**, 83–95.
5. Plummer,S.M., Holloway,K.A., Manson,M.M., Munks,R.J.L., Kaptein,A., Farrow,S. and Howells,L. (1999) Inhibition of cyclo-oxygenase 2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF-ΦB activation via the NIK/IKK signalling complex. *Oncogene*, **18**, 6013–6020.
6. Taketo,M.M. (1998) Cyclooxygenase-2 inhibitors in tumorigenesis (Part II). *J. Natl Cancer Inst.*, **90**, 1609–1620.
7. Eberhart,C.E., Coffey,R.J., Radhika,A., Giardiello,F.M., Ferrenbach,S. and DuBois,R.N. (1994) Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology*, **107**, 1183–1188.
8. Kargman,S.L., O'Neill,G.P., Vickers,P.J., Evans,J.F., Mancini,J.A. and Jothy,S. (1995) Expression of prostaglandin G/H synthase-1 and -2 protein in human colon cancer. *Cancer Res.*, **55**, 2556–2559.
9. Sheng,H.M., Shao,J.Y., Kirkland,S.C., Isakson,P., Coffey,R.J., Morrow,J., Beauchamp,R.D. and DuBois,R.N. (1997) Inhibition of human colon cancer cell growth by selective inhibition of cyclooxygenase-2. *J. Clin. Invest.*, **99**, 2254–2259.
10. Diczfalusy,U., Falardeau,P. and Hammarstrom,S. (1977) Conversion of prostaglandin endoperoxides to C17-hydroxyacids by human platelet thromboxane synthase. *FEBS Lett.*, **84**, 271–274.
11. Plastaras,J.P., Guengerich,F.P., Nebert,D.W. and Marnett,L.J. (2000) Xenobiotic-metabolizing cytochromes P450 convert prostaglandin endoperoxide to hydroxyheptadecatrienoic acid and the mutagen, malondialdehyde. *J. Biol. Chem.*, **275**, 11784–11790.
12. Hendrickse,C.W., Kelly,R.W., Radley,S., Donovan,I.A., Keighley,M.R.B. and Neoptolemos,J.P. (1994) Lipid peroxidation and prostaglandins in colorectal cancer. *Br. J. Surg.*, **81**, 1219–1223.
13. Brock,T.G., McNish,R.W. and Peters-Golden,M. (1999) Arachidonic acid is preferentially metabolised by cyclooxygenase-2 to prostacyclin and prostaglandin E_2. *J. Biol. Chem.*, **274**, 11660–11666.
14. Marnett,L.J., Basu,A.K., O'Hara,S.M., Weller,P.E., Rahman,A.F.M.M. and Oliver,J.P. (1986) Reaction of malondialdehyde with guanine nucleosides: formation of adducts containing oxadrazabicyclononene residues in the base-pairing region. *J. Am. Chem. Soc.*, **108**, 1348–1350.
15. Pool-Zobel,B.L., Abrahamse,S.L., Collins,A.R., Kark,W., Gugler,R., Oberreuther,D., Siegel,E.G., Treptow-van Lishaut,S. and Rechkemmer,G. (1999) Analysis of DNA strand breaks, oxidized bases and glutathione S-transferase P1 in human colon cells from biopsies. *Cancer Epidemiol. Biomarkers Prev.*, **8**, 609–614.
16. Sharma,R.A., Ireson,C.R., Verschoyle,R.D., Hill,K.A., Williams,M.L., Leuratti,C., Manson,M.M., Marnett,L.J., Steward,W.P. and Gescher,A. (2001) Effects of dietary curcumin on glutathione S-transferase and malondialdehyde–DNA adducts in rat liver and colon mucosa: Relationship with drug levels. *Clin. Cancer Res.*, **7**, 1452–1458.
17. Sevilla,C.L., Mahle,N.H., Eliezer,N., Uzieblom,A., O'Hara,S.M., Nokubo,M., Miller,R., Rouzer,C.A. and Marnett,L.J. (1997) Development of monoclonal antibodies to the malondialdehyde–deoxyguanosine adduct, pyrimidopurinone. *Chem. Res. Toxicol.*, **10**, 172–180.
18. Chapeau,M.C. and Marnett,L.J. (1991) Enzymatic synthesis of purine deoxynucleoside adducts. *Chem. Res. Toxicol.*, **4**, 636–638.
19. Ji,C., Rouzer,C.A., Marnett,L.J. and Pietenpol,J.A. (1998) Induction of cell cycle arrest by the endogenous product of lipid peroxidation, malondialdehyde. *Carcinogenesis*, **19**, 1275–1283.
20. Barnett,J., Chow,J. and Ives,D. (1994) Purification, characterization and selective inhibition of human prostaglandin G/H synthase 1 and 2 expressed in the baculovirus system. *Biochim. Biophys. Acta*, **1209**, 130–139.
21. Chaudhary,A.K., Nokubo,M., Reddy,G.R., Yeola,S.N., Morrow,J.D., Blair,I.A. and Marnett,L.J. (1994) Detection of endogenous malondialdehyde–deoxyguanosine adducts in human liver. *Science*, **265**, 1580–1582.
22. Erdelmeier,I., Gerard-Monnier,D., Yadan,J.C. and Chaudiere,J. (1998) Reactions of N-methyl-2-phenylindole with malondialdehyde and 4-hydroxyalkenals. Mechanistic aspects of the colorimetric assay of lipid peroxidation. *Chem. Res. Toxicol.*, **11**, 1184–1194.
23. Leuratti,C., Singh,R., Lagneau,C., Farmer,P.B., Marnett,L.J. and Shuker,D.E.G. (1998) Determination of malondialdehyde-induced DNA damage in human tissues using an immunoslot blot assay. *Carcinogenesis*, **19**, 1919–1924.
24. Everett,S.M., Singh,R., Leuratti,C., White,K.L.M., Neville,P., Greenwood,D., Marnett,L.J., Schorah,C.J., Forman,D., Shuker,D. and Axon,A.T.R. (2001) Levels of malondialdehyde–deoxyguanosine in the gastric mucosa: Relationship with lipid peroxidation, ascorbic acid and *Helicobacter pylori*. *Cancer Epidemiol. Biomarkers Prev.*, **10**, 369–376.
25. Dedon,P.C., Plastaras,J.P., Rouzer,C.A. and Marnett,L.J. (1998) Indirect mutagenesis by oxidative DNA damage: formation of the pyrimidopurinone adduct of deoxyguanosine by base propenal. *Proc. Natl Acad. Sci. USA*, **95**, 11113–11116.
26. Johnson,K.A., Fink,S.P. and Marnett,L.J. (1997) Repair of propano-deoxyguanosine by nucleotide excision repair in vivo and in vitro. *J. Biol. Chem.*, **272**, 11434–11438.
27. Canuto,R.A., Ferro,M., Muzio,G., Bassi,A.M., Leonarduzzi,G., Maggiora,M., Adamo,D., Poli,G. and Lindahl,R. (1994) Role of aldehyde metabolizing enzymes in mediating effects of aldehyde products of lipid peroxidation in liver cells. *Carcinogenesis*, **15**, 1359–1364.
28. Siu,G.M. and Draper,H.H. (1982) Metabolism of malonaldehyde *in vivo* and *in vitro*. *Lipids*, **17**, 349–355.

Received September 28, 2000; revised May 10, 2001; accepted May 11, 2001

APPENDIX 3: Abstracts of full papers submitted

APPENDIX 3A:

Plummer SM, Hill KA, Festing MFW, Steward WP, Gescher A, Sharma RA[1].

Clinical development of leukocyte cyclooxygenase-2 activity as a systemic biomarker for cancer chemopreventive agents.

Submitted to *Cancer Epidemiol Biomarkers Prev*, April 2001.

