

CANCER CHEMOPREVENTIVE PROPERTIES OF ANTHOCYANINS IN
PATIENTS WITH COLORECTAL CANCER AND COLORECTAL LIVER
METASTASES

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ABSTRACT

CANCER CHEMOPREVENTIVE PROPERTIES OF ANTHOCYANINS IN PATIENTS WITH COLORECTAL CANCER AND COLORECTAL LIVER METASTASES

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Anthocyanins, polyphenolic phytochemicals which render fruit and vegetables bright red or blue, possess anticarcinogenic properties in preclinical models of carcinogenesis. The aim of this study was to elucidate whether consumption of mirtocyan, a standardised anthocyanin extract, would cause pharmacodynamic changes consistent with chemoprevention and generate measurable levels of anthocyanins in blood, urine and target tissue.

Twenty-five patients with either primary colorectal cancer or colorectal liver metastases received 1.4, 2.8 or 5.6 g of mirtocyan (containing 0.5-2.0 g anthocyanins) daily for 7 days prior to colon/liver resection. Anthocyanin levels were measured by high performance liquid chromatography. Proliferation (Ki-67), apoptosis (caspase-3) and inflammation (COX-2) were measured in colorectal tumour tissue. Effects on the insulin-like growth factor (IGF) axis were evaluated in plasma and markers of oxidative DNA damage were assessed in blood and urine.

Consumption of up to 5.6 g of mirtocyan daily was well tolerated. Analysis of colorectal tumour tissue revealed that consumption of mirtocyan was associated with a 7% decrease in proliferation ($p=0.003$) and a 1.7% increase in apoptosis (0.044). A trend towards a reduction in circulating IGF-1 levels was observed ($p=0.168$). Mirtocyan anthocyanins and methyl and glucuronide metabolites were identified in plasma, urine and colorectal tissue, but not in liver. Anthocyanin concentrations in biomatrices were approximately dose-dependent. Following consumption of 5.6 g of mirtocyan daily anthocyanin levels in plasma, urine and colorectal tumour tissue were 117 ng/ml, 3 μ g/ml and 179 ng/g, respectively. Mirtocyan did not affect levels of COX-2 or markers of oxidative DNA damage.

Administration of mirtocyan furnished levels of anthocyanins in colorectal tumour tissue comparable to those capable of mediating chemopreventive effects *in vivo*. Consumption of only 1.4 g of mirtocyan daily may exert pharmacodynamic effects commensurate with colorectal cancer chemoprevention. These data support further clinical development of anthocyanins as potential colorectal cancer chemopreventive agents.

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LIST OF ABBREVIATIONS

8-oxo-dG	8-oxo-7,8-dihydro-2'-deoxyguanosine
ACF	Aberrant crypt foci
AIF	Apoptosis inducing factor
AP-1	Activator protein 1
ASA	American Society of Anesthesiologists
ATCC	American type tissue collection
C3G	Cyanidin-3-glucoside
CDK	Cyclin-dependent kinase
CDKI	Cyclin-dependent kinase inhibitor
C/EBP	CCAAT/enhancer-binding protein
C _{max}	Maximal concentration
COMT	Catechol-O-methyl transferase
COX	Cyclooxygenase
DAB	3,3'-diaminobenzidine
DMH	1,2 dimethylhydrazine
DPX	Dibutyl phthalate xylene
EGCG	Epigallocatechin gallate
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
Endo G	Endonuclease G
ERK	Extracellular signal-regulated kinase
FAP	Familial adenomatous polyposis

FCS	Foetal calf serum
FRAP	Ferric reducing ability of plasma
GMP	Good manufacturing practice
GTE	Green tea extract
H ₂ O ₂	Hydrogen peroxide
HPLC	High performance liquid chromatography
IC ₅₀	Half maximal inhibitory concentration
IEN	Intraepithelial neoplasia
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IMS	Industrial methylated spirit
JNK	c-Jun <i>N</i> -terminal kinase
LC/MS/MS	Liquid chromatography, tandem mass spectrometry
LPS	Lipopolysaccharide
LOH	Loss of heterozygosity
MAPK	Mitogen activated protein kinase
MDA	Malondialdehyde
MHRA	Medicines and healthcare products regulatory agency
MMP	Matrix metalloproteinase
NF-κB	Nuclear factor κB
NMDA	N-nitrosomethylbenzylamine
NSAID	Non-steroidal anti-inflammatory drug
ORAC	Oxygen radical absorbing capacity

PARP	Poly(ADP)ribose polymerase
PBS	Phosphate buffered saline
PhIP	2-amino-1-methyl-6-phenylimidazol[4,5- <i>b</i>]pyridine
ROS	Reactive oxygen species
Rt	Retention time
TBS	Tris(hydroxymethyl)aminomethane buffered saline
TEAC	Trolox equivalent antioxidant capacity
TIMP	Tissue inhibitor of metalloproteinase
TRIS	Tris(hydroxymethyl)aminomethane
U-PA	Urokinase-type plasminogen activator
VEGF	Vascular endothelial growth factor

CHAPTER 1

INTRODUCTION

1.1 CHEMOPREVENTION OF COLORECTAL CANCER

This thesis describes work in which a novel putative colorectal cancer chemopreventive intervention has been studied in humans. Fundamental issues in colorectal carcinogenesis and cancer chemoprevention are initially discussed.

Worldwide colorectal cancer is the third most common form of cancer with in excess of one million new cases and 500,000 deaths occurring annually (Parkin et al., 2005). The liver is the most common site for metastatic spread from colorectal cancer and approximately 50% of patients develop hepatic metastases. The significant morbidity and mortality associated with colorectal cancer has contributed towards much interest within the field of chemoprevention.

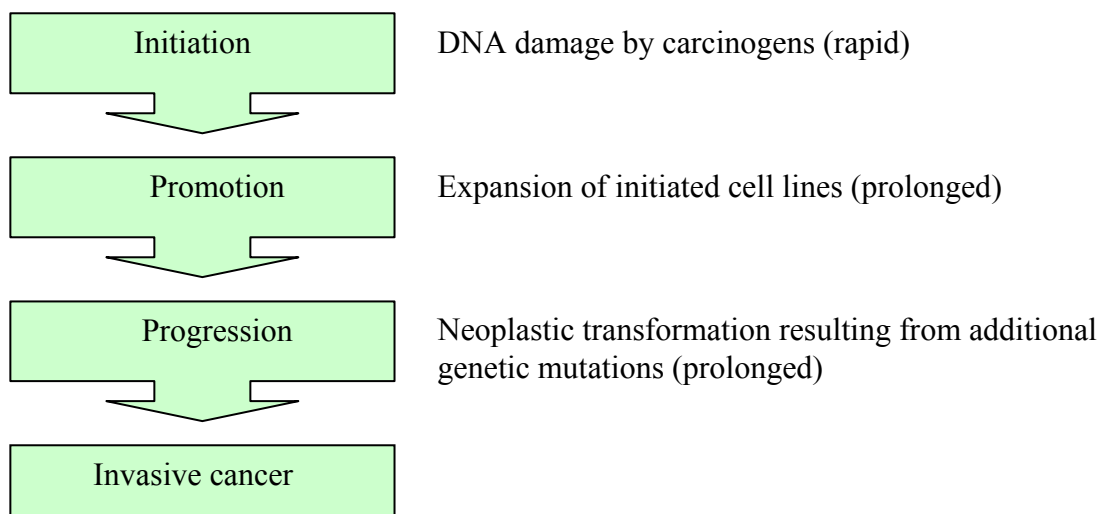
1.1.1 DEFINITION OF CARCINOGENESIS AND CHEMOPREVENTION

Carcinogenesis is a multistage process (*Figure 1.1*) which is characterised by the accumulation of genetic defects within a single cell line leading to a progressively dysplastic cellular appearance, deregulated cell growth and finally carcinoma (Tsao et al., 2004). With rare exceptions this process occurs over years, often decades.

Cancer chemoprevention is defined as the use of natural or synthetic agents to prevent, arrest or reverse the process of carcinogenesis (Greenwald, 2002). Several thousand agents with potential chemopreventive properties have been described. These can be broadly classified into those which prevent carcinogens interacting with DNA (“carcinogen-blocking agents”) and those which inhibit the carcinogenic

process after initiation (“suppressing agents”) (Chemoprevention Working Group, 1999). Chemoprevention strategies may be aimed at preventing disease in the healthy population (primary prevention), disease in high risk individuals (secondary prevention) or recurrent disease (tertiary prevention).

Figure 1.1: The multistage process of carcinogenesis



1.1.2 IDENTIFICATION OF CANDIDATE CHEMOPREVENTIVE AGENTS

Epidemiological studies which demonstrate an inverse correlation between cancer incidence and the intake of specific agents often provide the earliest indication of potential chemopreventive efficacy. For example, observational studies dating from the 1980's revealed a 40-50% reduction in the incidence of adenomas, colorectal cancer and colorectal cancer associated mortality in people consuming non-steroidal anti-inflammatory drugs (NSAIDs) (Umar et al., 2002). Further data to guide the

selection of potential chemopreventive agents for use in clinical trials is provided by *in vitro* and *in vivo* experiments and short-term pilot studies in healthy volunteers and patients with premalignant disease or cancer. The importance of thorough evaluation of potential chemopreventive agents is illustrated by the fateful results of trials involving β -carotene in the prevention of lung cancer. Two large clinical trials, initiated predominantly on the basis of epidemiological data, actually revealed an *increased* incidence of the disease in high risk individuals (Omenn et al., 1996) (The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group, 1994). Pre-clinical experiments and small pilot studies reduce, but do not eliminate, the risk of adverse events occurring during clinical chemoprevention trials. Strong preclinical data and a favourable initial experience in patients with familial adenomatous polyposis (FAP) provided the basis for three major colorectal cancer chemoprevention trials of cyclooxygenase-2 (COX-2) inhibitors. In all three trials a significant reduction in adenoma formation in patients at high risk for colorectal cancer was accompanied by an unexpected increase in serious cardiovascular events (relative risk 1.3-3.4) (Arber et al., 2006; Baron et al., 2006; Bertagnolli et al., 2006).

Cancer chemoprevention frequently involves administering agents to healthy individuals for prolonged periods and safety is a crucial consideration. The expected low toxicity of diet-derived compounds has generated interest in their potential role as cancer chemopreventive agents. Phytochemicals have frequently been administered as formulated, isolated agents in chemoprevention studies, at doses in excess of those

normally consumed within the diet, and reports of severe toxicity have been rare (Thomasset et al., 2007).

1.1.3 BIOMARKERS OF CHEMOPREVENTIVE EFFICACY

Whilst a reduction in cancer incidence and mortality is the ultimate efficacy criterion for a chemopreventive agent, the use of surrogate biomarkers of chemopreventive activity, minimising trial size, length and cost, is an appealing alternative goal in the clinical development of chemopreventive agents. Criteria for the acceptability of potential surrogate end-point biomarkers of carcinogenesis are their (Kelloff et al., 2000):

- Differential expression in normal and high-risk tissue
- Differential expression along the carcinogenic pathway
- Capability of being modulated by chemopreventive agents
- Ability to be detected early in the carcinogenic pathway
- Sensitivity, specificity and accuracy
- Ease of measurement
- Modulation in a manner which correlates with cancer incidence

Intraepithelial neoplasia (IEN), such as colorectal adenomas and those involving the prostate and cervix, are on a causal pathway to cancer and generally considered suitable biomarkers for following the process of carcinogenesis (Kelloff et al., 2004; O'Shaughnessy et al., 2002). In 2006, the American Association for Cancer Research (AACR) IEN Task Force proposed a number of clinical trial designs for chemoprevention studies. A proposed end-point in colorectal chemoprevention

studies was adenoma prevention/regression in FAP patients/carriers and patients previously treated for colon cancer or adenomas (Kelloff et al., 2006).

Many cellular, biochemical, molecular and genetic events have been proposed as potentially useful surrogate end-point biomarkers for colorectal cancer chemoprevention trials. Such biomarkers require validation to establish their relationship with cancer risk. Preclinical models may provide strong supportive evidence for validation, but ideally this process should be undertaken in clinical studies (Kelloff et al., 2000).

1.1.4 PRE-CLINICAL EVALUATION OF CHEMOPREVENTIVE AGENTS

In vitro experiments utilising potential chemopreventive agents may provide valuable mechanistic data, initial information on agent toxicity, organ specificity and concentrations that may be required to observe pharmacological efficacy *in vivo*. Animal studies yield further data on agent efficacy and toxicity, in addition to providing information on pharmacokinetics.

Two types of rodent models exist to assess the activity of chemopreventive agents. These are chemical models, in which cancer is induced by exposure to high concentrations of genotoxic carcinogens (e.g. dimethylhydrazine or its metabolite azoxymethane) and transgenic models in which animals spontaneously develop tumours secondary to germline genetic mutations (e.g. Apc gene). Although the tumours formed are histologically similar to those in humans, both models are

associated with a number of limitations. The genetic mutations observed in rodent models vary significantly from those in human tumours and the mechanism by which tumours are induced in chemical models is very different to the multifactorial aetiology of human cancer. In addition, Apc mutant rodents predominantly develop tumours in the small intestine, not the colon. They die rapidly due to haemorrhage from many adenomas, before advanced disease develops. A recent meta-analysis concluded that the results of rodent studies do not accurately predict the efficacy of all chemopreventive agents in humans (Corpet & Pierre, 2005).

1.1.5 CLINICAL EVALUATION OF CHEMOPREVENTIVE AGENTS

Agents that show efficacy and low toxicity in preclinical studies are considered for clinical cancer chemoprevention trials. Clinical chemoprevention trials have traditionally been divided into three phases (Gescher et al., 2001). Phase I trials are conducted to determine dose-related safety and pharmacokinetics. Doses and schedules should be based on efficacy data obtained in preclinical studies. Phase II trials use a randomised, blinded, placebo-controlled design to evaluate dose-response and common toxicities likely to result from prolonged administration, preferably 3 months or longer. These trials involve measurement of biomarkers previously validated in preclinical and clinical studies. If safety and efficacy are judged to be satisfactory, large-scale, randomised, prospective phase III trials are undertaken. These trials are the ultimate tests of efficacy and evaluate effects on the incidence of primary tumours as well as biomarkers, in relation to dose and toxicity.

1.2 ANTHOCYANINS: CHEMISTRY AND PHARMACOKINETICS

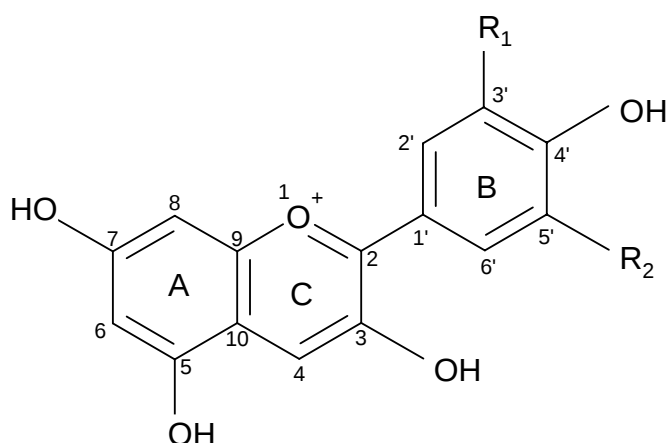
Anthocyanins are members of the flavonoid class of polyphenolic phytochemicals. They occur ubiquitously in the plant kingdom and are responsible for the intense red or blue coloration of berries and other fruits and vegetables. In recent years the potential health benefits associated with the consumption of phytochemicals, including anthocyanins, has been the focus of much research. Numerous studies have described the anti-carcinogenic properties of anthocyanins (*Sections 1.3 and 1.4*). Furthermore, evidence suggests a possible role for anthocyanins in the prevention/treatment of cardiovascular disease (Xia et al., 2006), neurodegenerative disease (Andres-Lacueva et al., 2005) and ocular disease (Lee et al., 2005b).

1.2.1 CHEMICAL PROPERTIES OF ANTHOCYANINS

Anthocyanins are water-soluble glycosides of anthocyanidins, molecules with a diphenylpropane based structure. Six anthocyanidins are commonly found in higher plants: cyanidin (50%), pelargonidin (12%), peonidin (12%), delphinidin (12%), petunidin (7%) and malvidin (7%). These occur almost exclusively as glycosides (Kong et al., 2003) (*Figure 1.2*). Individual anthocyanins vary in: 1) the number and position of hydroxyl and methoxyl groups attached to the aglycone, 2) the nature, number and position of sugars attached to the aglycone and 3) the nature, number and position of aliphatic or aromatic acids attached to the sugars. The most common sugar components of anthocyanins are glucose, galactose and arabinose, which are usually conjugated to the C3 hydroxyl group in the C ring (Cooke et al., 2005). To date, in

excess of 400 naturally occurring anthocyanins have been identified (Kong et al., 2003).

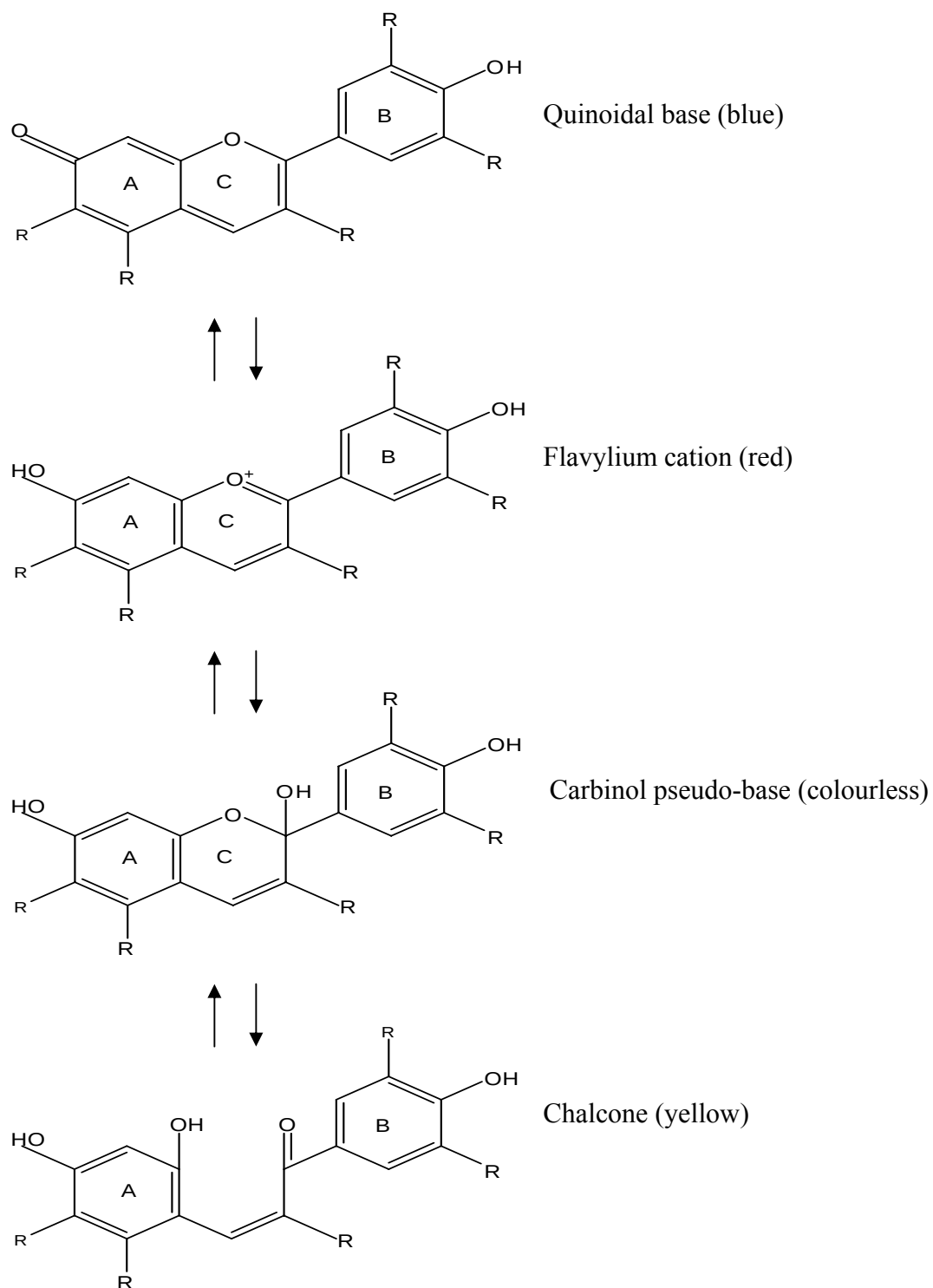
Figure 1.2: Structures of commonly occurring anthocyanidins



Cyanidin	$R_1 = \text{OH}$	$R_2 = \text{H}$
Delphinidin	$R_1 = \text{OH}$	$R_2 = \text{OH}$
Malvidin	$R_1 = \text{OCH}_3$	$R_2 = \text{OCH}_3$
Pelargonidin	$R_1 = \text{H}$	$R_2 = \text{H}$
Peonidin	$R_1 = \text{OCH}_3$	$R_2 = \text{H}$
Petunidin	$R_1 = \text{OCH}_3$	$R_2 = \text{OH}$

The stability of anthocyanins (anthocyanidins and anthocyanins) is highly dependent on temperature, light, oxygen, enzymes and pH. At pH values of <2 a relatively stable flavylium compound predominates, giving a characteristic red colouration. Increasing pH is accompanied by rapid loss of a proton generating a blue quinoidal base. Concurrently, a much slower hydration of the flavylium cation occurs which yields a colourless carbinol pseudo-base. This species tautomerises, through an opening in the C-ring to generate a yellow chalcone (*Figure 1.3*).

Figure 1.3: pH-dependent conformational rearrangement of the anthocyanidin molecule



The ratio of different molecular forms of anthocyanins coexisting in aqueous solution at any given time is dependent on pH, temperature and time (McGhie & Walton, 2007).

1.2.2 PHARMACOKINETICS

ABSORPTION

Most animal studies have found that anthocyanins appear rapidly in the circulatory system following oral administration, with C_{max} frequently occurring after only 15 minutes (Ichiyanagi et al., 2006; Ichiyanagi et al., 2005; Miyazawa et al., 1999). This observation implies that anthocyanins may be absorbed from the upper gastrointestinal tract, a suggestion which is supported by the results of animal *in situ* perfusion studies and a recent *in vitro* chamber study. Following direct administration into the stomach and small intestine of rats high concentrations of anthocyanins were detected in plasma derived from the gastric and mesenteric veins, respectively (Talavera et al., 2003; Talavera et al., 2004). When grape anthocyanins were administered into the stomachs of anaesthetised rats which had been ligated at the cardia and pylorus malvidin-3-glucoside appeared in both portal and systemic plasma after only 6 minutes (Passamonti et al., 2003). Matuschek et al. recently investigated anthocyanin absorption using an *in vitro* chamber model mounted with murine intestinal tissue. Minor absorption occurred with duodenal tissue and no absorption was recorded when tissues from the ileum or colon were used (Matuschek et al., 2006).

Human and animal studies have consistently revealed intact glycosides in the peripheral circulation and urine following oral anthocyanin administration, suggesting that these compounds may be absorbed without prior chemical alteration (Matsumoto et al., 2001; McGhie et al., 2003). Given the size and hydrophilic nature of anthocyanins passive diffusion has been considered an unlikely mechanism of absorption. However, definitive evidence to support the role of specific transporter molecules is currently lacking. Absorption efficiency is influenced by the nature of the aglycone and sugar moiety. A recent study which investigated transport of blueberry extract through a human Caco-2 intestinal cell monolayer revealed that glycoside-based anthocyanins were transported more efficiently than galactosides. In addition, malvidin-3-glucoside was transported more efficiently than delphinidin-3-glucoside, indicating that more free hydroxyl groups and less methoxyl groups may decrease absorption (Yi et al., 2006). This finding has been replicated *in vivo* where urinary excretion (taken to indicate absorption) of malvidin-based anthocyanins was superior to that of delphinidin-based anthocyanins (McGhie et al., 2003).

Pharmacokinetic studies have shown that anthocyanins are rapidly absorbed and eliminated but that absorption efficiency is low (*Table 1.1*). However, bioavailability may have been underestimated in these studies for two important reasons. Firstly, anthocyanins which are not absorbed in the upper gastrointestinal tract may be degraded/transformed by gut microflora into compounds which are not detected by currently employed analytical methods (Aura et al., 2005). Protocatechuric acid, an anthocyanin metabolite known to result from the action of microflora (Aura et al.,

2005), has recently been detected in human plasma at concentrations far in excess of the anthocyanin consumed (cyanidin-3-glucoside) (Vitaglione et al., 2007). Secondly, most analytical methods involve acidification to transform anthocyanins which are present at neutral pH (colourless carbinol pseudo-base and chalcone forms) into the flavylum cation. Metabolic conversion which impedes this transformation would result in failure to detect these compounds (McGhie et al., 2003). Given these issues a study utilising radiolabelled anthocyanins would be extremely useful to confirm bioavailability data.