[1] Corresponding author

Abstract

Advancement of cancer prevention and therapy requires clinical development of systemic biomarkers of pharmacological efficacy of the agent under scrutiny, as potential surrogates of the premalignant or malignant tissue target. Curcumin, a polyphenol derived from *Curcuma spp.*, has shown wide-ranging chemopreventive activity in preclinical carcinogenic models, in which it inhibits cyclooxygenase 2 (COX-2) at the transcriptional level. COX-2 has been implicated in the development of many human cancers. In order to explore the inhibition of COX-2 activity as a systemic biomarker of drug efficacy, of potential use in clinical trials of many chemopreventive drugs known to inhibit this enzyme, we measured COX-2 protein induction and prostaglandin E_2 (PGE_2) production in human blood following incubation with lipopolysaccharide (LPS). When 1 μM curcumin was added *in vitro* to blood from healthy volunteers, LPS-induced COX-2 protein levels and concomitant PGE_2 production were reduced by 24% and 41% respectively ($p < 0.05$ by ANOVA). To test whether effects on COX-2 activity could also be measured following oral dosing in humans, we conducted a dose-escalation pilot study of a standardised formulation of *Curcuma* extract in 15 patients with advanced colorectal cancer. Basal and LPS-mediated PGE_2 production was measured in blood, twice pre-treatment and on days 1, 2, 8, and 29 of treatment. Analysis of basal and LPS-induced PGE_2 production during treatment demonstrated a trend towards dose-dependent inhibition ($p < 0.005$ by regression analysis), but there was no significant difference to values from pre-treatment timepoints. Future trials should consider pre-treatment stratification of subjects by baseline PGE_2 levels. The results demonstrate the feasibility of measuring leukocyte COX-2 activity as a systemic biomarker in

clinical trials of agents shown to inhibit this isozyme when added to human blood *in vitro*.

APPENDIX 3B:

Perkins S, Sharma RA[1], Marnett LJ, Steward WP, Gescher A.

Intestinal adenoma levels of malondialdehyde-deoxyguanosine adducts: chemopreventive biomarker modified by dietary curcumin.

Submitted to *J Natl Cancer Inst*, May 2001.

No abstract available ("brief communication")

[1] Corresponding author

APPENDIX 4: Titles of other papers published by the author during the PhD registration period, not related to work submitted in this thesis

4A: Sharma RA. Diabetic eye disease in southern India. *Ophthalmic Nursing* 1999: **3**: 20-23.

4B: Thomas AL, Cox G, Sharma RA, Steward WP, Shields F, Jeyapalan K, Muller S, O'Byrne KJ. Gemcitabine and paclitaxel associated pneumonitis in non-small cell lung cancer: Report of a phase I/II dose-escalating study. *Eur J Cancer* 2000: **36**: 2329-2334.

4C: Sharma RA. The Handshake. *J Royal Soc Med* 1999: **92**: 548.

4D: Sharma RA, Symonds RP, O'Byrne KJ, Cheater F, Abrams K, Steward WP. Involving patients in treatment decisions: Can we learn from clinical trials? *Clin Oncol*, in press.

4E: Sharma RA, Hemingway D, West KP, Steward WP. Toxic megacolon: Remember cytomegalovirus. *Br J Hospital Med* 2001: **62**: 178-179.

4F: Sharma RA. Thinking before prescribing. *Br J Hospital Med* 2001, **62**: 305.

4G: Sharma RA, Eatock MM, Twelves C, Brown G, McLelland HR, Clayton K, O'Byrne KJ, Moyses C, Carmichael J, Steward WP. Bioavailability study of oral and

intravenous OGT 719, a novel nucleoside analogue. *Cancer Chemother Pharmacol*, in press.

4H: Sharma RA, Harris AL, Dalgleish A, Steward WP, O'Byrne KJ. Angiogenesis as biomarker and target for cancer chemoprevention. *Lancet Oncol*, in press.

APPENDIX 5: Titles of published abstracts

5A: Sharma RA, Plummer SM, Shepherd PC, Leuratti C, Singh R, Marnett LJ, Gescher A, Steward WP. Malondialdehyde-DNA adduct levels do not correlate with basal or induced cyclo-oxygenase 2 protein in human colonic cell lines. *Proc Am Assoc Cancer Res* 2000, **41**: 686.

5B: Sharma RA, Ireson CR, Perkins S, Leuratti C, Singh R, Verschoyle R, Marnett LJ, Gescher A, Steward WP. Effect of curcumin on basal and induced malondialdehyde-DNA adduct levels in F344 rats. *Proc Am Assoc Cancer Res* 2000, **41**: 412.

5C: Edwards JG, Faux SP, Sharma RA, Shepherd PC, Plummer SM, Walker RA, Waller DA, O'Byrne K. Cyclooxygenase-2 and prostaglandin E_2 in malignant mesothelioma. *Proc Am Assoc Cancer Res* 2000, **41**: 204.

5D: Sharma RA, Plummer SM, Leuratti C, Singh R, Gallacher-Horley B, Marnett LJ, Gescher A, Steward WP. Cyclooxygenase-2, prostaglandin-E_2, malondialdehyde and malondialdehyde-DNA adducts in human colon cells. *Br J Cancer* 2000, **83** (Suppl 1): 24 **(Oral Presentation).**

5E: Sharma RA, Ireson CR, Verschoyle RD, Hill KA, Leuratti C, Singh R, Williams ML, Manson MM, Gescher A, Steward WP. Preclinical pharmacokinetic study of

dietary curcumin and its effects on biomarkers of cancer chemoprevention. *Clin Cancer Res* 2000, **6S**: 63.

5F: Ireson CR, Veschoyle RD, Orr S, Oustric S, Jones DJL, Donald S, Sharma RA, Hill KA, Williams ML, Lim CK, Steward WP, Gescher A. Disposition of the chemopreventive agent curcumin in rats and in human liver and gut cells. *Proc Am Assoc Cancer Res* 2001: **42**: 21-22.

5G: Plummer SM, Sharma RA, Hill KA, Ireson CR, Euden S, Shepherd PC, Gescher A, Steward WP. Curcuminoids inhibit cyclooxygenase-mediated prostaglandin E_2 production and COX-2 expression in human blood. *Proc Am Assoc Cancer Res* 2001: **42**: 17-18.

5H: Faux SP, Houghton CE, Swain WA, Edwards JG, Sharma RA, Plummer SM, O'Byrne KJ. EGFR induced activation of NF-kB in mesothelial cells by asbestos is important in cell survival. *Proc Am Assoc Cancer Res* 2001: **42**: 244.

5I: Sharma RA, Ireson CR, McLelland HR, Hill KA, Euden SA, Leuratti C, Williams ML, Plummer SM, Manson MM, Gescher A, Steward WP. Phase I pharmacokinetic study of *Curcuma* extracts (P54FP) in patients with colorectal cancer including measurement of chemotherapeutic and chemopreventive indices. *Proc Am Assoc Cancer Res* 2001: **42**: 697 **(Minisymposium Presentation).**

5J: Sharma RA, Perkins S, Steward WP, Gescher A. Elevated cyclooxygenase-2 protein and pyrimidopurinone-deoxyguanosine adducts in adenomas of Apc^{Min} mice and suppression by dietary curcumin. *Br J Cancer*: **85** (Suppl 1): 40.

5K: Sharma RA, McLelland HR, Singh R, Jones DJL, Hill KA, Euden SA, Ireson CR, Plummer SM, Manson MM, Gescher A, Steward WP. Phase I clinical trial of *Curcuma* extracts. *Br J Cancer*: **85** (Suppl 1): 40.

APPENDIX 6: Other proferred papers

6A: Sharma RA, Williams ML, Verschoyle RD, Manson MM, Plummer SM, Ireson CR, Shepherd PC, Howells L, Perkins S, Jones DJL, Orr S, Leuratti C, Singh R, Shuker DEG, Gescher A, Steward WP. Development of curcumin as an anticancer agent - bench to bedside. Winter meeting of Pharmacokinetics and Molecular Mechanisms group of European Organization for Research and Treatment of Cancer 2000: **Young Investigator Prize.**

6B: Sharma RA, Plummer SM, Gallacher-Horley B, Gescher A, Steward WP. Link between malondialdehyde and its DNA adduct levels in human colon adenocarcinoma cells of differing cyclooxygenase-2 activities. Winner of **Sidney King Prize** for cancer research in Leicestershire 2000.

6C: Sharma RA, Ireson CR, McLelland HR, Hill KA, Euden SA, Rush N, Sanganee C, Leuratti C, Singh R, Williams ML, Plummer SM, Manson MM, Gescher A and Steward WP. Phase I trial of *Curcuma* extracts (P54FP) in patients with cancer. Winter meeting of Pharmacokinetics and Molecular Mechanisms group of European Organization for Research and Treatment of Cancer 2001.