Table 1.1: Human pharmacokinetic studies involving consumption of a single dose of anthocyanins (Studies > 5 individuals)

Source	Total anthocyanin dose (mg)*	C _{max} plasma (nmol/L)*	T _{max} plasma (h)	Urinary excretion (% of intake)	Maximal urinary excretion (h)	Reference
Black currant concentrate	3.58 mg/kg	5.0-73.4 [†]	1.25-1.75 [†]	0.060-0.11 (8 h) [†]	0-4 [†]	(Matsumoto et al., 2001)
Black currant juice	144.8	2.91 ng/ml	1.0	0.043 (7 h)	1.5	(Bitsch et al., 2004)
Black currant juice	716	16 ng/ml	0.75	0.048 (4 h)		(Nielsen et al., 2003)
Black current juice	1029.2	3.5-51.3 [†]	1.0	0.007-0.133 (30.5)	1.0	(Rechner et al., 2002)
Black currant juice	1239	53 ng/ml	0.75	0.072 (4 h)		(Nielsen et al., 2003)
Blood orange juice	71	1.9 (PCA 492)	0.5 (PCA 2)	1.2 (24)	2-12 [†]	(Vitaglione et al., 2007)
Blueberry	690	Not detected		0.004 (6 h)	2-4	(Wu et al., 2002)
Cranberry juice	0.65			5 (24 h)	3-6	(Ohnishi et al., 2006)
Elderberry concentrate	1900			0.003-0.012 (6 h)	1-3	(Mulleter et al., 2002)
Elderberry extract	147.3	15.6 ng/ml	1.5	0.37 (7 h)	1.5	(Bitsch et al., 2004)
Elderberry extract						(Frank et al., 2007)
Elderberry extract						(Frank et al., 2007)
Elderberry juice	3570			0.06 (24 h)	0.5	(Frank et al., 2005)
Purple carrots (cooked)	321	5.3	1.5-2.5	0.038 (24 h)	4	(Kurilich et al., 2005)
Purple carrots (raw)	416	5.8	1.5-2.5	0.030 (24 h)	4	(Kurilich et al., 2005)
Purple carrots (cooked)	643	5.0	1.5-2.5	0.020 (24 h)	4	(Kurilich et al., 2005)
Red cabbage	138.2 µmol			0.083 (24 h)	2-4	(Charron et al., 2007)
Red cabbage	276.5 µmol			0.050 (24 h)	2-4	(Charron et al., 2007)
Red cabbage	414.7 µmol			0.043 (24 h)	2-4	(Charron et al., 2007)
Red fruit extract	2.95 mg/kg	29	1			(Miyazawa et al., 1999)
Red grape juice	169.3	2.8 [†]	2.0 [†]	0.02 (6 h) [†]	0-3 [†]	(Bub et al., 2001)
Red grape juice	283.5	100.1 ng/ml	0.5	0.23 (7 h)	0.5	(Frank et al., 2003)
Red wine (dealcoholised)	72.4	1.7 [†]	1.5 [†]	0.02 (6 h) [†]	0-3 [†]	(Bub et al., 2001)
Red wine	85.6	1.4 [†]	0.8 [†]	0.02 (6 h) [†]	0-3 [†]	(Bub et al., 2001)
Red wine	279.6	42.9 ng/ml	1.5	0.18 (7 h)	1.5	(Frank et al., 2003)
Strawberry	15 µmol			2.1 (24 h)	2	(Carkeet et al., 2008)
Strawberry	30 µmol			2.2 (24 h)	2	(Carkeet et al., 2008)
Strawberry	60 µmol			1.9 (24 h)	2	(Carkeet et al., 2008)
Strawberry	179 µmol			1.8 (24 h)	0-4 [†]	(Felgines et al., 2003)
Strawberry	241.2 µmol	274	1.1	0.75 (24 h)	0-2	(Mullen et al., 2008)

* If not stated otherwise; [†] Depending on the anthocyanin considered; PCA, Protocatechuric acid

METABOLISM

The two major metabolites of anthocyanins are glucuronidated and methylated conjugates. The results of human pharmacokinetic studies have shown either intact anthocyanins or glucuronidated metabolites to be the predominant compounds present in urine and plasma samples (Kay et al., 2004; Kay et al., 2005). Methylated metabolites have been detected in human urine and plasma (Kay et al., 2004; Kay et al., 2005). In addition, aglycones and sulphate conjugates have been identified in human urine (Felgines et al., 2003; Felgines et al., 2005; Mullen et al., 2008). The polyphenol protocatechuric acid has recently been identified in human plasma following ingestion of blood orange juice (Vitaglione et al., 2007).

Glucuronidated metabolites of anthocyanins were first detected in a study by Wu et al. After four healthy women ingested 12 g of elderberry extract, which predominantly contained cyanidin-3-glucoside and cyanidin-3-sambubioside, cyanidin-3-glucoside monoglucuronide and peonidin monoglucuronide were identified in urine (Wu et al., 2002). Two possible pathways to explain the formation of glucuronidated metabolites were proposed. The first possibility is that glucosides are directly converted to glucuronides by the action of UDP-glucose dehydrogenase, an enzyme located in a variety of tissues including the liver, kidney and intestinal mucosa (Cappiello et al., 1991). A second possibility is that glycosides are initially hydrolysed by intestinal β -glucosidases to yield aglycones which are then subject to glucuronidation in the liver (Wu et al., 2002). Animal studies have shown the presence of aglycones in jejunal tissue and plasma following oral ingestion of

anthocyanins (Talavera et al., 2005). In addition, a recent study investigating anthocyanin metabolism in pigs provided further support for glucuronidation subsequent to the formation of aglycones. Pigs consumed a chokeberry extract which although plentiful in cyanidin glycosides contained little cyanidin-3-glucoside (2.5%). Levels of cyanidin monoglucuronide observed in the urine suggested formation from glycosides other than cyanidin-3-glucoside (Wu et al., 2005).

Miyazawa et al. first detected methylated metabolites in rat liver following consumption of red fruit anthocyanins (Miyazawa et al., 1999). Formation of methylated metabolites primarily occurs in the liver, but also to a lesser extent in the kidney and small intestine, sites where catechol-O-methyl transferase (COMT) is located. Early animal studies revealed high levels of methylated metabolites in the liver but failed to detect these in the plasma, suggesting biliary excretion (Miyazawa et al., 1999). More recently animal studies have confirmed that the predominant anthocyanins extracted from bile samples are methylated metabolites (Talavera et al., 2003; Talavera et al., 2004). Following direct gastric administration anthocyanins have been detected in the bile of rats after only 20 minutes (Talavera et al., 2003).

TISSUE DISTRIBUTION

A number of animal studies have been conducted to assess the tissue distribution of anthocyanins. El Mohsen et al. demonstrated that pelargonidin, or its metabolites, were present in the gastrointestinal tract, liver, kidneys, brain and lungs of rats at 2 hours post-gavage. Anthocyanins were not detected in the heart or spleen at any time

point and were not detected in any tissue at 18 hours post-gavage (El Mohsen et al., 2006). Intact anthocyanins were recently identified in ocular tissue, the cortex and cerebellum of pigs whose diet had been supplemented with blueberry powder for 4 weeks. An unanticipated result was the detection of anthocyanins in tissues from control pigs and tissues obtained at the beginning of the study (Kalt et al., 2008).

1.2.3 SAFETY

Data from animal and human studies suggests that consumption of high doses of anthocyanins is safe. When the diet of rats was supplemented with up to 5% purple corn colour for 90 days no mortalities or cases of systemic toxicity occurred (Nabae et al., 2008). Stoner et al. recently conducted a pharmacokinetic study in which 11 healthy volunteers consumed 45 g of freeze-dried black raspberry powder for 7 days. Four subjects reported a total of 5 minor adverse events. Those events probably related to the consumption of raspberry powder were constipation and dark stool (Stoner et al., 2005).

1.3 CANCER CHEMOPREVENTIVE EFFECTS OF ANTHOCYANINS

1.3.1 CHEMOPREVENTION IN RODENT MODELS

Anthocyanins have exhibited cancer chemopreventive properties in xenograft models and animal models of colorectal, breast, skin, oesophageal, oral and multi-organ carcinogenesis (Afaq et al., 2005; Casto et al., 2002; Cooke et al., 2006a; Ding et al., 2006; Jung et al., 2006; Kresty et al., 2001; Yamagishi et al., 2003). This section will focus on studies which have involved models of colorectal carcinogenesis.

When the diet of Apc^{Min} mice (*Section 1.1.4*) was supplemented with 0.3% cyanidin-3-glucoside (C3G) or mirtoselect, a commercially available standardised bilberry extract, adenoma numbers were reduced by 45% ($p < 0.001$) and 30% ($p < 0.05$), respectively. Total anthocyanin levels in the intestinal mucosa of mice that received C3G were 43 ng/g and levels in those that received mirtoselect were 8.1 $\mu\text{g/g}$ (Cooke et al., 2006a). A similar effect was observed when the diet of Apc^{Min} mice was supplemented with suboptimal levels of sulindac, with and without the addition of up to 0.3% anthocyanin-rich extract derived from tart cherries. Mice that received the anthocyanin extract in combination with sulindac developed 22% fewer tumours in the small intestine than those that received sulindac alone ($p = 0.02$). Although total tumour area in the small intestine was reduced by the addition of anthocyanin extract (20%, $p = 0.04$) average tumour size in both groups was similar (Bobe et al., 2006). In another study Apc^{Min} mice received either a mixture of anthocyanins or pure cyanidin in their drinking water at 800 mg/l or 200 mg/l, respectively. A third group received dietary supplementation with 20% freeze-dried tart cherries. The number and volume

of caecal adenomas was reduced in all mice that received anthocyanins compared to those that consumed a control diet or sulindac ($p < 0.05$). Numbers of adenomas in the small intestine were unaffected by anthocyanins (Kang et al., 2003).

Anthocyanins have also exhibited chemopreventive properties in chemically induced models of colorectal carcinogenesis. Rats have received 1,2 dimethylhydrazine (DMH) to induce the formation of colorectal adenomas and carcinomas followed by dietary supplementation with 5% purple corn, purple sweet potato or red cabbage colourings and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), a known carcinogen. The average number of adenomas/adenocarcinomas was significantly reduced in dye treated rats compared to controls. Furthermore, the induction of aberrant crypt foci (ACF) by PhIP was significantly inhibited in a subgroup of rats that received purple corn or red cabbage colourings but not DMH (Hagiwara et al., 2001; Hagiwara et al., 2002). In a similar study dietary supplementation with up to 10% lyophilized black raspberries significantly reduced adenocarcinoma and ACF multiplicity in azoxymethane-induced rats by up to 80% and 36%, respectively (Harris et al., 2001). The development of azoxymethane-induced ACF in rats has also been inhibited by dietary supplementation with bilberry, chokeberry and grape anthocyanin-rich extracts (Lala et al., 2006).

1.3.2 CHEMOPREVENTION IN HUMANS

A number of clinical trials designed to investigate the role of anthocyanins in patients with existing cancer or patients at high risk for developing cancer have recently been

published or are currently underway. Shumway et al. recently published a study on the effects of topically applied freeze-dried black raspberry (FBR) gel in patients with oral intraepithelial neoplasia (IEN). After a period of 6 weeks, during which time 10% FBR gel was applied 4 times daily, pre and post treatment biopsies were evaluated for change in histopathological grade by two maxillofacial pathologists. Seven patients showed histopathological improvement, 6 exhibited stable disease and 4 showed evidence of progression. A reduction in loss of heterozygosity (LOH) at tumour suppressor gene-associated loci was also observed, which is linked to the development of many human cancers, including oral squamous cell carcinoma. Interestingly, there was an association, although relatively weak, between reduction in LOH and improvement in histopathological grade ($r = 0.3504$, $p < 0.05$) (Shumway et al., 2008).

In terms of clinical chemoprevention trials involving colorectal cancer, in addition to the pre-surgery model described in the present study, trials of anthocyanins are currently ongoing in patients with familial adenomatous polyposis who have undergone a subtotal colectomy with ileorectal anastomosis. Over a period of 9 months patients are being treated with 2 black raspberry powder suppositories daily in combination with an oral dose of 20 g of black raspberry powder or placebo. Endoscopic biopsies are obtained at baseline, 18 weeks and 36 weeks. Preliminary data from 18 weeks suggests that consumption of black raspberry powder is associated with an approximate rate of rectal polyp regression of 50% (Stoner et al., 2007).

Stoner et al. are also currently conducting a clinical trial involving patients with Barrett's oesophagus. Twenty patients are consuming up to 45 g of black raspberry powder daily for 6 months and endoscopic biopsies are obtained before and after treatment. To date, only limited data from 10 patients has been reported which showed the length of Barrett's lesions were unaltered by treatment. In addition, urinary markers of oxidative stress were assessed. A significant reduction in 8-epi-prostaglandin F2 α (8-Iso-PGF2) was observed, but there was no significant change in mean urinary levels of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG) (Kresty et al., 2006; Stoner et al., 2007).

1.4 MOLECULAR MECHANISMS OF THE CHEMOPREVENTIVE

EFFECTS OF ANTHOCYANINS

1.4.1 CHEMOPREVENTION IN VITRO

Numerous studies have shown that anthocyanins (anthocyanidins and anthocyanins) possess antiproliferative properties in human cell lines derived from a variety of malignant tissues, including the colon, breast, lung and uterus (Lazze et al., 2004; Olsson et al., 2004; Zhang et al., 2005). Antiproliferative activity *in vitro* may be determined by anthocyan structure. Delphinidin and cyanidin have shown potent growth inhibitory effects *in vitro*, and it has been suggested that free hydroxyl groups on the B ring, particularly at position 3', may contribute to antiproliferative activity (Figure 1.2) (Meiers et al., 2001). This explanation, however, is not definitive, as malvidin has exhibited superior growth inhibitory effects in some cell lines (Zhang et al., 2005).

Anthocyanidins occur relatively infrequently *in vivo* but have been associated with greater antiproliferative activity than anthocyanins *in vitro* (Zhang et al., 2005). Anthocyanins have demonstrated IC₅₀ values for growth inhibition in the range of 100-800 µM, whereas plasma C_{max} in human intervention studies has been < 300 nM (Table 1.1) (Cooke et al., 2005). Few investigators have followed anthocyan concentrations in cell culture experiments. However, a recent study by Kern et al. has shown that anthocyanidins are highly unstable in cell culture conditions. Delphinidin and cyanidin were not detectable after only 30 minutes and 3 hours of incubation, respectively. In addition, less than 10% of the applied pelargonidin, peonidin and

malvidin were detectable after 3 hours. The degradation products of anthocyanins may therefore also possess growth inhibitory properties *in vitro*. The IC₅₀ of gallic acid, a degradation product of delphinidin, was 42 µM when incubated with human HT-29 colon carcinoma cells, but also possessed limited stability (Kern et al., 2007). Definitive data on the role of degradation products *in vitro* is currently lacking.

1.4.2 INHIBITION OF CYCLOOXYGENASE

Cyclooxygenase enzymes, which exist in three isoforms, are the rate-limiting step in the conversion of arachidonic acid to prostaglandins. COX-1 is expressed constitutively in most normal tissues and is responsible for prostaglandin production under physiological conditions. COX-3 is a variant of COX-1 which is predominantly expressed in the cerebral cortex (Chandrasekharan et al., 2002). COX-2 is induced by pro-inflammatory stimuli and is expressed in most colorectal cancers, particularly in large tumours, tumours with lymph node involvement and those with an advanced Duke's stage (Sheehan et al., 1999). COX-2 plays a key role in the development of colorectal cancer and is involved in tumour proliferation, resistance to apoptosis, invasion and angiogenesis (Pai et al., 2002; Tsujii & DuBois, 1995; Tsujii et al., 1997; Tsujii et al., 1998). Recent trials of COX-2 inhibitors have provided strong evidence that pharmacological modulation of COX-2 activity can impact on clinical outcome (Section 1.1.2).

Several reports suggest that anthocyanidins, anthocyanins and certain berry extracts can inhibit COX activity. For example, 40 µM anthocyanidins inhibited the activity of

COX-1 and COX-2 in a cell-free system by 20.4-52.2% and 30.9-74.2%, respectively. The overall order of COX inhibitory activity was cyanidin > malvidin > peonidin > pelargonidin > delphinidin (Seeram et al., 2003). In a similar study the ability of anthocyanins from a number of sources to inhibit COX activity were compared. Raspberry and sweet cherry anthocyanins at concentrations of 125 µg/ml showed the highest degree of COX-1 (45.8%) and COX-2 (47.4%) inhibition, respectively (Seeram et al., 2001).

Focusing on colorectal carcinogenesis, a study by Lala et al. found that COX-2 expression was significantly reduced in the colonic mucosa of rats treated with azoxymethane that consumed bilberry and grape anthocyanin-rich extracts compared to those that were fed a control or chokeberry diet ($p = 0.009$). No significant difference was observed in expression of the COX-1 gene (Lala et al., 2006). In a separate study application of 50 µg/ml chokeberry extract to human HT-29 colorectal carcinoma cells for 24 hours produced a 60% growth inhibition. A 35% decrease in COX-2 expression was observed within 24 hours of exposure, however, this effect was transient and increased levels of COX-2 protein and prostaglandin-E2 were observed. No other anthocyanin compounds were tested, and it may be that chokeberry extract suppresses the growth of colorectal cancer cells in a COX-independent manner (Malik et al., 2003).

Hou et al. have shown that delphinidin potently inhibits COX-2 expression and reduces COX-2 protein levels in lipopolysaccharide (LPS)-induced murine RAW264

macrophage cells. A number of transcription factors which bind to the promoter region of the COX-2 gene have been identified, including nuclear factor κ B (NF- κ B), CCAAT/enhancer-binding protein (C/EBP) and activator protein 1 (AP-1). Delphinidin suppressed LPS-induced activation of these transcription factors. Evidence suggests that LPS induces the activation of mitogen-activated protein kinases (MAPK), including c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p38 kinase which subsequently activate COX-2 transcription factors (Mestre et al., 2001). Delphinidin caused dose-dependent inhibition of LPS-induced phosphorylation of these kinases. In addition, when cells were treated with a combination of three MAPK inhibitors there was complete inhibition of COX-2 induction. In summary, delphinidin suppressed LPS-induced COX-2 expression by blocking MAPK pathways and the subsequent activation of NF- κ B, C/EBP and AP-1 (Hou et al., 2005b).

1.4.3 INDUCTION OF APOPTOSIS

Inhibition of apoptosis is a fundamental event in tumour development (Evan & Vousden, 2001). Anthocyanidins, anthocyanins and a number of berry extracts have induced apoptosis in many malignant cell lines, including human lines derived from leukaemia, the colon, breast, prostate and uterus (Chen et al., 2005; Katsube et al., 2003; Lazze et al., 2004; Reddivari et al., 2007; Seeram et al., 2006; Yi et al., 2005). *In vivo*, the topical application of delphinidin to SKH-1 hairless mice inhibited UVB-induced formation of apoptotic cells as determined by TUNEL staining (Afaq et al., 2007).

With reference to colorectal carcinogenesis, an increase in apoptosis, as determined by DNA fragmentation and a caspase-3 colorimetry assay, was observed when HT-29 cells were treated with several varieties of blueberry extract at concentrations ranging from 50-150 µg/ml (Srivastava et al., 2007). In a similar study HT-29 cells were exposed to either bilberry or cloudberry extracts, with high and low anthocyanin contents, respectively. These extracts also induced apoptosis, but pronounced DNA fragmentation was only evident when cells were exposed to 20-60 mg/ml of bilberry extract or 40-60 mg/ml of cloudberry extract (Wu et al., 2007). Exposure of human Caco-2 colorectal carcinoma cells to blueberry anthocyanins has also resulted in increased DNA fragmentation (Yi et al., 2005). In addition, exposure of Caco-2 cells to 200 µM delphinidin caused induction of apoptosis, although non-significant, as determined by analysis of cell morphology and flow cytometry (Lazze et al., 2004).

Reddivari et al. have recently shown that anthocyanins derived from potato extracts can induce apoptosis in prostate cells via caspase-dependent and caspase-independent pathways. Anthocyanins inhibited growth and induced apoptosis in both LNCaP (androgen dependent) and PC-3 (androgen independent) human prostate cells. Cleaved caspase-3, cleaved caspase-9 and cleaved poly(ADP)ribose polymerase (PARP), a substrate for caspase-3, were increased in LNCaP but not PC-3 cells treated with anthocyanins. Mitochondrial factors, such as apoptosis inducing factor (AIF) and endonuclease G (Endo G) are also proapoptotic and contribute to caspase-independent cell death after being released from mitochondria and translocating to the nucleus. Nuclear levels of AIF and Endo G were increased in both cell lines following

exposure to anthocyanins. In summary, these results indicate that caspase-dependent apoptosis occurred only in LNCaP cells, whereas caspase-independent apoptosis occurred in both LNCaP and PC-3 cells (Reddivari et al., 2007).

Two major pathways which initiate the activation of caspases are the mitochondrial and apoptotic death receptor pathways. The mitochondrial pathway is controlled by members of the Bcl-2 family, including the antiapoptotic Bcl-2 and Bcl-X_L proteins and the proapoptotic Bax, Bid, Bim, Bak and Bad proteins. Changes in the ratios of these proteins can stimulate the release of cytochrome *c* from mitochondria which in turn leads to activation of caspase-9. The apoptotic death receptor pathway is controlled by a number of cell surface membrane proteins, including Fas and the TNF receptor. Binding of a complementary protein, Fas ligand or TNF, leads to the activation of caspase-8. The mitochondrial and death receptor pathways converge at caspase-3 activation which in turn induces downstream caspases leading to apoptosis.

When human HL-60 leukaemia cells were treated with cyanidin-3-rutinoside (C3R) dose dependent increases in caspase-3 and caspase-9 activity were detected. Cytosolic cytochrome *c* was detected along with activated mitochondrial Bax and Bak. Certain species of Bim were increased, and HL-60 cells which overexpressed Bcl-2 or Bcl-X_L were significantly resistant to C3R induced apoptosis and caspase activation (Feng et al., 2007). In another study the effect of *Hibiscus* anthocyanins on HL-60 cells was investigated. Protein and mRNA levels of both Fas and Fas ligand were increased along with caspase-8 and caspase-3 (Chang et al., 2005). These data suggest that

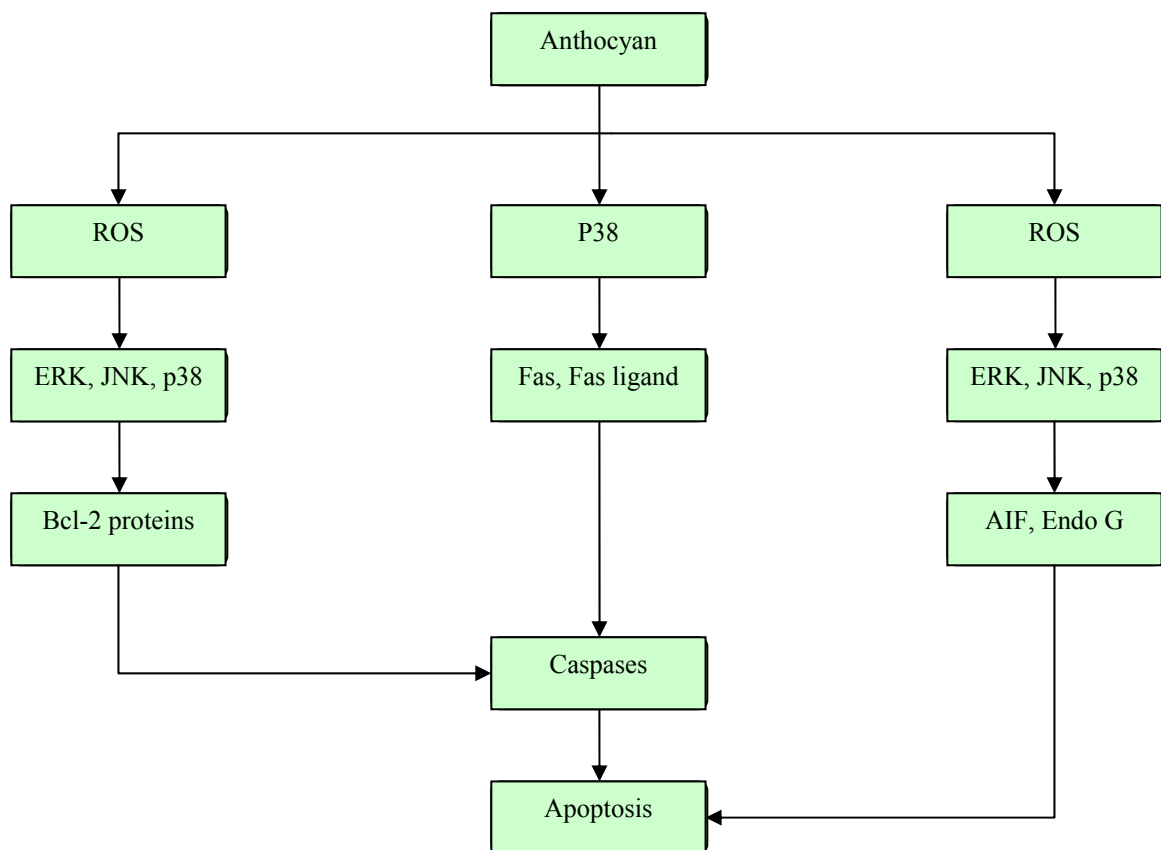
anthocyanins may exert effects on both the mitochondrial and death receptor pathways of caspase activation.

Studies have shown that anthocyanins may regulate apoptosis, in addition to COX-2 expression (1.4.2), via MAPK pathways. Treatment of HL-60 cells with C3R resulted in a rapid increase in the phosphorylation of MAPKs. Inhibitors of p38 and JNK prevented mitochondrial release of cytochrome *c*, caspase activation and apoptosis induced by C3R (Feng et al., 2007). Inhibitors of JNK and ERK blocked caspase-dependent apoptosis in LNCaP cells and caspase-independent apoptosis in LNCaP and PC-3 cells exposed to anthocyanins from potato extract (Reddivari et al., 2007). In addition, p38 inhibitor reduced the expression of Fas and Fas ligand in HL-60 cells exposed to *Hibiscus* anthocyanins (Chang et al., 2005).

Despite strong evidence that anthocyanins can function as antioxidants, a number of studies have paradoxically suggested that anthocyanin treatment may lead to the generation of reactive oxygen species (ROS) which subsequently induce apoptosis. This effect has been observed in human hepatoma (HepG₂) and leukaemia (HL-60) cells treated with anthocyanidins, delphinidin-3-sambubioside and cyanidin-3-rutinoside (Feng et al., 2007; Hou et al., 2003; Hou et al., 2005a; Yeh & Yen, 2005) but was not observed in prostate cells (Reddivari et al., 2007). Furthermore, treatment of HL-60 cells with the antioxidant N-acetyl-cysteine and the H₂O₂ scavenger catalase suppressed activation of p38, ERK and JNK (Feng et al., 2007). It may be that apoptotic pathways in certain cell lines are triggered when ROS activate MAPKs.

Mechanisms by which anthocyanins may act to induce apoptosis are illustrated in *Figure 1.4*.

Figure 1.4: Simplified mechanisms by which anthocyanins may act to induce apoptosis



ERK, extracellular signal-regulated kinase; JNK, c-Jun *N*-terminal kinase; ROS, reactive oxygen species; AIF, apoptosis inducing factor; Endo G, endonuclease G.

1.4.4 THE MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) PATHWAY

Mitogen-activated protein kinases (MAPKs), including ERK, JNK and p38, are major signalling transduction molecules that play a crucial role in modulating a variety of cellular responses, including proliferation, differentiation and apoptosis. MAPK pathways consist of three tiers of activation in which a MAPK is phosphorylated by a MAPK kinase (MAPKK) which is itself phosphorylated by a MAPKK kinase (MAPKKK).

Evidence described in *Sections 1.4.2 and 1.4.3* suggests that MAPK pathways play an important role in both the suppression of COX-2 and induction of apoptosis by anthocyanins. At first glance these data may appear contradictory: MAPKs are suppressed by anthocyanins to inhibit COX-2 production but activated to induce apoptosis. The ‘dual role’ of MAPKs is well recognised, for example ERK, JNK and p38 are capable of inducing either apoptosis or cell proliferation (Wu, 2007). In addition, for the most part, suppression of MAPK pathways has been observed when cells have been exposed to both anthocyanins and inducing agents, for example LPS, UVB radiation and 12-O-tetradecanolyphorbol-13-acetate (TPA) (Afaq et al., 2005; Ding et al., 2006; Hou et al., 2005b). Activation of MAPK pathways has been observed when cells have only been treated with anthocyanins (Chang et al., 2005; Feng et al., 2007; Reddivari et al., 2007). Specific mechanistic data on the modulation of MAPKs by anthocyanins which subsequently influence COX-2 and apoptosis is currently lacking.