CHAPTER 8

REFERENCES

1. Sporn MB, Newton DL. Chemoprevention of cancer with retinoids. *Fed Proc* 1979: **38**: 2528-2534.
2. Omenn GS Goodman GE, Thornquist MD, Balmes J, Cullen MR, Glass A. Risk factors for lung cancer and for intervention effects in CARET, the beta-carotene and retinol efficacy trial. *J Natl Cancer Inst* 1996: **88**: 1550-1559.
3. Knekt P, Järvinen R, Teppo L, Aromaa A, Seppänen R. Role of various carotenoids in lung cancer prevention. *J Natl Cancer Inst* 1999: **91**: 182-184.
4. Kelloff GJ, Boone CW, Steele VE, Crowell JA, Lubet R, Sigman CC. Progress in cancer chemoprevention: perspectives on agent selection and short-term clinical intervention trials. *Cancer Res* 1994: **54S**: 2015s-2024s.
5. Einspahr JG, Alberts DS, Gapstur SM, Bostick RM, Emerson SS, Gerner EW. Surrogate end-point biomarkers as measures of colon cancer risk and their use in cancer chemoprevention trials. *Cancer Epidemiol Biomarkers Prev* 1997: **6**: 37-48.
6. Potter JD. Colorectal cancer: Molecules and populations. *J Natl Cancer Inst* 1999: **91**: 916-932.
7. Kronberg O, Fenger C, Olsen J. Randomised study of screening for colorectal cancer with fecal occult blood test. *Lancet* 1996: **348**: 1467-71.
8. Loeb L. Cancer cells exhibit a mutator phenotype. *Adv Cancer Res*, 1998, **72**: 25-56.
9. Powell SM, Zilz N, Beazer-Barclay Y, Bryan TM, Hamilton SR, Thibodeau SN. APC mutations occur early during colorectal tumorigenesis. *Nature* 1992: **359**: 235-237.

10. He TC, Sparks AB, Rago C. Identification of c-MYC as a target of the APC pathway. *Science* 1998: **281**: 1509-1512.

11. Rosenwald IB, Chen J-J, Wang S, Savas L, London IM, Pullman J. Upregulation of protein synthesis initiation factor eIF-4E is an early event during colon carcinogenesis. *Oncogene* 1999: **18**: 2507-2517.

12. Sparks AB, Morin PJ, Vogelstein B, Kinzler KW. Mutational analysis of the APC/β-catenin/Tcf pathway in colorectal cancer. *Cancer Res* 1998: **58**: 1130-1134.

13. Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B. Activation of β-catenin-Tcf signaling in colon cancer by mutations in β-catenin or *APC*. *Science* 1997: **275**: 1787-1790.

14. Clapper ML, Szarka CE, Pfeiffer GR, Graham TA, Balshem AM, Litwin S, Goosenberg EB, Frucht H, Engstrom PF. Preclinical and clinical evaluation of broccoli supplements as inducers of glutathione S-transferase activity. *Clin Cancer Res* 1997: **3**: 25-30.

15. Love RR, Jacoby R, Newton MA, Tutsch KD, Simon K, Pomplun M, Verma AK. A randomized placebo-controlled trial of low-dose α-difluoromethylornithine in individuals at risk for colorectal cancer. *Cancer Epid Biomarkers Prev* 1998: **7**: 989-992.

16. Meyskens FL Jr, Gerner EW, Emerson S, et al. Effect of α-difluoromethylornithine on rectal mucosal levels of polyamines in a randomised, double-blinded trial of colon cancer prevention. *J Natl Cancer Inst* 1998: **90**: 1212-1218.

17. Krishnan K, Ruffin T, Brenner DE, et al. Colon cancer chemoprevention : Clinical development of aspirin as a chemopreventive agent. *J Cell Biochem* 1997: **28/29**(Suppl): 148-158.

18. Szarka CE, Pfeiffer GR, Hum ST, et al. Glutathione S-transferase activity and glutathione S-transferase μ expression in subjects at risk for colorectal cancer. *Cancer Res* 1995: **55**: 2789-2793.

19. Carbone PP, Douglas JA, Larson DO, et al. Phase I Chemoprevention study of piroxicam and α-difluoromethylornithine. *Cancer Epid Biomarkers Prev* 1998: **7**: 907-912.

20. Mitchell MF, Tortolero-Luna G, Lee JJ, et al. Phase I dose de-escalation trial of α-difluoromethylornithine in patients with grade 3 cervical intraepithelial neoplasia. *Clin Cancer Res* 1998: **4**: 303-310.

21. Rutzky LP, Moyer MP. Human cell lines in colon cancer research. Academic Press, 1990: pp. 155-202.

22. Sharma S, Stutzman JD, Kelloff GJ, Steele VE. Screening of potential chemopreventive agents using biochemical markers of carcinogenesis. *Cancer Res* 1994: **54**: 5848-5855.

23. Bird R. Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: Preliminary findings. *Cancer Lett* 1987: **37**: 147-151.

24. Foulds L. Neoplastic development, Vol 2. New York: Academic Press, 1975: 9-10.

25. Wargovich MJ, Jimenez A, McKee K, Steele VE, Velasco M, Woods J, Price R, Gray K, Kelloff GJ. Efficacy of potential chemopreventive agents on rat colon aberrant crypt formation and progression. *Carcinogenesis* 2000: **21**: 1149-1155.

26. Moser AR, Pitot HC, Dove WF. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science* 1989: **247**: 322-324.

27. Goss KH, Groden J. Biology of the adenomatous polyposis coli tumor suppressor. *J Clin Oncol* 2000: **18**: 1967-1979.

28. Ritland SR, Gendler SJ. Chemoprevention of intestinal adenomas in the Apc^{Min} mouse by piroxicam: kinetics, strain effects and resistance to chemosuppression. *Carcinogenesis* 1999: **20**: 51-58.

29. Mahmoud NM, Carothers AM, Grunberger D, Bilinski RT, Churchill MR, Martucci C, Newmark HL and Bertagnolli MM. Plant phenolics decrease intestinal tumors in an animal model of familial adenomatous polyposis. *Carcinogenesis* 2000: **21**: 921-927.

30. Kelloff GJ, Johnson JR, Crowell JA, et al. Approaches to the development and marketing approval of drugs that prevent cancer. *Cancer Epid Biomarkers Prev* 1995, **4**: 1 – 10.

31. Arbiser JL, Klauber N, Rohan R, van Leeuwen R, Huang MT, Fisher C, Flynn E, Byers HR. Curcumin is an in vivo inhibitor of angiogenesis. *Mol Med* 1998: 4: 376-383.

32. Jiang MC, Yang Yen HF, Yen JJY, Lin JK. Curcumin induces apoptosis in immortalized NIH 3T3 and malignant cancer cell lines. *Nutr Cancer* 1996: **26**: 111-120.

33. Kuttan R, Sudheeran PC, Josph CD. Turmeric and curcumin as topical agents in cancer therapy. *Tumori* 1987: **73**: 29-31.

34. Israel L, Breau JL, Morere JF, et al. Evaluation of the antiprostaglandin agent piroxicam in 87 patients with advanced cancer. *Semaine des hopitaux* 1993: **69**: 1293-1297.

35. Brouk B. *Plants consumed by man.* Academic Press, New York, 1975: p. 331.

36. Eigner D, Sholz D. Ferula asa-foetida and curcuma longa in traditional medical treatment and diet in Nepal. *J Ethnopharmacol* 1999: **67**: 1-6.

37. Milobedzka J, Kostanecki V and Lampe V. Structure. *Chem Ber* 1910: **43**: 2163.

38. Bhavant Shanker TN, Shantha NV, Raniesh HP, Murthy IAS, Murthy VS. Toxicity studies on turmeric: Acute toxicity studies in rats, guinea-pigs and monkeys. *Indian J Exp Biol* 1980: **18**: 73-75.

39. Wang YJ, Pan MH, Cheng AL, Lin LI, Ho YS, Hsieh CY, Lin JK. Stability of curcumin in buffer solutions and characterization of its degradation products. *J Pharm Biomed Analysis* 1997: **15**: 1867-1876.