Upstream of the MAPK pathway is the epidermal growth factor receptor (EGFR). Delphinidin and cyanidin have potently inhibited the tyrosine kinase activity of the EGFR isolated from a human A431 vulva carcinoma cell line. Malvidin, cyanidin-3-galactoside and malvidin-3-glucoside were inactive at concentrations up to 100 μ M (Meiers et al., 2001). Another study which also utilised A431 cells found that the ability of anthocyanidins to inhibit EGFR tyrosine kinase activity declined in the order cyanidin $\approx$ delphinidin > pelargonidin > peonidin > malvidin. Potency of EGFR inhibition may relate to the presence of hydroxyl groups and absence of methoxyl groups on the B ring (*Figure 1.2*) (Marko et al., 2004).

1.4.5 CELL CYCLE ARREST

Anthocyanins induce cell cycle arrest in both the G₀/G₁ and G₂/M phases. For example, treatment with 200 μ M of malvidin for 24 hours caused 79% of human AGS gastric adenocarcinoma cells to accumulate in the G₀/G₁ phase (Shih et al., 2005). When human HS578T breast carcinoma cells were exposed to 5 μ M of cyanidin-3-glucoside or 10 μ M of peonidin-3-glucoside for 48 hours significant increases in the percentage of cells in the G₂/M phase were observed (Chen et al., 2005).

Cell cycle progression is regulated by a number of cyclin/cyclin-dependent kinase (CDK) complexes and by cyclin-dependent kinase complex inhibitors (CDKI). The G₁ phase of the cell cycle is regulated by cyclin D/CDK 4 and cyclin D/CDK 6, transition from G₁/S is controlled by cyclin E/CDK 2 and G₂/M is regulated by cyclin

A/CDK 2 and cyclin B/CDK 1. Examples of CDKIs are p21^{WAF1} and p27^{KIP1} which bind to cyclin/CDK complexes and cause arrest in the G₁ phase of the cell cycle.

A small number of studies have investigated alterations in cyclins, CDKs and CDKIs in an attempt to explain anthocyanin induced cell cycle arrest in the G₀/G₁ and G₂/M phases. Exposure of HT-29 cells to chokeberry extract for up to 72 hours produced arrest in the G₀/G₁ and G₂/M phases of the cell cycle. This coincided with increased expression of the p21^{WAF1} and p27^{KIP1} genes and decreased expression of the cyclin A and B genes (Malik et al., 2003). When HT-29 cells were incubated with a variety of berry extracts for 24 hours a 2.2-14 fold increase in p21^{WAF1} was evident (Wu et al., 2007). G₂/M arrest observed when human HS578T breast cells were incubated with anthocyanins coincided with down-regulation of CDK-1, CDK-2, cyclin B₁ and cyclin E when treated with peonidin-3-glucoside and down-regulation of CDK-1, CDK-2, cyclin B₁ and cyclin D when treated with cyanidin-3-glucoside (Chen et al., 2005).

1.4.6 ANTIOXIDANT PROPERTIES

Numerous studies, both *in vitro* and *in vivo*, have demonstrated the antioxidant properties of anthocyanins. For example, *in vitro*, the antioxidant capacities of the glycosides of delphinidin, malvidin and petunidin were 2-2.5 times greater than that of ascorbic acid, as determined by the 'ferric reducing ability of plasma' (FRAP) assay. Similarly, utilising the 'trolox equivalent antioxidant capacity' (TEAC) and 'oxygen radical absorbing capacity' (ORAC) methods, the antioxidant capacities of anthocyanins were 3-6 times greater than that of the control antioxidant trolox. The

FRAP and TEAC values declined in the order delphinidin-3-glucoside > malvidin-3-glucoside = petunidin-3-glucoside, suggesting that free hydroxyl groups in the B ring may improve antioxidant capacity (*Figure 1.2*) (Garcia-Alonso et al., 2004).

Studies investigating the antioxidant capacity of anthocyanins have also been conducted in healthy volunteers. However, interpretation of these studies is complicated by the fact that non-extracted substances have frequently been consumed, which are rich in vitamin C and other polyphenols, in addition to anthocyanins (Duthie et al., 2006; Kay & Holub, 2002; Mazza et al., 2002; Riso et al., 2005; Weisel et al., 2006). Increased plasma antioxidant capacity, as determined by photon emission (PE) intensity in a H₂O₂-acetaldehyde system, has been observed following consumption of a single dose of black currant anthocyanins by healthy volunteers. Following anthocyanin consumption, PE intensity reached a maximal level at 2-4 hours and remained elevated in plasma samples obtained at 8 hours (Matsumoto et al., 2002).

OXIDATIVE DNA DAMAGE

In addition to general improvements in antioxidant capacity observed both *in vitro* and *in vivo*, anthocyanins have also exerted effects on markers of oxidative DNA damage. Reactive oxygen species, such as hydroxyl radicals, H₂O₂ and superoxide anions can attack both DNA bases and the deoxyribosyl backbone of DNA. One of the most extensively studied oxidative DNA adducts is 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG). It can induce G→T and A→C base substitutions,

changes which are frequently identified in oncogenes and tumour suppressor genes (Cheng et al., 1992).

In a rodent model of colorectal carcinogenesis rats received azoxymethane and dietary supplementation with 2.5%, 5% or 10% lyophilized black raspberries. Urinary 8-oxo-dG levels after 23 weeks of dietary supplementation were reduced by 73%, 81% and 83% compared to control rats, respectively ($p < 0.01$) (Harris et al., 2001). When rats were maintained on a vitamin E deficient diet for 10 weeks, to enhance susceptibility to oxidative damage, levels of hepatic 8-oxo-dG were lower in rats whose diets were subsequently supplemented with anthocyanins (1g/kg diet) than in those which continued to consume the depleted diet (Ramirez-Tortosa et al., 2001). However, when the diet of vitamin E deficient rats was supplemented with a more nutritionally relevant concentration of anthocyanins (100 mg/kg diet, cyanidin-3-glucoside), levels of 8-oxo-dG in the liver and colon were unaffected (Duthie et al., 2005). The interim results of an ongoing trial of black raspberry powder in patients with Barrett's oesophagus have recently been published. Although analysis of data from the first 10 recruits has shown no significant change in mean urinary levels of 8-oxo-dG, substantial declines were noted in five patients (Kresty et al., 2006).

Malondialdehyde (MDA), a compound generated during lipid peroxidation, and its DNA adducts, including M₁dG, have also been utilised as potential surrogate biomarkers of chemopreventive efficacy in chemoprevention studies (Sharma & Farmer, 2004). *In vitro* the effect of anthocyanins on the formation of

malondialdehyde has been investigated using oxidised calf thymus DNA. Oxidation in the presence of trolox, callistephin, kerayanin, pelargoinidin and cyanidin reduced the formation of malondialdehyde by 41.2%, 45%, 33.2%, 25.1% and 10.2%, respectively (Matsufuji & Shibamoto, 2004). A recent study conducted by Cooke et al. assessed M₁dG levels in the adenomas of Apc^{Min} mice after dietary supplementation with up to 0.3% cyanidin-3-glucoside (C3G) or a standardised bilberry extract. M₁dG levels were 78% ± 69% and 58% ± 35% of that observed in control mice, respectively. However, due to high variability between individual mice levels did not reach statistical significance (Cooke et al., 2006a).

1.4.7 INSULIN-LIKE GROWTH FACTOR SYSTEM

The insulin-like growth factor system, which includes insulin-like growth factors (IGF-1 and IGF-2), IGF receptors (IGF-1R and IGF-2R) and IGF binding proteins (IGFBP 1-6), regulates cell proliferation, apoptosis and differentiation. Epidemiological studies have suggested that an increase in IGF-1 and decrease in IGFBP-3 may be associated with the development of colorectal cancer (Ma et al., 1999; Nomura et al., 2003).

To date no studies have investigated the impact of anthocyanins on the IGF system, however, a number of other flavonoids have been shown to affect this system. Incubation of human SW837 colorectal carcinoma cells with 20 µg/ml epigallocatechin gallate (EGCG) produced a decrease in phosphorylated IGF-1R and IGF-1 and an increase in IGFBP-3 within 12 hours. Incubation with only 1.0 µg/ml

for 96 hours also produced the same changes (Shimizu et al., 2005). In addition, genistein, but not daidzein, reduced levels of IGF-1R in human HT-29 colorectal carcinoma cells (Kim et al., 2005).

1.4.8 ANGIOGENESIS AND INVASION

Tumours induce angiogenesis, a process crucial for growth and expansion, by secreting growth factors, such as vascular endothelial growth factor (VEGF). *In vitro* when human (HUVEC) umbilical vein endothelial cells were incubated with delphinidin, phosphorylation of VEGFR-2 and subsequent downstream signalling pathways (ERK-1/2) were inhibited (Lamy et al., 2006). Angiogenesis has been studied *in vivo* in rats treated with N-nitrosomethylbenzylamine (NMBA) to induce the formation of oesophageal tumours. Rats that received black raspberry powder for 19 weeks (5% /diet) showed significantly lower levels of VEGF-C expression in the oesophagus than control rats ($p < 0.005$). Microvessel density in the oesophageal epithelium, determined by immunohistochemical analysis, was also significantly depressed in rats that consumed black raspberry powder ($p < 0.0001$) (Chen et al., 2006c).

The ability of anthocyanins to inhibit tumour cell invasion has been addressed in a small number of studies. Invasion of human HT115 colorectal carcinoma cells into a basement membrane preparation was significantly reduced, in a dose-dependent manner, by incubation with raspberry extract which had initially been exposed to an *in vitro* digestion procedure (Coates et al., 2007).

Degradation of extracellular matrix by tumour cells is mediated by a number of proteolytic enzymes, including matrix metalloproteinases (MMPs) and urokinase-type plasminogen activator (u-PA). The activities of MMPs and u-PA may be inhibited by endogenous tissue inhibitor of metalloproteinases (TIMPs) and plasminogen activator inhibitor (PAI). Exposure of highly metastatic human A549 lung carcinoma cells to up to 100 μ M of cyanidin-3-glucoside or cyanidin-3-rutinoside suppressed the activity of MMP-2 and u-PA and enhanced the activity of TIMP-2 and PAI (Chen et al., 2006a). In a similar study cyanidin-3-glucoside and peonidin-3-glucoside reduced the activity of MMP-9 and u-PA in human SKHep-1 hepatocellular carcinoma cells. Cyanidin-3-glucoside inhibited the activity of TIMP-2 but PAI was unaffected by either anthocyanin (Chen et al., 2006b).

In summary, anthocyanins exert effects on a number of molecular mechanisms which are fundamental to the process of carcinogenesis. Pleiotropic activity is a characteristic shared by many phytochemicals and contributes towards their appeal as potential cancer chemopreventive agents.

CHAPTER 2

AIMS

Preclinical studies conducted with anthocyanins in the last 10 years have provided convincing evidence of chemopreventive effects both *in vitro* and *in vivo*, coupled with mechanistic data (*Sections 1.3.1, 1.4*). This evidence supports the tentative clinical development of anthocyanins as potential cancer chemopreventive agents. Although a number of pharmacokinetic studies have been undertaken in healthy volunteers, published clinical trials with a cancer chemoprevention related rationale are currently scarce (*Section 1.3.2, Table 1.1*). The overall aim of this project was to accrue clinical information in order to help adjudge whether further development of anthocyanins as potential cancer chemopreventive agents is warranted. To that end a clinical study utilising mirtocyan, a standardised bilberry extract, was designed, approved and conducted.

The study had the following three specific objectives:

1. To measure levels of anthocyanins and their metabolites in human biomatrices following a seven-day course of oral mirtocyan. Recruiting patients with operable colorectal cancer and liver metastases provided access to samples of colon/liver tissue, bile and portal blood, in addition to peripheral blood and urine. The hypothesis was tested that concentrations of anthocyanins achieved would be within an order of magnitude to those associated with pharmacological effects *in vitro* and in animal models (*Sections 1.3.1, 1.4.1*).
2. To gain detailed information on the absorption of anthocyanins in humans. Data from animal studies suggests that anthocyanins are primarily absorbed from the stomach (*Section 1.2.2*). To test whether the site for absorption is comparable in

humans, patients were consented to receive a dose of mirtocyan via a nasogastric or nasojejunal tube intraoperatively. Levels of anthocyanins were measured in serial peripheral and portal blood, bile and urine samples.

3. To gain information on the pharmacodynamics of anthocyanins. Surrogate biomarkers of chemopreventive activity were selected on the basis of existing mechanistic information (*Section 1.4*). In particular, the hypotheses were tested that consumption of mirtocyan effects levels of oxidative DNA damage in whole blood (M₁dG) and/or urine (8-oxo-dG) and tissue proliferation (Ki-67), apoptosis (cleaved caspase-3) and inflammation (COX-2). Furthermore, the notion was tested that anthocyanins affect the insulin-like growth factor system (IGF-1 and IGFBP-3) (*Section 1.4*).