40. Jovanovic SV, Steenken S, Boone CW, Simic MG. H-Atom transfer is a preferred antioxidant mechanism of curcumin. *J Am Chem Soc* 1999: **121**: 9677-9681.

41. Lin JK, Pan MH, Shiau SYL. Recent studies on the biofunctions and biotransformations of curcumin. *Biofactors* 2000: **13**: 153-158.

42. Wahlstrom B and Blennow G. A study on the fate of curcumin in the rat. *Acta Pharmacol Toxicol* 1978: **43**: 86-92.

43. Holder GM, Plummer JL and Ryan AJ. The metabolism and excretion of curcumin [1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] in the rat. *Xenobiotica* 1978: **8**: 761-768.

44. Ravindranath V and Chandrasekhara N. Absorption and tissue distribution of curcumin in rats. *Toxicol* 1980: **16**: 259-265.

45. Ireson CR, Orr S, Jones DL, Verschoyle R, Lim CK, Luo JL, Howells L, Plummer SP, Jukes R, Williams M, Steward WP, and Gescher A. Characterization of metabolites of the chemopreventive agent curcumin in humans and rat hepatocytes and in the rat in vivo, and evaluation of their ability to inhibit phorbol ester-induced prostaglandin E_2 production. *Cancer Res* 2001: **61**: 1058-1064.

46. Pan MH, Huang TM and Lin JK. Biotransformation of curcumin through reduction and glucuronidation in mice. *Drug Met Dispos* 1999: **27**: 486-494.

47. Shoba G, Joy D, Joseph T, Majeed M, Rajendran R and Srinivas PSSR. Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers. *Planta Medica* 1998: **64**: 353-356.

48. Cheng AL, Lin JK, Hsu MM, Shen TS, Ko JY, Lin JT, Wu MS, Yu HS, Jee SH, Chen GS, Chen TM, Chen CA, Lai MK, Pu YS, Pan MH, Wang UJ, Tsai CC and Hsieh CY. Phase I chemoprevention clinical trial of curcumin. *Proc Am Soc Clin Oncol* 1998: **17**: 558a.

49. Brenner DE, Shureiqi I, Bailey J, Normolle D, Kelloff G, Crowell J, Ireson C, Gescher A, Rock CL, Ruffin MT. A phase I study of curcumin: Bioavailability and safety profile for low doses. Submitted.

50. NCI, DCPC. Clinical development plan: Curcumin. *J Cell Biochem* 1996: **26S**: 72-85.

51. Gupta B, Kulshrestha CK, Sristava RK, Prasad DN. Mechanisms of curcumin induced gastric ulcer in rats. *Indian J Med Res* 1980: **71**: 806-814.

52. Deodhar SD, Sethi R and Srimal RC. Preliminary study on antirheumatic activity of curcumin (diferuloyl methane). *Indian J Med Res* 1980: **71**: 632-634.

53. Ammon HTP, Wahl MA. Pharmacology of curcuma longa. *Planta Med* 1991: **57**: 172-179.

54. Taketo MM. Cyclooxygenase-2 inhibitors in tumorigenesis (part II). *J Natl Cancer Inst* 1998: **90**: 1609-1620.

55. Khafif A, Schantz SP, Chou TC, Edelstein D, Sacks PG. Quantitation of chemopreventive synergism between (-)-epigallocatechin-3-gallate and curcumin in normal, premalignant and malignant human oral epithelial cells. *Carcinogenesis* 1998: **19**: 419-424.

56. Zhang F, Altorki NK, Mestre JR, et al. Curcumin inhibits cyclooxygenase-2 transcription in bile acid- and phorbol ester-treated human gastrointestinal epithelial cells. *Carcinogenesis* 1999: **20**: 445-451.

57. Plummer SM, Holloway KA, Manson MM, Munks RJL, Kaptein A, Farrow S and Howells L. Inhibition of cyclo-oxygenase 2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF-κB activation via the NIK/IKK signalling complex. *Oncogene* 1999: **18**: 6013-6020.

58. Yin M-J, Yamamoto Y, Gaynor RB. The anti-inflammatory agents aspirin and salicylate inhibit the activity of IκB kinase-β. *Nature* 1998: **396**: 77-78.

59. Huang TS, Lee SC and Lin JK. Suppression of c-Jun/AP-1 activation by an inhibitor of tumor promotion in mouse fibroblast cells. *Proc Natl Acad Sci USA* 1991: **88**: 5292-5296.

60. Chen YR and Tan TH. Inhibition of the c-Jun N-terminal kinase (JNK) signaling pathway by curcumin. *Oncogene* 1998: **17**: 173-178.

61. Firozi PF, Aboobaker VS, Bhattacharya RK. Action of curcumin on the cytochrome P450-system catalysing the activation of aflatoxin B1. *Chemico-Biol Interactions* 1996: **100**: 41-51.

62. Ciolino HP, Daschner PJ, Wang TT, Yeh GC. Effect of curcumin on the aryl hydrocarbon receptor and cytochrome P450 1A1 in MCF-7 breast carcinoma cells. *Biochem Pharmacol* 1998: **56**: 197-206.

63. Singh SV, Hu X, Srivastava SK, Singh M, Xia H, Orchard JL and Zaren HA. Mechanism of inhibition of benzo[*a*]pyrene-induced forestomach cancer in mice by dietary curcumin. *Carcinogenesis* 1998: **19**: 1357–1360.

64. Susan M and Rao MNA. Induction of glutathione S-transferase activity by curcumin in mice. *Drug Res* 1992: **42**: 962-964.

65. Piper T, Singhal SS, Salameh M, Torman RT, Awasthi YC and Awasthi S. Mechanisms of anticarcinogenic properties of curcumin: the effect of curcumin on glutathione linked detoxification enzymes in rat liver. *Int J Biochem Cell Biol* 1998: **30**: 445-456.

66. Nijhoff WA, Groen GM, Peters WHM. Induction of rat hepatic and intestinal glutathione S-transferases and glutathione by dietary naturally occurring anticarcinogens. *Int J Oncol* 1993: **3**: 1131-1139.

67. Dinkova-Kostova AT, Talalay P. Relation of structure of curcumin analogs to their potencies as inducers of Phase 2 detoxification enzymes. *Carcinogenesis* 1999: **20**: 911-914.

68. Kensler TW, Trush MA, Guyton KZ. Free radicals as targets for cancer chemoprevention: Prospects and problems. In *Cellular and Molecular Targets for Chemoprevention,* Steele VE, Stoner GDM, Boone CW, Kelloff GJ. (eds) pp 173-191. CRC Press, Boca Raton, FL, USA: 1992.

69. Joe B, Lokesh BR. Role of capsaicin, curcumin and dietary n-3 fatty acids in lowering the generation of reactive oxygen species in rat peritoneal macrophages. *Biochim Biophys Acta* 1994: **1224**: 255-263.

70. Tonnesen HH and Greenhill JV. Studies on curcumin and curcuminoids. XXII. Curcumin as a reducing agent and as a radical scavenger. *Int J Pharmaceut* 1992, **87**: 79-87.

71. Kunchandy E and Rao MNA. Oxygen radical scavenging activity of curcumin. *Int J Pharmaceut* 1990: **58**: 237-240.

72. Pulla Reddy AC, Lokesh BR. Studies on anti-inflammatory activity of spice principles and dietary n-3 polyunsaturated fatty acids on carcinogen-induced inflammation in rats. *Ann Nutr Met* 1994: **38**: 349-358.

73. Ahsan H, Parveen N, Khan NU, Hadi SM. Pro-oxidant, anti-oxidant and cleavage activities on DNA of curcumin and its derivatives demethoxycurcumin and bisdemethoxycurcumin. *Chem Biol Interact* 1999: **121**: 161-75.

74. Lala PK, Chakraborty C. Role of nitric oxide in carcinogenesis and tumour progression. *Lancet Oncol* 2001: **2**: 149-156.

75. deRojas-Waker T, Tamir S, Ji H. Nitric oxide induces oxidative damage in addition to deaminiation in macrophage DNA. *Chem Res Toxicol* 1995: **8**: 473-477.