CHAPTER 3
MATERIALS

3.1 SUPPLY OF MIRTOCYAN

Mirtocyan (formerly mirtoselect), a standardised bilberry extract containing 15 anthocyanins (36%), was kindly supplied by Drs P Morazzoni and A Riva (Indena SpA, Milan, Italy). The five predominant anthocyanin constituents are the 3-galactoside, 3-glucoside and 3-arabinoside of delphinidin and the 3-galactoside and 3-glucoside of cyanidin. Other anthocyanins in mirtocyan are cyanidin-3-arabinoside, petunidin-3-galactoside, petunidin-3-glucoside, petunidin-3-arabinoside, peonidin-3-galactoside, peonidin-3-glucoside, peonidin-3-arabinoside, malvidin-3-galactoside, malvidin-3-glucoside and malvidin-3-arabinoside. Furthermore mirtocyan contains other polyphenols (phenolic acids, flavonols, proanthocyanidins, ~18%), carbohydrates and aliphatic organic alcohols (~29%), fats (~0.04%), nitrogen compounds (~1%) and ash (~0.7%). Mirtocyan was encapsulated in accordance with Good Manufacturing Practice (GMP) standards by Nova Laboratories Ltd, Leicestershire, UK. Gelatine capsules were produced which each contained 470 mg of mirtocyan and had a shelf life of one year.

3.2 CHEMICALS AND INSTRUMENTS

All chemicals were purchased from Sigma-Aldrich (Dorset, UK) and from Fisher Scientific (Leicestershire, UK) unless stated otherwise. Plastic ware for cell culture was purchased from Nunc (Denmark).

Supplier	Chemical/instrument
<u>Agilent Technologies</u> West Lothian, UK	HPLC instrument (1100 series)
<u>Applied Biosystems</u> Cheshire, UK	Mass spectrometer (AP12000)
<u>Beckman Coulter</u> Buckinghamshire, UK	Particle counter and size analyzer (Z2)
<u>Becton Dickinson</u> Oxfordshire, UK	Flow cytometer (FACScan)
<u>BMG Labtech</u> Buckinghamshire, UK	Plate reader (FLUOstar OPTIMA)
<u>Dako</u> Cambridgeshire, UK	Strept AB complex/HRP duet. Mouse/rabbit (Caspase-3 and COX-2 staining)
<u>Hielscher</u> Germany	Cell sonicator (UP 50H)
<u>Jencons</u> West Sussex, UK	Glass tissue homogeniser
<u>Leica</u> Buckinghamshire, UK	Digital camera for microscopy
<u>Leitz</u> Germany	Mircroscope (Orthoplan)
<u>Merck Biosciences</u> Nottinghamshire, UK	RNase A

Micromass
Manchester, UK

Mass spectrometer
(Micromass Quattro-BioQ)

Perbio Science
Northumberland, UK

Supersignal West Dura Substrate

Qiagen
West Sussex, UK

DNA Midi kit

R&D Systems
Oxfordshire, UK

Human IGF-1 ELISA kit

Streptavidin horseradish peroxidase
(IGFBP-3 ELISA)

Substrate reagent pack
(IGFBP-3 ELISA)

Roche Diagnostics
East Sussex, UK

Proteinase K

RNase T₁

Sarstedt
Leicestershire, UK

Lithium-heparin blood bottles

Schleicher and Schuell
Surrey, UK

Gel-blotting paper

Nitrocellulose filter

Syngene
Cambridgeshire, UK

Image acquisition system
(ChemiGenius2)

Thermo Electron Corporation
Cheshire, UK

Shandon Sequenza slide rack

Shandon cover plates

Varian
Oxfordshire, UK

HPLC instrument
(230 pump, 410 auto-sampler, 325 UV-vis
detector)

Vision Biosystems
Tyne and Wear, UK

NovoLink Polymer Detection kit
(Ki-67 staining)

Waters
Hertfordshire, UK

Oasis solid phase extraction cartridges
(30 and 150 mg)

Xtera Phenyl column
(3.5 μ m, 4.6 x 250 mm)

Xtera Phenyl guard column
(3.5 μ m, 2.1 x 50 mm)

Ystral
Germany

Blade homogeniser
(X-1020)

3.3 ANTIBODIES

Supplier

Antibody

Dako
Cambridgeshire, UK

Goat anti-mouse IgG horseradish peroxidase
conjugate
(Secondary antibody in M₁dG analysis)

Monoclonal mouse anti-human COX-2 antibody

Monoclonal mouse anti-human Ki-67 antibody

New England Biolabs
Hertfordshire, UK

Polyclonal cleaved caspase-3 (Asp 175)
antibody

R&D systems
Oxfordshire, UK

Monoclonal anti-human IGFBP-3 antibody

Biotinylated anti-human IGFBP-3 antibody

Primary antibody for M₁dG analysis was supplied by Prof. Lawrence Marnett
(Vanderbilt University, USA).

3.4 BUFFERS

Buffers were prepared as listed below.

Citrate buffer (Caspase-3 and COX-2 staining)

2.1g of citric acid in 1 litre of water, pH 6.0

TBS buffer concentrate (x20) (Ki-67 staining)

60.57 g of TRIS and 87.66g NaCl in 500 ml water, pH 7.65

TE buffer concentrate (x100) (Ki-67 staining)

60.57g of TRIS and 18.6g EDTA in 500 mls of water, pH 9.0

(TRIS; Tris(hydroxymethyl)aminomethane, EDTA; Ethylenediaminetetraacetic acid)

3.5 COLORECTAL CELLS

Non-malignant (HCEC) and malignant (HT-29) colorectal cells were kindly provided by Dr Lynne Howells and Mr Chris Neal (Department of Cancer Studies and Molecular Medicine, Biocentre, University of Leicester). HCEC cells were originally obtained from the Nestle Research Centre, Lausanne, Switzerland. HT-29 cells were originally obtained from American Type Culture Collection (ATCC), Middlesex, UK.

CHAPTER 4

METHODS

4.1 ACKNOWLEDGEMENTS

I undertook all tasks relating to the clinical trial, including applications to the Ethics Committee and MHRA, recruitment of patients, consent and collection of samples. In addition, I conducted the *in vitro* experiments involving assessment of mirtocyan stability, and I studied the effects of mirtocyan on cell growth and the cell cycle. I performed anthocyanin extraction from all biological samples and subsequent HPLC analysis. I also performed all the immunohistochemistry described in this thesis myself, with assistance in method optimization from Richard Edwards (MRC, Toxicology Unit Leicester) and Angie Gillies (Department of Cancer Studies and Molecular Medicine, University of Leicester). Dr Kevin West kindly provided advice on evaluating histological sections. I conducted all statistical analysis of results.

Mass spectrometry and analysis was kindly conducted by Dr Hong Cai (Department of Cancer Studies and Molecular Medicine, University of Leicester). The following analyses of blood/urine samples were conducted by colleagues in the department: IGF-1/IGFBP-3 (ELISA) by Dr Debbie Marsden, M₁dG (immunoslot blot) by Ankur Karmokar and Manijeh Maleki-Dizaji, 8-oxo-dG (HPLC/MS/MS) by Dr Frieda Teichert.

4.2 CLINICAL METHODS

4.2.1 OVERVIEW OF THE CLINICAL TRIAL

A phase I trial of oral mirtocyan in patients with resectable colorectal cancer and colorectal liver metastases was undertaken. Consent was sought to obtain blood, urine and tissue samples during routine colonoscopy/staging laparoscopy. Mirtocyan was then consumed for 7 days prior to colon/liver resection and further blood, urine and tissue samples were collected. Concentrations of anthocyanins were measured in biomatrices obtained before and after the consumption of mirtocyan and pharmacodynamic effects of the intervention were evaluated. Clinical trial approval was obtained from the Leicestershire, Northamptonshire and Rutland Research Ethics Committee (REC reference numbers: 06/Q2502/75 and 06/Q2502/76) and the Medicines and Healthcare Products Regulatory Agency (MHRA) (EudraCT numbers: 2006-004226-82 and 2006-004245-42).

4.2.2 MIRTOCYAN DOSES

All patients were randomly assigned to receive 1.4, 2.8 or 5.6 g of mirtocyan daily, which were consumed in three divided doses, for 7 days prior to colon/liver resection (*Table 4.1*). These doses were derived on the basis of comparison with an Apc^{Min} mice study, in which consumption of mirtocyan reduced adenoma number by 30% (Cooke et al., 2006a) (~ 450 mg/kg mouse $\approx$ 2.6 g per 80 kg human, extrapolated by dose surface area comparison) (Freireich et al., 1966).

Table 4.1: Doses of oral mirtocyan consumed in the study

Number of capsules consumed daily	Total quantity of mirtocyan consumed daily (mg)	Total quantity of anthocyanins consumed daily (mg)
3	1410	508
6	2820	1015
12	5640	2030

4.2.3 PATIENT RECRUITMENT

PATIENTS WITH COLORECTAL CANCER

Patients were recruited from the Leicester Royal Infirmary and Leicester General Hospital. When the trial initially commenced patients were approached prior to colonoscopy and consent sought to obtain additional biopsies for research, if a malignancy was detected. Patient recruitment in this way was far more difficult than initially envisaged, and therefore substantial amendment applications were made to the MHRA and Ethics Committee in order to alter the study design. Approval was granted for patients to be approached in outpatient clinics, following a diagnosis of colorectal cancer, and consent sought to utilise archived biopsy material which had been obtained during their colonoscopy. A relative disadvantage of the amended method of recruitment was that normal tissue could not be procured prior to the consumption of mirtocyan.

PATIENTS WITH COLORECTAL LIVER METASTASES

It is routine practice at Leicester General Hospital for patients with colorectal liver metastases to undergo a staging laparoscopy prior to liver resection, the aim of which is to exclude disseminated intra/extrahepatic disease below the resolution of computer tomography (CT). Consent was sought to obtain biopsies of normal and malignant liver tissue during laparoscopy.

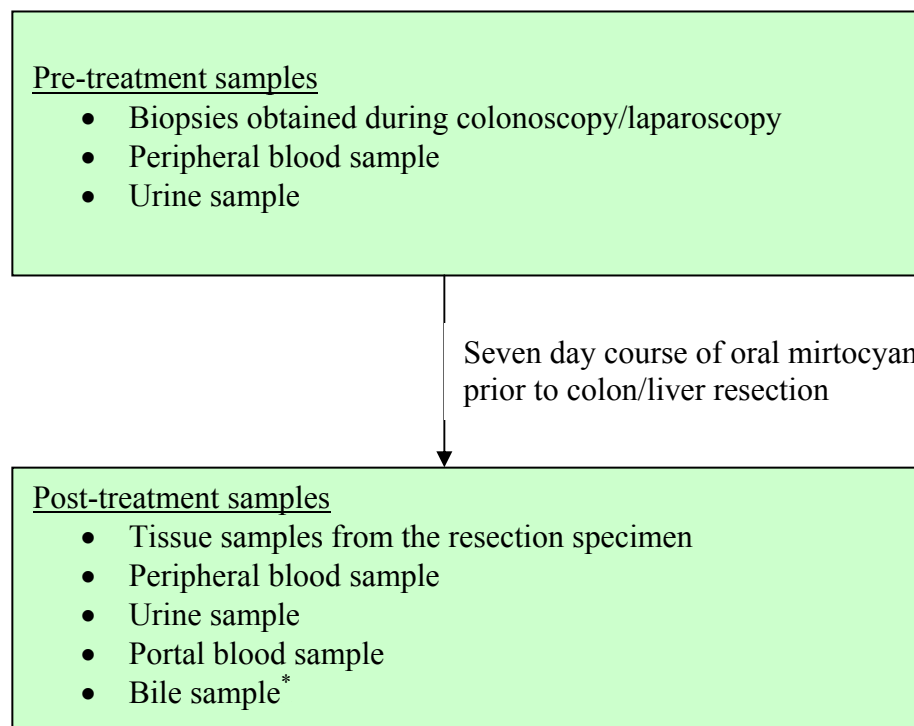
INCLUSION/EXCLUSION CRITERIA

Patients were considered suitable for the study if they were over 18 years of age, able to provide written informed consent and had disease which was considered amenable to surgical resection. Patients were excluded if they were unfit for general anaesthesia, had gastrointestinal disease which may have impaired absorption or were unable/unwilling to comply with the study protocol.

4.2.4 OBTAINING SAMPLES

Patients consented to enable biopsy material taken during colonoscopy/laparoscopy to be utilised in this trial. At enrolment urine and peripheral blood (25 ml) samples were also provided. The final dose of a seven day course of mirtocyan was consumed on the morning of the patients' colon/liver resection (06:00). A second peripheral blood sample (25 ml) was obtained an hour after consumption of the penultimate dose of mirtocyan. Urine was collected between consumption of the penultimate and final doses. When patients underwent open colon/liver resection a sample of portal blood (5-10 ml) was obtained intraoperatively. An intra-operative bile sample was also

obtained from the hepatic cohort of patients (5 ml). Samples of normal and tumour tissue were obtained from resection specimens, in accordance with pathology guidelines. In the case of colorectal specimens normal tissue was obtained from sites 5 and 10 cm proximal and distal to the tumour, whenever possible. Up to 1g of normal and malignant tissue were harvested from resection specimens. Individual tissue samples were divided, with half being flash frozen in liquid nitrogen and half being placed in formalin and later embedded in paraffin wax.



* Hepatic cohort only

Figure 4.1: Summary of samples obtained from patients during the trial

Blood samples were collected in lithium-heparin tubes. Approximately 15 ml of peripheral blood samples and the entire volume of portal blood samples were centrifuged to generate plasma (3300g, 10 min, 4°C). The remainder of peripheral samples were stored as whole blood. All blood, urine and bile samples were collected on ice. These samples, in addition to flash frozen tissue, were stored at -80°C.