76. Laval F, Wink DA. Inhibition of nitic oxide of the repair protein O^6-methylguanine-DNA-methyltransferase. *Carcinogenesis* 1994: **15**: 443-447.

77. Graziewicz M, Wink DA, Laval F. Nitric oxide inhibits DNA ligase activity: potential mechanisms for NO-mediated DNA damage. *Carcinogenesis* 1996: **17**: 2501-2505.

78. Ambs S, Bennett WP, Merrium WG. Relationship between p53 mutations and inducible nitric oxide synthase expression in human colorectal cancer. *J Natl Cancer Inst* 1999: **91**: 1509-1510.

79. von Knethen A, Brune B. Cyclooxygenase-2: an essential regulator of NO-mediated apoptosis. *FASEB J* 1997: **11**: 887-895.

80. von Knethen A, Callsen D, Brune B. NF-kB and AP-1 activation by nitric oxide attenuated apoptotic death in RAW 264.7 macrophages. *Mol Biol Cell* 1999: **10**: 361-372.

81. Brouet I, Ohshima H. Curcumin, an anti-tumour promoter and anti-inflammatory agent, inhibits induction of nitric oxide synthase in activated macrophages. *Biochem Biophys Res Comm* 1995: **206**: 533-540.

82. Chan MM, Huang HI, Fenton MR, Fong D. *In vivo* inhibition of nitric oxide synthase gene expression by curcumin, a cancer preventive natural product with anti-inflammatory properties. *Biochem Pharmacol* 1998: **55**: 1555-1562.

83. Tsao SM, Yin MC. Enhanced inhibitory effect from interaction of curcumin with amphotericin B or fluconazole against candida species. *J Food Drug Anal* 2000: **8**: 208-212.

84. de Clercq E. Current lead natural products for the chemotherapy of human immunodeficiency virus (HIV) infection. *Med Res Rev* 2000: **20**: 323-349.

85. Jiang MC, Yang Yen HF, Yen JJY, et al. Curcumin induces apoptosis in immortalized NIH 3T3 and malignant cancer cell lines. *Nutr Cancer* 1996: **26**: 111-120.

86. Chen H, Zhang ZS, Zhang YL, Zhou DY. Curcumin inhibits cell proliferation by interfering with the cell cycle and inducing apoptosis in colon carcinoma cells. *Anticancer Res* 1999: **19**: 3675-3680.

87. Simon A, Allais DP. Duroux JL, Basly JP, Delage C. Inhibitory effect of curcuminoids on MCF-7 cell proliferation and structure-activity relationships. *Cancer Lett* 1998: **129**: 111-116.

88. Gautam SC, Yong YX, Pndolia KR, Chapman RA. Nonselective inhibition of proliferation of transformed and nontransformed cells by the anticancer agent curcumin. *Biochem Pharmacol* 1998: **55**: 1333-1337.

89. Plummer S, Wakelin D, Bouer M, Shepherd P, Howells L, Gescher A, Chow S, Manson M. Inhibition of growth of colon tumour cells by curcumin correlates with inhibition of the IkB kinase and down regulation of cyclin D1. *Br J Cancer* 2000: **83** Suppl 1: 20.

90. Han SS, Chung ST, Robertson DA, Ranjan D, Bondada S. Curcumin causes the growth arrest and apoptosis of B cell lymphoma by downregulation of egr-1, c-myc, bcl-XL, NF-kappa B, and p53. *Clin Immunol* 1999: **93**: 152-161.

91. Bhaumik S, Jyothi MD, Khar A. Differential modulation of nitric oxide production by curcumin in host macrophages and NK cells. *FEBS Lett* 2000: **483**: 78-82.

92. Churchill M, Chadburn A, Bilinski RT, Bertagnolli MM. Inhibition of intestinal tumors by curcumin is associated with changes in the intestinal immune cell profile. *J Surg Res* 2000: **89**: 169-175.

93. Dorai T, Gehani N, Katz A. Therapeutic potential of curcumin in human prostate cancer cells. *Prostate Cancer Dis* 2000: **3**: 84-93.

94. Jee SH, Shen SC, Tseng CR, Chiu HC, Kuo ML. Curcumin causes a p53-dependent apoptosis in human basal cell carcinoma cells. *J Inv Dematol* 1998: **111**: 656-661.

95. Kuo ML, Huang TS, Lin JK. Curcumin, an antioxidant and anti-tumor promoter, induces apoptosis in human leukaemia cells. *Biochim Biophys Acta* 1996: **1317**: 95-100.

96. Ozaki K, Kawata Y, Amano S, Hanazawa S. Stimulatory effect of curcumin on osteoclast apoptosis. *Biochem Pharmacol* 2000: **59**: 1577-1581.

97. Ranjan D, Johnston TD, Reddy KS Chen CG. Ehanced apoptosis mediates inhibtion of EBV-transformed lymphoblastoid cell line proliferation by curcumin. *J Surg Res* 1999: **87**: 1-5.

98. Samaha HS, Kelloff GJ, Steele V, Rao CV, Reddy BS. Modulation of apoptosis by sulindac, curcumin, phenylethyl-3-methylcaffeate, and 6-phenylhexyl isothiocyanate: Apoptotic index as a biomarker in colon cancer chemoprevention and promotion. *Cancer Res* 1997**: 57**: 1301-1305.

99. Kelloff GJ, Crowell JA, Hawk ET, Steel VE, Lubet RA, Boone CW, Covey JM, Doody LA, Omenn GS, Greenwald P, Hong WK, Parkinson DR, Bagheri D, Baxter GT, Blunden M, Doeltz MK, Eisenhauer KM, Johnson K, Knapp GG, Longfellow DG, Malone WF, Nayfield SG, Seifried HE, Swall LM and Sigman CC. Strategy and planning for chemopreventive drug development: clinical development plans II. *J Cell Biochem* 1996: **26***S*: 54-71.

100. Chuang SE, Kuo ML, Hsu CH, Chen CR, Lin JK, Lai GM, Hsieh CY, Cheng AL. Curcumin-containing diet inhibits diethylnitrosamine-induced murine hepatocarcinogenesis. *Carcinogenesis* 2000: **21**: 331-335.

101. Folkman J. Clinical applications of research on angiogenesis. *N Engl J Med* 1995: **333**: 1757-63.

102. Thaloor D, Singh AK, Sidhu GS, Prasad PV, Kleinman HK, Maheshwari RK. Inhibition of angiogenic differentiation of human umbilical vein endothelial cells by curcumin. *Cell Growth Differentiation* 1998: **9**: 305-312.

103. Menon LG, Kuttan R, Kuttan G. Anti-metastatic activity of curcumin and catechin. *Cancer Lett* 1999: **141**: 159-165.

104. Chan MMY. Inhibition of tumor necrosis factor by curcumin, a phytochemical. *Biochem Pharmacol* 1995: **49**: 1541-1556.

105. Abe Y, Hashimoto S, Horie T. Curcumin inhibition of inflammatory cytokine production by human peripheral blood monocytes and alveolar macrophages. *Pharmacol Res* 1999: **39**: 41-47.

106. Gupta B, Ghosh B. Curcuma longa inhibits TNF-alpha induced expression of adhesion molecules on human umbilical vein endothelial cells. *Int J Immnopharmacol* 1999: **21**: 745-757.

107. Satoskar RR, Shah SJ and Shenoy SG. Evaluation of anti-inflammatory property of curcumin (diferuloyl methane) in patients with post-operative inflammation. *Int J Clin Pharmacol Ther Toxicol* 1986: **24**: 651-654.

108. Soni KB and Kuttan R. Effect of oral curcumin administration on serum peroxides and cholesterol levels in human volunteers. *Indian J Physiol Pharmacol* 1992: **36**: 273-275.

109. Ramirez Bosca A, Soler A, Gutierrez MAC, Alvarez JL and Almagro EQ. Antioxidant curcuma extracts decrease the blood lipid levels of human subjects. *Age* 1995: **18**: 167–169.

110. Raysid A, Lelo A. The effect of curcumin and placebo on human gall-bladder function: an ultrasound study. *Aliment Pharmacol Ther* 1999: **13**: 245-249.

111. Gelman KA, Eisenhauer EA, Harris AL, Ratain MJ, Workman P. Anticancer agents targeting signaling molecules and cancer cell environment: Challenges for drug development? *J Natl Cancer Inst* 1999: **91**: 1281-1287.