4.2.5 NASOGASTRIC AND NASOJEJUNAL ADMINISTRATION OF MIRTOCYAN

Two patients consented to receive a dose of mirtocyan via a nasogastric or nasojejunal tube intraoperatively, during a liver resection. Patients received 1880 mg of mirtocyan, the same quantity as that consumed by individuals in the high dose group, three times daily. The mirtocyan was administered in approximately 150 ml of water. Prior to administration the position of the nasogastric/nasojejunal tube was confirmed by the operating surgeon.

Samples of peripheral blood, portal blood, bile and urine were obtained prior to the administration of mirtocyan. Following administration peripheral blood, portal blood and bile samples were obtained at 15 min intervals for 2 hr, whenever possible. Urine samples were obtained at 2 hr intervals for 12 hr. All samples were collected on ice prior to storage at -80°. Blood samples were collected in lithium heparin bottles and centrifuged (3300g, 10 min, 4°C) to generate plasma for storage.

4.2.6 STANDARD TRIAL PROCEDURE

In accordance with ethical guidelines detailed case report forms were completed for every trial participant. Information collected included demographic data, past medical history, regular medications, smoking status, examination findings, results of routine pre-operative blood tests and the exact times that samples were collected. Adverse events in trial participants were reported to the trial sponsor (University Hospitals of Leicester NHS Trust).

4.3 LABORATORY METHODS

4.3.1 EXTRACTION OF ANTHOCYANINS FROM BIOLOGICAL SAMPLES

Anthocyanins were extracted from urine, plasma, colorectal tissue and bile by a solid phase method. A liquid-liquid method was used for liver tissue. Anthocyanins were quantified on the basis of standard curves which were run contiguously. These were established for cyanidin-3-glucoside (C3G) in the appropriate biophases. Volunteer urine and plasma were utilized along with surplus tissue and bile from a previous project conducted within the Department of Cancer Studies and Molecular Medicine, University of Leicester. Quantification was performed assuming that individual anthocyanins in the mirtocyan mixture possess absorption coefficients similar to that of C3G. All samples were analyzed within 2 months of collection. Preliminary experiments, conducted by Dr Hong Cai (Department of Cancer Studies and Molecular Medicine, University of Leicester), showed that absorption coefficients for mirtocyan anthocyanins differed from that of C3G by factors of 0.7 to 1.4, so were

roughly similar to that of C3G. It was also demonstrated that anthocyanins were stable in biological samples stored at -80°C for up to 2 months.

URINE AND PLASMA

Anthocyanin extraction from urine and plasma was performed in accordance with a previously validated method (Cooke et al., 2006b). Oasis solid phase extraction cartridges (30 mg) were initially conditioned with 1 ml of acetone:formic acid (9:1) followed by 1 ml of water:formic acid (9:1). Aliquots of urine (1 ml) and plasma (2 ml) were centrifuged (16000 g, 10 min, 4°C) and then loaded onto the extraction cartridges. Anthocyanins were eluted sequentially with 0.2 and then 0.1 ml of acetone:formic acid (9:1). At all stages of the solid phase extraction procedure, a vacuum was used to draw solvents through the cartridge. Pooled eluates were evaporated to dryness under a stream of nitrogen. Residues were reconstituted in 75 µl of water:formic acid (9:1), centrifuged (16000 g, 25 min, 4°C) and the supernatant transferred to HPLC vials. The volume injected for HPLC analysis was 50 µl.

BILE

Analysis of bile was undertaken using a method based on that described above. Oasis solid phase extraction cartridges (150 mg) were initially conditioned with 5 ml of acetone:formic acid (9:1) followed by 5 ml of water:formic acid (9:1). Bile samples were centrifuged (16000 g, 10 min, 4°C) and aliquots (100 µl) were diluted in ultrapure water (400 µl) then loaded onto the extraction cartridges. Anthocyanins were eluted with 3 ml of acetone:formic acid (9:1). At all stages, a vacuum was used

to draw solvents through the cartridge. Pooled eluates were evaporated to dryness under a stream of nitrogen. Residues were reconstituted in 75 µl of water:formic acid (9:1), centrifuged (16000 g, 25 min, 4°C) and the supernatant transferred to HPLC vials. The volume injected for HPLC analysis was 50 µl.

COLORECTAL TISSUE

The method of extraction from colorectal tissue was based on that previously published in an Apc^{Min} mice study (Cooke et al., 2006a). Samples of colorectal tumour tissue and normal mucosa (80–165 mg) were mixed with PBS (w/v 1:10), and centrifuged (16000 g, 10 min, 4 °C) to remove any surface anthocyanin contamination. The supernatant was discarded, fresh PBS added (m/v 1:10), and the sample homogenized using a blade homogenizer at full speed at full speed for 1 min. A cell sonicator was then used for a further min and PBS was added to give a total volume of 2 ml. The samples were mixed, centrifuged (16000 g, 10 min, 4 °C) and the supernatant loaded onto Oasis cartridges (30 mg) which had initially been conditioned with 1 ml of acetone:formic acid (9:1), followed by 1 ml of water:formic acid (9:1). 2 ml of PBS was added to the remaining pellet of colorectal tissue and the extraction procedure was repeated. Anthocyanins were eluted sequentially with 0.2 and then 0.1 ml of acetone:formic acid (9:1). Pooled eluates were evaporated to dryness under a stream of nitrogen. Residues were reconstituted in 75 µl of water:formic acid (9:1), centrifuged (16000 g, 25 min, 4°C) and the supernatant transferred to HPLC vials. The volume injected for HPLC analysis was 50 µl.

Analysis of colorectal tissue samples spiked with 5 µg of C3G revealed an extraction efficiency of 59.6%. Lower limits of detection ($S/N > 3$) and quantification ($S/N > 7$) were 5.4 and 6.0 ng per 100 mg of tissue, respectively. Values for intraday variation when colorectal tissue samples were spiked with 10 ng, 1 µg and 5 µg of C3G were 7.7, 2.9 and 4.6 %, respectively. Equivalent values for interday variation were 9.0, 2.5 and 4.3 %. These experiments were performed in triplicate.

LIVER TISSUE

Samples of liver tissue from resection specimens (120-180 mg) and laparoscopic biopsies (20-125 mg) were homogenized by hand, using a glass homogenizer, in 2% formic acid in PBS (w/v 1:2.5) on ice. 5 ml of cold (-20°C) acetone:formic acid (9:1) was added to the samples, which were then mixed thoroughly before being transferred to a freezer at -20°C for 10 min. The samples were then mixed again and centrifuged (16000 g, 30 min, 4°C). The supernatant was dried down under a stream of nitrogen, reconstituted in 150 µl water:formic acid (9:1) and centrifuged (16000 g, 25 min, 4°C). The volume injected for HPLC analysis was 50 µl.

Analysis of liver samples spiked with 5 µg of C3G revealed an extraction efficiency of 35%. Preliminary experiments revealed lower extraction efficiencies with solid phase methods and liquid-liquid techniques involving various volumes of other solvents (including non-acidified acetone, acetone:methanol (50:50), non-acidified and acidified (2% formic acid) methanol and acetonitrile).

4.3.2 SEPARATION OF ANTHOCYANINS BY HPLC

The method for separation of anthocyanins by HPLC was previously developed by Drs Darren Cooke and Tim Marczyklo (Department of Cancer Studies and Molecular Medicine, University of Leicester). A Varian HPLC system was used with a Xterra Phenyl column and matching guard column. Biomatrices were analyzed for anthocyanin content at 520 nm and 40 °C. The gradient elution system ran at 1 ml/min and comprised two solvents: A, water:formic acid (9:1), pH≈1.6, and B, acetonitrile. The gradient employed was as follows: 99 to 97% A for 12 min, 97 to 90% A for 7.5 min, unchanged for 6.5 min, 90% to 60% A for 2 min and unchanged for 2 min.

4.3.3 IDENTIFICATION OF ANTHOCYANINS AND METABOLITES BY LC/MS/MS

Mass spectrometric analysis was undertaken by Dr Hong Cai, in accordance with a previously published method (Cooke et al., 2006b). An Agilent Technologies HPLC system was used to separate anthocyanins prior to injection into the mass spectrometer (Applied Biosystems). A Xterra Phenyl column and guard column were used for HPLC separation and the gradient elution system involved the same solvents as described above (*Section 4.3.2*). The gradient was as follows: 99 to 97% A for 5 min, 97 to 90% A for 3 min, unchanged for 9 min, 90 to 70% A for 1 min and unchanged for 2 min. When a gradient of 90% A was achieved the flow rate was reduced from 1 ml/min to 0.31 ml/min to allow direct injection into the mass spectrometer, eliminating the need for eluate splitting. Mass spectrometric analyses were performed in positive ion mode, and anthocyanins identified using multiple

reaction monitoring (MRM). Samples were analyzed for 28 mass transitions, spanning anthocyanins present in mirtocyan and possible metabolites generated by methylation, glucuronidation and sulphation.

4.3.4 CELL CULTURE

MAINTENANCE OF CELL LINES

HT-29 and HCEC cells were grown in Dulbecco's modified eagle's media (4500 mg glucose/L), supplemented with 10% foetal calf serum (FCS). In the case of HCEC cells, plastic ware required pre-coating prior to plating. The pre-coating solution comprised 50 ml of media, 0.5 ml of Vitrogen 100 (Collagen Corp.), 125 µl of fibronectin and 65 µl of bovine serum albumin. Cells were maintained in a warm (37°C) humidified atmosphere supplemented with 5% CO₂.

ROUTINE CELL PASSAGE AND TREATMENT

Cells were grown to approximately 70% confluency. Medium was aspirated and cells were washed twice in warm (37°C) sterile PBS. Cells were harvested using trypsin EDTA in PBS (1% for HCEC cells and 5% for HT-29 cells). After a 5 min incubation at 37°C the cells were gently agitated and detachment confirmed by microscopy. Warm sterile media was added to the flasks and the cell suspension centrifuged (15000 RPM, 3 min). The supernatant was discarded, the cells suspended in fresh media and subsequently seeded into new flasks. The passage number of cells used in experiments did not exceed 20.

In all experiments mirtocyan was directly added to media, containing 10% FCS. The solution was filtered prior to incubation with cells.

STABILITY OF MIRTOCYAN UNDER CELL CULTURE CONDITIONS

10 ml of media (10% FCS) containing 100 µg/ml mirtocyan were added to petri dishes and transferred to an incubator. At 0, 3, 6, 12 and 24 hr 2 ml of media was removed from a petri dish, which was subsequently discarded. Media was stored at -80°C (< 1 week) and subsequently analyzed by HPLC (*Section 4.3.2*). 0.5 ml of media from each time point was extracted, using the method detailed for urine/plasma in *Section 4.3.1*. HPLC traces from 0 hr were compared to those obtained from subsequent time points. The experiment was repeated in the presence of HT-29 cells (1 million cells in each petri dish). These experiments were performed in triplicate.

EFFECTS OF MIRTOCYAN ON CELL GROWTH

HT-29 and HCEC cells were seeded in 24-well plates, at a density of 10,000 cells per well. After an incubation period of 24 hr, media was discarded and cells treated with concentrations of mirtocyan ranging from 0 to 200 µg/ml. In separate experiments cells were exposed to single and daily doses of mirtocyan. In the case of daily dosing, media was discarded from each well, and replaced daily. Cells were harvested by trypsinisation and counted at time points ranging from 96 to 192 hr following plating, using a Coulter Counter. A total of 8 wells were analyzed for each concentration and time point for both cell lines. IC₅₀ values were calculated from a graph of cell number, as a percentage of control, versus mirtocyan concentration, after 168 hr of incubation.

CELL CYCLE ANALYSIS

HT-29 and HCEC cells were seeded in pertri dishes at densities of 1×10^6 and 5×10^5 per dish, for cell cycle analysis at 24 and 48 hr, respectively. Cells were allowed to adhere overnight prior to dosing with fresh media containing mirtocyan at concentrations ranging from 0 to 200 $\mu\text{g/ml}$. After incubation with mirtocyan for 24 or 48 hr, media was discarded, adherent cells harvested by trypsinisation, washed twice in PBS and resuspended in 200 μl of PBS. 2 ml of ice cold 70% ethanol in PBS was then added to each sample whilst being mixed vigorously. At this stage cells were stored at 4°C for up to a week. Just prior to analysis, cells were centrifuged (600 g, 10 min) and resuspended in 800 μl PBS. 100 μl of RNAase (1 mg/ml) and propidium iodide (50 $\mu\text{g/ml}$) were added to the cell suspensions which were then incubated overnight at 4°C . They were analyzed by flow cytometry using Modfit LT software. Cell cycle analysis was undertaken in triplicate, on 2 separate occasions.

4.3.5 INSULIN-LIKE GROWTH FACTOR SYSTEM

IGF-1 AND IGFBP-3

Analysis of IGF-1 and IGFBP-3 were undertaken using enzyme-linked immunosorbent (ELISA) assays. The assays were performed by Dr Debbie Marsden (Department of Cancer Studies and Molecular Medicine, University of Leicester), according to the manufacturer's instructions (R&D Systems). 20 and 100 μl of plasma were used in the analysis of IGF-1 and IGFBP-3, respectively.

The principles of the assays are as follows. ELISA plates are pre-coated with monoclonal antibodies specific for IGF-1 and IGFBP-3. Standards and plasma samples are pipetted into wells on the plates and any IGF-1 or IGFBP-3 binds to the immobilized antibody. After a wash step, an enzyme-linked polyclonal antibody specific for IGF-1 or IGFBP-3 is added to the wells. Following another wash step, a substrate solution is added and colour develops in proportion to the amount of IGF-1 or IGFBP-3 which bound in the initial step. After 30 min a solution is added to stop colour development and the optical density of each well is determined at 450 nm using a plate reader. Standards are assayed allowing generation of a calibration curve, from which IGF-1 and IGFBP-3 concentrations are extrapolated.