112. Hayes JD and Pulford DJ. The glutathione S-transferases supergene family. *Crit Rev Biochem Mol Biol* 1995: **30**: 445-600.

113. Katoh T, Nagata N, Kuroda Y, et al. Glutathione S-transferase M1 and T1 genetic polymorphisms and susceptibility to gastric and colorectal adenocarcinoma. *Carcinogenesis* 1996: **17**: 1855-1859.

114. Mulder TPJ, Verspaget HW, Sier CFM, et al. Glutathione S-transferase π in colorectal tumours is predictive for overall survival. *Cancer Res* 1995: **55**: 2696-2702.

115. Mulder TPJ, Roelofs HMJ, Peters WHM, et al. Glutathione S-transferases in liver metastases of colorectal cancer. *Carcinogenesis* 1994: **15**: 2149-2153.

116. Clapper ML and Szarka CE. Glutathione S-transferases: Biomarkers of cancer risk and chemopreventive response. *Chemico-Biol Interactions* 1998: **111/112**: 377-388.

117. van Lieshout EMM, Bedaf MMG, Pieter M, et al. Effects of dietary anticarcinogens on rat gastrointestinal glutathione S-transferase theta levels. *Carcinogenesis* 1998: **19**: 2055-2077.

118. van Lieshout EMM, Tiemessen DM, Roelofs HMJ, et al. Nonsteroidal anti-inflammatory drugs enhance glutathione S-transferase theta levels in rat colon. *Biochim Biophys Acta* 1998: **1381**: 305-311.

119. Clapper ML. Chemopreventive activity of oltipraz. *Pharmacol Ther* 1998: **77**

120. Seiler N, Atanassov CL, Raul F, et al. Polyamine metabolism as target for cancer chemoprevention. *Int J Oncol* 1998: **13**: 993-1006.

121. Takenoshita S, Matsuzaki S, Nakano G, et al. Selective elevation of the N1-acetylspermidine level in human colorectal adenocarcinomas. *Cancer Res* 1984: **44**: 845-847.

122. Weiss T, Bernhardt G, Buschauer A, et al. High-resolution reversed-phase high-performance liquid chromatography analysis of polyamines and their monoacetyl conjugates. *Ann Biochem* 1997: **247**: 294-304.

123. Luk GD, Baylin SB. Ornithine decarboxylase as a biological marker in familial colonic polyposis. *N Engl J Med* 1984: **311**: 80-83.

124. Pegg AE. Ornithine decarboxylase as a target for chemoprevention. *J Cell Biochem* 1995: **22S**: 132-138.

125. Giardiello FM, Hamilton SR, Hylind LM, et al. Ornithine decarboxylase and polyamines in familial adenomatous polyposis. *Cancer Res* 1997: **57**: 199-201.

126. Auvinen M, Paasinen A, Andersson LC, et al. Ornithine decarboxylase activity is critical for cell transformation. *Nature* 1992: **360**: 355-358.

127. Wang WQ, Liu LQ, Higuchi CM, et al. Mucosal polyamine measurements and colorectal cancer risk. *J Cell Biochem* 1996: **63**: 252-257.

128. Rao CV, Simi B, Reddy BS, et al. Inhibition by dietary curcumin of azoxymethane-induced ornithine decarboxylase, tyrosine protein kinase, arachidonic acid metabolism and aberrant crypt foci formation in the rat colon. *Carcinogenesis* 1993: **14**: 2219-2225.

129. Lu YP, Chang RL, Huang MT, et al. Inhibitory effect of curcumin on 12-O-tetradecanoylphorbol-13-acetate-induced increase in ornithine decarboxylase mRNA in mouse epidermis. *Carcinogenesis* 1993: **14**: 293-297.

130. Manson MM, Hudson EA, Ball HWL, et al. Chemoprevention of aflatoxin B1-induced carcinogenesis by indole-3-carbinol in rat liver - predicting the outcome using early biomarkers. *Carcinogenesis* 1998: **19**: 1829-1835.

131. Halline AG, Dudeja PK, Lasher BA, et al. Urinary excretion of N1-acetlyspermidine and other acetylated and free polyamines in the 1,2-dimethylhydrazine model of experimental rat colon cancer. *Cancer Res* 1989: **49**: 4721-4723.

132. O'Brien BL, Hankewych M, McCormick D, et al. Urinary N1-acetlyspermidine and N8-acetylspermidine excretion in normal humans and in patients with colorectal cancer. *Digestive Dis Sci* 1995: **40**: 1269-1274.

133. Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN. Up-regulation of cyclo-oxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterol* 1994: **107**: 1183-1186.

134. Kargman SL, O'Neill GP, Vickers PJ, Evans JF, Mancini JA, Jothy S. Expression of prostaglandin G/H symthase 1 and 2 in human colon cancer. *Cancer Res* 1995: **55**: 2556-2558.

135. Bennett A, Tacca MD, Stamford IF, Zebro T. Prostaglandins form tumours of the large bowel. *Brit J Surg* 1977: **35**: 882-890.

136. Narisawa M, Kusaka H, Yamazaki Y, et al. Relationship between blood plasma prostaglandin E_2 and liver and lung metastases in colorectal cancer. *Dis Colon Rectum* 1990: **33**: 840-843.

137. Boolbol SK, Dannenberg AJ, Chadburn A, Martucci C, Guo XJ, Ramonetti JT. Cyclo-oxygenase 2 overexpression and tumor formation are blocked by sulindac in a murine model of familial adenomatous polyposis. *Cancer Res* 1996: **56**: 2556-2559.

138. Oshima M, Dinchuk JE, Kargman SL, Oshima H, Hancock B, Kwong E, Trzaskos JM, Evans JF, Taketo MM. Suppression of intestinal polyposis in Apc delta 716 knockout mice by inhibition of COX-2. *Cell* 1996: **87**: 803-809.

139. Patrignani P, Panara MR, Greco A, Fusco O, Natoli C., Iacobelli S, Cipollone F, Ganci A, Créminion C, Maclouf J and Patrono C. Induction of prostaglandin endoperoxide synthase-2 in human monocytes associated with cyclo-oxygenase-dependent E2-isoprostane formation. *Brit J Pharmacol* 1996: **118**: 1285-1293.

140. Warner TD, Giuliano F, Vojnovic I, Bukasa A, Mitchell JA, Vane JR. Nonsteroidal drug selectivities for cyclooxygenase-1 rather than cyclooxygenase-2 are associated with human gastrointestinal toxicity: a full in vitro analysis. *Proc Natl Acad Sci USA* 1999: **96**: 7563-68.

141. Vane JR, Warner TD. Nomenclature for COX-2 inhibitors. *Lancet* 2000: **356**: 1373-74.

142. Brock TG, McNish RW, Peters-Golden M. Arachidonic acid is preferentially metabolized by cyclooxygenase-2 to prostacyclin and prostaglandin E_2. *J Biol Chem* 1999: **274**: 11660-11666.

143. Plastaras JP, Guengerich FP, Nebert DW and Marnett LJ. Xenobiotic-metabolizing cytochromes P450 convert prostaglandin endoperoxide to hydroxyheptadecatrienoic acid and the mutagen, malondialdehyde. *J Biol Chem* 2000: **275**: 11784-11790.

144. Ozturk HS. Activities of the enzymes participating in purine and free-radical metabolism in cancerous human colorectal tissues. *Cancer Biochem Biophys* 1998: **16**: 157-168.

145. Hendickse CW, Kelly RW, Radley S, Donovan IA, Keighley MRB and Neoptolemos JP. Lipid peroxidation and prostaglandins in colorectal cancer. *Brit J Surg* 1994: **81**: 1219-1223.

146. Marnett LJ. Lipid peroxidation - DNA damage by malondialdehyde. *Mut Res* 1999: **424**: 83-95.

147. Marnett LJ. Oxyradicals and DNA damage. *Carcinogenesis* 2000: **21**: 361-370.

148. Wang M, Dhingra K, Hittleman WN, Liehr JG, de Andrade M, and Li D. Lipid peroxidation induced putative malondialdehyde-DNA adducts in human breast tissues. *Cancer Epid Biomarkers Prev* 1996: **5**: 705-710.

149. Venkatesan P, Rao MNA. Structure-activity relationships for the inhibition of lipid peroxidation and the scavenging of free radicals by synthetic symmetrical curcumin analogues. *J Pharm Pharmacol* 2000: **52**: 1123-1128.

150. Dedon PC, Plastaras JP, Rouzer CA and Marnett LJ. Indirect mutagenesis by oxidative DNA damage: formation of the pyrimidopurinone adduct of deoxyguanosine by base propenal. *Proc Nat Acad Sci USA* 1998: **95**: 11113-11116.

151. Parker J, Kaplon MK, Alvarez CJ, Krishnaswamy G. Prostaglandin H-synthase expression is variable in human colorectal adenocarcinoma cell lines. *Expt Cell Res* 1997: **236**: 321-329.

152. Kirkland SC. Dome formation by a human colonic adenocarcinoma cell line (HCA-7). *Cancer Res* 1985: **45**: 3790-3795.

153. Wight NJ, Stack WA, Pathmakanthan S, Curtis HS, Jones JIW, Atherton CT, Stevens M, Stott C, Rubin P, Hawkey CJ. A crossover comparison of the gastric

mucosal effects of P54, a novel turmeric compound with aspirin and ibuprofen in healthy volunteers. *Gut* 2000: **46S**: A88.

154. Erdelmeier I, Gerard-Monnier D, Yadan JC and Chaudiere J. Reactions of N-methyl-2-phenylindole with malondialdehyde and 4-hydroxyalkenals. Mechanistic aspects of the colorimetric assay of lipid peroxidation. *Chem Res Toxicol* 1998: **11**: 1184-1194.

155. Gerard-Monnier D, Erdelmeier I, Chaudiere J. Reactions of 1-methly-2-phenylindole with malondialdehyde and 4-hydroxyalkenals: analytical applications to a colorimetric assay of lipid peroxidation. *Chem Res Toxicol* 1998: **11**: 1176-1183.

156. Sevilla CL, Mahle NH, Eliezer N, Uzieblom A, O'Hara SM, Nokubo M, Miller R, Rouzer CA and Marnett LJ. Development of monoclonal antibodies to the malondialdehyde-deoxyguanosine adduct, pyrimidopurinone. *Chem Res Toxicol* 1997 **10**: 172-180.

157. Sheng H, Shao J, Kirkland SC, Isakson P, Coffey RJ, Morrow J, Beauchamp RD, DuBois RN. Inhibition of human colon cancer cell growth by selective inhibition of cyclooxygenase-2. *J Clin Invest* 1997: **99**: 2254-2259.

158. Chapeau MC and Marnett LJ. Enzymatic synthesis of purine deoxynucleoside adducts. *Chem Res Toxicol* 1991: **4**: 636-638.

159. Ji C, Rouzer CA, Marnett LJ, Pietenpol JA. Induction of cell cycle arrest by the endogenous product of lipid peroxidation, malondialdehyde. *Carcinogenesis* 1998: **19**: 1275-1283.

160. Barnett J, Chow J and Ives D. Purification, characterization and selective inhibition of human prostaglandin G/H synthase 1 and 2 expressed in the baculovirus system. *Biochim Biophys Acta* 1994: **1209**: 130-139.

161. Fang JL, Vaca CE, Valsta LM, Mutanen M. Determination of DNA adducts of malondialdehyde in humans: effects of dietary fatty acid composition. *Carcinogenesis* 1996: **17**: 1035-1040.

162. Draper HH, Agarwal S, Nelson DE, Farber E. Effects of peroxidative stress and age on the concentration of a deoxyguanosine-malondialdehyde adduct in rat DNA. *Lipids* 1995: **30**: 959-961.

163. Chaudhary AK, Nokubo M, Reddy GR, Yeola SN, Morrow JD, Blair IA and Marnett LJ. Detection of endogenous malondialdehyde-deoxyguanosine adducts in human liver. *Science* 1994: **265**: 1580-1582.

164. de Zwart LL, Hermanns RCA, Meerman JHN, et al. Evaluation of urinary biomarkers for radical-induced liver damage in rats treated with carbon tetrachloride. *Toxicol Applied Pharmacol* 1998: **148**: 71-82.

165. Chatterjee S, Variyar PS, Gholap AS, PadwalDesai SR, Bongirwar DR. Effect of gamma-irradiation on the volatile oil constituent of turmeric (Curcuma longa). *Food Res Int* 2000: **33**: 103-106.

166. Negi PS, Jayaprakasha GK, Jagan Mohan Rao L, Sakariah KK. Antibacterial activity of turmeric oil: a byproduct from curcumin manufacture. *J Agric Food Chem* 1999: **47**: 4297-4300.

167. Garg SN, Bansal RP, Gupta MM, Kumar S. Variation in the rhizome essential oil and curcumin contents and oil quality in the land races of turmeric curcuma longa of north Indian plains. *Flavour Fragr J* 1999: **14**: 315-318.

168. Aaronson NK, Ahmedzai S, Bergman B, Bullinger M, Cull A, Duez NJ, Filiberti A, Flechtner H, Fleishman SB, de Haes JC. The European organization for research and treatment of cancer QLQ-C30: A quality of life instrument for

use in international clinical trials in oncology. *J Natl Cancer Inst* 1993: **85**: 365-376.

169. Habig WH, Pabst MJ and Jakoby WB. Glutathione S-transferases. *J Biol Chem* 1974: **249**: 7130-7139.

170. DeSousa M, Pirmohamed M, Kitteringham NR, Woolf T and Park BK. No association between tacrine transaminitis and the glutathione transferase theta genotype in patients with Alzheimer's disease. *Pharmacogenetics* 1998: **8**: 353-355.

171. Harries LW, Stubbins MJ, Forman D, Howard GC, and Wolf CR. Identification of genetic polymorphisms at the glutathione S-transferase Pi locus and association with susceptibility to bladder, testicular and prostate cancer. *Carcinogenesis* 1997: **18**: 641-644.

172. Wang LH, Groves MJ, Hepburn MD, Bowen DT. Gluathone S-trnasferase enzyme expression in hematopoietic cell lines implies a differential protective role for T1 and A1 isoenzymes in erythroid and lymphoid lineages. *Haematologica* 2000: **85**: 573-579.

173. Bruhn C, Brockmoller J, Borchert HH. Concordance between enzyme activity and genotype of glutathione S-transferase theta (GSTT1). *Biochem Pharmacol* 1998: **56**: 1189-1193.

174. Lubich WP, Marton LJ. Solid-phase extraction and determination of dansyl derivatives of uncojugated and acetylated polyamines by reversed-phase liquid chromatography. *J Chromat* 1986: **380**: 19-32.

175. Aboul-enein HY, Al-Duraibi IA. Separation of several free polyamines and their acetyl derivatives by ion-pair reversed-phase high performance liquid chromatography. *Biomed Chromat* 1998: **12**: 291-293.

176. Leuratti C, Singh R, Lagneau C, Farmer PB, Marnett LJ and Shuker DEG. Determination of malondialdehyde-induced DNA damage in human tissues using an immunoslot blot assay. *Carcinogenesis* 1998: **19**: 1919-1924.

177. Kautiainen A, Vaca CE, Granath F. Studies on the relationship between hemoglobin and DNA adducts of malonaldehyde and their stability *in vivo*. *Carcinogenesis* 1993: **14**: 705-708.

178. Hull MA, Booth JK, Tisbury A, Scott N, Bonifer C, Markham AF, Coletta PL. Cyclooxygenase 2 is upregulated and localised to macrophages in the intestine of Min mice. *Br J Cancer* 1999: **79**: 1399-1405.

179. Diczfalusy U, Falardeau P and Hammarstrom S. Conversion of prostaglandin endoperoxides to C17-hydroxyacids by human platelet thromboxane synthase. *FEBS Lett* 1977: **84**: 271-274.

180. Canuto RA, Ferro M, Muzio G, Bassi AM, Leonarduzzi G, Maggiora M, Adamo D, Poli G and Lindahl R. Role of aldehyde metabolizing enzymes in mediating effects of aldehyde products of lipid peroxidation in liver cells. *Carcinogenesis* 1994: **15**: 1359-1364.

181. Siu GM and Draper HH. Metabolism of malonaldehyde *in vivo* and *in vitro*. *Lipids* 1982: **17**: 349-355.

182. Huang MT, Newmark HL and Frenkel K. Inhibitory effects of curcumin on tumorigenesis in mice. *J Cell Biochem* 1994: **67**: 26-34.

183. Rao CV, Rivenson A, Simi B and Reddy BS. Chemoprevention of colon carcinogenesis by dietary curcumin, a naturally occurring plant phenolic compound. *Cancer Res* 1995: **55**: 259-266.

184. Kawamori T, Lubet R, Steele VE, Kellof GJ, Kaskey RB, Rao CV and Reddy BS. Chemopreventative effect of curcumin, a naturally occurring anti-

inflammatory agent, during the promotion/progression stages of colon cancer. *Cancer Res* 1999: **59**: 597-601.

185. Park EJ, Jeon CH, Ko G, Kim J and Sohn DH. Protective effect of curcumin in rat liver injury induced by carbon tetrachloride. *J Pharm Pharmacol* 2000: **52**: 437-440.

186. Pool-Zobel BL, Abrahamse SL, Collins AR, Kark W, Gugler R, Oberreuther D, Siegel EG, Treptow van Lishaut S and Rechkemmer G. Analysis of DNA strand breaks, oxidized bases, and glutathione S-transferase P1 in human colon cells from biopsies. *Cancer Epidemiol Biomarkers Prev* 1999: **8**: 609-614.

187. Hawk ET, Viner JL. Chemoprevention in ulcerative colitis: narrowing the gap between clinical practice and research. *Ann Int Med* 2001: **134**: 158-160.

188. Oetari S, Sudibyo M, Commandeur JNM, Samhoedi R and Vermeulen NPE. Effect of curcumin on cytochrome P450 and glutathione S-transferase activities in rat liver. *Biochem Pharmacol* 1996: **51**: 39-45.

189. Plastaras JP, Riggins JN, Otteneder M, Marnett LJ. Reactivity and mutagenicity of endogenous DNA oxopropenylating agents: Base propenals, malondialdehyde, and N^{ε}-oxopropenyllysine. *Chem Res Toxicol* 2000: **13**: 1235-1242.

190. Ireson CR, Veschoyle RD, Orr S, Oustric S, Jones DJL, Donald S, Sharma RA, Hill KA, Williams ML, Lim CK, Steward WP, Gescher A. Disposition of the chemopreventive agent curcumin in rats and in human liver and gut cells. *Proc Am Assoc Cancer Res* 2001: **42**: 21-22.

191. Lin JK, Pan MH, Lin-Shiau SY. Suppression of iNOS by curcumin and its hydrogenated metabolites through down regulation of IkB kinase and NFkB activation in macrophages. *Proc Am Assoc Cancer Res* 2001: **42**: 235.

192. HernandezVargas P, LopezArmada MJ, Benito M J, SanchezPernaute O, Palacios I, Egido J, HerreroBeaumont G. PGE_2 stimulates cyclooxygenase (COX)-2 expression through activation of transcription factor kappa B (NF-kappa B) in rabbit synovial cells. *Arthritis Rheum* 1998: **41**: 971.

193. Anto RJ, Maliekal TT, Karunagaran D. L-929 cells harboring ectopically expressed RelA resist curcumin-induced apoptosis. *J Biol Chem* 2000: **275**: 15601-4.

194. Lu X, Xie W, Reed D, Bradshaw WS, Simmons DL. Nonsteroidal antiinflammatory drugs cause apoptosis and induce cyclooxygenases in chicken embryo fibroblasts. *Proc Natl Acad Sci USA* 1995: **92**: 177961-177965.

195. Ward C, Chilvers ER, Lawson MF, Pryde JG, Fujihara S, Farrow SN, Haslett C, and Rossi AG. NF-kB activation is a critical regulator of human granulocyte apoptosis in vitro. *J Biol Chem* 1999: **274**: 4309-4318.

196. Kelloff GJ. Perspectives on cancer chemoprevention research and drug development. *Adv Cancer Res* 2000: **78**: 199-334.

197. Calaluce R, Earnest DL, Heddens D, Einspahr JG, Roe D, Bogert CL, Marshall JR, Alberts DS. Effects of piroxicam on prostaglandin E2 levels in rectal mucosa of adenomatous polyp patients: A randomised phase IIb trial. *Cancer Epid Biomarkers Prev* 2000: **9**: 1287-1292.

198. Are rofecoxib and celecoxib safer NSAIDs? *Drug Ther Bulletin* 2000: **38**: 81-86.

199. Evaluation of certain food additives: 51st Report of the joint FAO/WHO Expert Committee on Food Additives. Geneva: WHO Technical Report Series 891, 2000.

200. Chow HHS, Earnest DL, Clark D, Mason-Liddil N, Kramer CB, Einspahr JG, Guillen-Rodriguez JM, Roe DJ, Malone W, Crowell JA, Alberts DS. Effect of subacute ibuprofen dosing on rectal mucosal prostaglandin E_2 levels in healthy subjects with a history of resected polyps. *Cancer Epid Biomarkers Prev* 2000: **9**: 351-356.

201. van Lieshout EMM and Peters WHM. Age and gender dependent levels of glutathione and glutathione S-transferases in human lymphocytes. *Carcinogenesis* 1998: **19**: 1873-75.

202. Liu YH, Taylor J, Linko P, Lucier GW and Thompson CL. Gluathione S-transferase mu in human lymphocyte and liver: role in modulating formation of carcinogen-derived DNA adducts. *Carcinogenesis* 1991: **12**: 2269-2275.

203. Everett SM, Singh R, Leuratti C, White KLM, Neville P, Greenwood D, Marnett LJ, Schorah CJ, Forman D, Shuker D, Axon ATR. Levels of malondialdehyde-deoxyguanosine in the gastric mucosa: Relationship with lipid peroxidation, ascorbic acid, and helicobacter pylori. *Cancer Epid Biomarkers Prev* 2001: **10**: 369-376.

204. Singhal SS, Awasthi S, Pandya U, Piper JT, Awasthi YC. Effect of curcumin on glutathione-linked enzymes in K562 human leukaemia cells. *Toxicol Lett* 1999: **109**: 87-95.

205. Pereira MA, Grubbs CJ, Barnes LH, Li H, Olson GR, Eto I, Juliana M, Whitaker LM, Kelloff GJ, Steele VE, Lubet RA. Effects of the phytochemicals, curcumin and quercetin, upon azoxymethane-induced colon cancer and 7,12-dimethylbenz[a]anthracene-induced mammary cancer in rats. *Carcinogenesis* 1996: **17**: 1305-1311.

206. Alberts DS. Advanced Clinical Trial Design: The use of Surrogate End Point Biomarkers in Cancer Chemoprevention Trials. *In:* Perry MC (ed.) American Society of Clinical Oncology Educational Book, pp 76-80. Baltimore (USA): Lippincott, 1999.

207. Vigushin DM, Poon GK, Boddy A, Jarman M, Coombes RC. Phase I and pharmacokinetic study of D-limonene in patients with advanced cancer. *Cancer Chemother Pharmacol* 1998: **42**: 111-117.

208. Ripple GH, Gould MN, Arzoomanian RZ, Alberti D, Feierabend C, Simon K, Binger K, Wilding G, Bailey HH. Phase I clinical and pharmacokinetic study of perillyl alcohol administered four times a day. *Clin Cancer Res* 2000: **6**: 390-396.

209. Pisters KMW, Newman RA, Coldman B, Shin DM, Khuri FR, Hong WK, Glisson BS, Lee JS. Phase I trial of oral green tea extract in adult patients with solid tumors. *J Clin Oncol* 2001: **19**: 1830-1838.

210. Prevention of cancer in the next millennium: Report of the chemoprevention working group to the American association for cancer research. *Cancer Res* 1999: **59**: 4743-4758.

211. Lippman SM, Lee JJ, Sabichi AL. Cancer chemoprevention: progress and promise. *J Natl Cancer Inst* 1998: **90**: 1514-1528.

212. Kelsen DP. Surrogate endpoints in assessment of new drugs in colorectal cancer. *Lancet* 2000: **356**: 353-354.