IGF-1:IGFBP-3

The molar ratio of IGF-1 to IGFBP-3, was calculated according to previous publications (Lee et al., 2005a; Max et al., 2008), as follows:

$$\text{IGF-1:IGFBP-3} = (\text{IGF-1 conc. [ng/ml]} \times 0.130) / (\text{IGFBP-3 conc. [ng/ml]} \times 0.036)$$

This calculation is based on 1 ng/ml IGF-1 = 0.130 nmol IGF-1 and 1 ng/ml IGFBP-3 = 0.036 nmol IGFBP-3 (Max et al., 2008).

4.3.6 MALONDIALDEHYDE DNA ADDUCT

DNA EXTRACTION FROM WHOLE BLOOD

DNA was extracted from whole blood samples using a Qiagen Midi kit. This was performed by Manijeh Maleki-Dizaji (Department of Cancer Studies and Molecular Medicine, University of Leicester). The method of DNA extraction from whole blood for analysis of M₁dG had previously been optimized by Dr Raj Singh (Department of Cancer Studies and Molecular Medicine, University of Leicester).

DNA extraction was from 3 ml of whole blood. An equal volume of ice-cold C1 buffer, spiked with 5 mM deferoxamine, was initially added to the blood samples, along with 9 ml of ice-cold HPLC grade water. This was mixed by inversion until the suspension became translucent and then incubated on ice for 10 min. The role of C1 buffer was to lyse cells but stabilize and preserve nuclei. Deferoxamine was added as an iron-chelating agent, it bound Fe²⁺ ions preventing the formation of free radicals. The lysed blood samples were centrifuged (3000 RPM, 15 min, 4°C) and the supernatant discarded. 1 ml of ice-cold C1 buffer, spiked with 5 mM deferoxamine, was then added to the pellet along with 3 ml of ice-cold HPLC water. The pellet was resuspended, centrifuged (3000 RPM, 15 min, 4°C) and the supernatant discarded to leave a pellet of nuclei. The pellet was resuspended in 1 ml of G2 buffer, spiked with 5 mM deferoxamine, and transferred to a 15 ml falcon tube. Samples were mixed for 10 seconds and mixed on a rotating wheel for 5 min. 8.5 ml of G2 buffer, spiked with 5mM deferoxamine, was then added, along with 100 µl of proteinase K (25 mg/ml of water). The samples were incubated at 37°C for 4 hr and mixed every 30 min. They

were then incubated overnight on a rotating wheel at 4°C. G2 buffer and proteinase K in combination stripped DNA of all bound proteins.

After an overnight incubation, 25 µl of RNase (10 µg/µl of water, heat activated for 10 min at 80°C) and 10 µl of RNase T₁ (5U/µl of PBS) were added. Samples were then incubated at 37°C for 60 min and mixed midway through this incubation. The Midi column was equilibrated with 4 ml of QBT buffer, the sample mixed and loaded onto the column. It was washed twice with 7.5 ml of QC buffer and DNA eluted with 5 ml of QF buffer kept at 50°C (and modified to a pH of 7.4 and NaCl concentration of 1.6 M). DNA was precipitated by adding 3.5 ml of cold isopropanol. This was then centrifuged (4000 RPM, 20 min, 4°C) and the supernatant discarded. The pellet was washed sequentially with 1 ml of cold 70% ethanol and absolute ethanol which were allowed to evaporate. The pellet was resuspended in 0.5 ml of HPLC grade water and stored at -20°C pending M₁dG analysis.

IMMUNOSLOT BLOT FOR M₁dG

M₁dG levels in DNA extracted from whole blood samples were analyzed by immunoslot blot. The assay was performed by Ankur Karmokar (Department of Cancer Studies and Molecular Medicine, University of Leicester), as previously described (Leuratti et al., 1998) (*Figure 4.2*). Each blot was performed using 1 µg of DNA from each sample/standard, in triplicate. Nine standards which contained different quantities of malondialdehyde (MDA) DNA provided a calibration curve for quantification of adduct levels. Discrepancies in the amount of DNA in each slot were

corrected for by staining the nitrocellulose filter with propidium iodide and performing UV light densitometry. The limits of detection and quantification of this method were 2.5 and 20 adducts per 10^8 nucleotides, respectively.

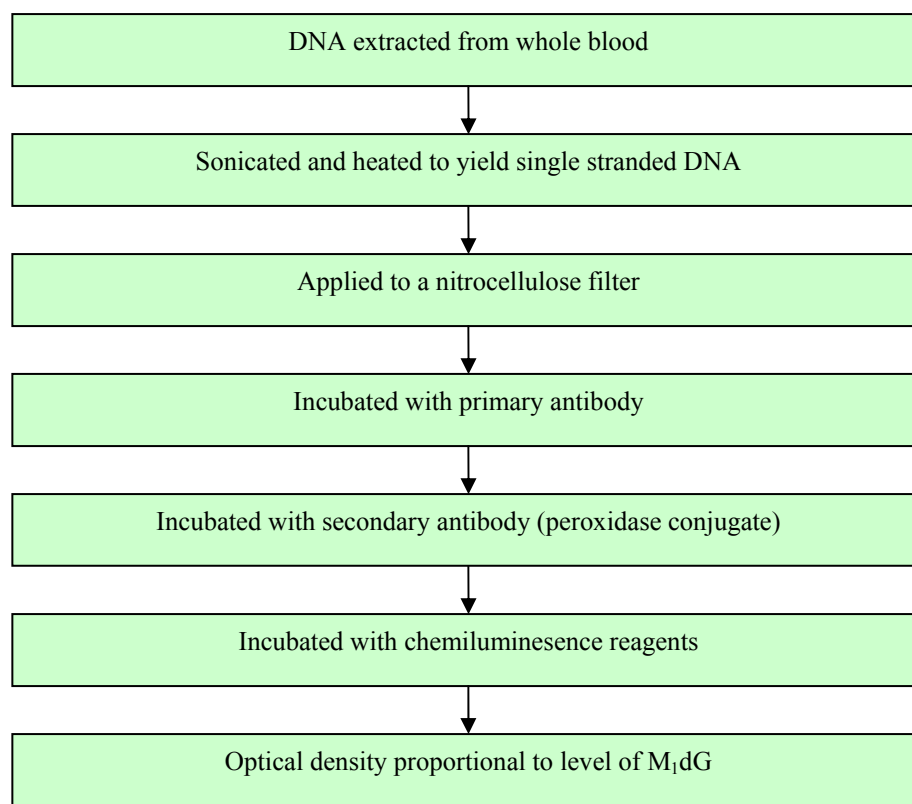


Figure 4.2: An outline of the method for M₁dG analysis

4.3.7 URINARY 8-oxo-dG

Levels of 8-oxo-dG in urine samples were measured by Frieda Teichert (Department of Cancer Studies and Molecular Medicine, University of Leicester), in accordance with a previously published method (Singh et al., 2003). In brief, an internal standard $^{15}\text{N}_5$ -8-oxo-dG (8-oxo-7,8-dihydro-2'-deoxyguanosine) was added to urine samples

prior to solid phase extraction. The eluate was analyzed by LC/MS/MS (Micromass), and levels of 8-oxo-dG were calculated by comparing the areas of peaks produced by the internal standard, with those produced by the adduct.

Levels of 8-oxo-dG were standardized to urinary creatinine. Levels of creatinine were measured in urine samples by the Department of Biochemistry, Leicester Royal Infirmary.

4.3.8 IMMUNOHISTOCHEMISTRY

Sections of paraffin embedded tissue were cut and mounted on slides by Angie Gillies (Department of Cancer Studies and Molecular Medicine, University of Leicester). Sections of colorectal tissue (cleaved caspase-3 and Ki-67) and breast tissue (COX-2) served as positive controls in these experiments. Negative controls were sections not exposed to the primary antibody. Assistance in optimizing primary antibodies was provided by Richard Edwards (MRC, Toxicology Unit, Leicester) (caspase-3 and COX-2) and Angie Gillies (Ki-67).

CLEAVED CASPASE-3 AND COX-2

Tissue sections were de-waxed by immersion in xylene, to allow antibody access (3x, 5 min). They were then immersed in IMS (3x, 5 min), followed by 70% IMS (5 min), running tap water (5 min) and distilled water (3 min). Slides were then transferred to a pressure cooker which contained citrate buffer and microwaved at 700 W in order to retrieve antigen. The microwave times were 20 min prior to caspase-3 staining and 25

min prior to COX-2 staining. Following microwaving the slides were cooled in two changes of distilled water and immersed in hydrogen peroxide for 20 min, to block endogenous peroxidase activity (33 ml hydrogen peroxide and 300 ml of distilled water). Sections were washed in tap water for 5 min and transferred into distilled water then PBS. The slides were placed into Shandon cover plates and then into a Shandon Sequenza rack. The reservoir was filled with PBS and inspected to ensure there was no leakage between the slide and coverplate. 100 µl of optimally diluted primary antibody was then incubated with appropriate sections for 3 hr at room temperature. Dilution factors were 1:200 and 1:25 in PBS, for cleaved caspase-3 and COX-2, respectively.

Following incubation with the primary antibody, sections were washed in PBS (3x, 3 min). To visualize the primary antibody a commercial kit was used (Dako Duet kit) and applied according the manufacturer's instructions. Slides were then washed in PBS (3x, 3 min) and 180 µl of freshly prepared 3,3'-diaminobenzidine solution (DAB) was applied. DAB solution was prepared as follows: 4.5 ml PBS, 0.5 ml of stock DAB (5mg/ml in PBS) and 50 µl of distilled water containing 3% hydrogen peroxide. Slides were exposed to the DAB solution for 5 min and then washed in PBS and running tap water. They were counter stained with haematoxylin and then dehydrated by immersion in 70% IMS (5 min), IMS (3x, 5 min) and finally transferred to xylene (3x, 5 min). Slides were mounted using dibutyl phthalate xylene (DPX).

Ki-67

Tissue sections were de-waxed by immersion in xylene, to allow antibody access (2x, 3 min). They were then immersed in 99% IMS (2x, 3 min), followed by 95% IMS (2x, 3 min) and running tap water. The slides were transferred to a plastic box which contained 500 ml of TE buffer and microwaved at 800 W for 18.45 min, in order to retrieve antigen. Following microwaving slides were allowed to cool at room temperature, whilst still immersed in TE buffer, for 50 min. The slides were then rinsed in TBS buffer. A commercial kit was utilized for the remainder of the procedure (NovoLink Polymer Detection kit), in accordance with the manufacturer's instructions. Peroxidase block was applied to the slides for 5 min, and they were then washed in TBS (2x, 5 min). Slides were incubated with protein block for 5 min and then washed again in TBS (2x, 5 min). Appropriate sections were incubated with 100 µl of Ki-67 antibody (1:100 in blocking solution) on a slide tray (covered) at room temperature for 3 hr. Blocking solution was 3% bovine serum albumin and 0.1% Triton-X-100 in TBS.

Following incubation with the Ki-67 antibody slides were washed in TBS (2x, 5 min) and incubated with post primary block for 30 min. After another wash in TBS (2x, 5 min) they were incubated with NovoLink Polymer for 30 min and washed again (TBS, 2x, 5 min). 3,3'-diaminobenzidine solution (DAB) solution was applied to the slides for 5 min and they were then washed in tap water. Slides were counter stained with haematoxylin and dehydrated by immersion in 95% IMS (2x, 3 min), 99 % IMS

(2x, 3 min) and finally transferred to xylene (2x, 3 min). They were mounted using dibutyl phthalate xylene (DPX).

EVALUATION OF SLIDES

Methods for the evaluation of immunohistochemical stains were developed with Dr Kevin West (Consultant Pathologist, Leicester Royal Infirmary). In the case of caspase-3 and Ki-67, the total number of epithelial cells and number of positively staining epithelial cells, were counted in six, representative, adjacent high power fields (x 400 magnification). In the case of COX-2, slides were scored in accordance with a previous publication in which colorectal biopsies were compared to samples obtained from resection specimens (Watwe et al., 2005). Slides were graded as follows – (negative), +/- (weakly positive), + (positive), ++ (strongly positive). A number of other publications describe methods for the evaluation of COX-2 in colorectal tissue samples, based on both the intensity and extent of staining (Masunaga et al., 2000; Soumaoro et al., 2004). Considering the discrepancy in volume between biopsies and samples obtained from resection specimens, the extent of staining was not evaluated in this study. To ensure accuracy, all results were reviewed with Dr West.

4.3.9 STATISTICAL ANALYSIS

Statistical analyses were undertaken using SPSS for Windows (Version 13.0). The tests utilized were the Student's t test, Paired student's t test, Wilcoxon rank test and

ANOVA, as shown in the results chapters. A p-value <0.05 was considered statistically significant.

CHAPTER 5

RESULTS

5.1 INTRODUCTION

Human and animal studies to date have shown that anthocyanins are safe and well tolerated. When the diet of rats was supplemented with up to 5% purple corn colour for 90 days no mortalities or systemic toxicity occurred. In rats staining of fur and black faeces were evident after consumption of 1.5 and 5% purple corn colour in the diet. In addition, brown urine and black material in the gastrointestinal tract were noted in rats who consumed the high dose colorant. These features were considered secondary to high anthocyanin content (Nabae et al., 2008). Stoner et al. recently conducted a study in which safety and tolerability of consumption of 45 g of lyophilized black raspberries were assessed in eleven healthy volunteers. A total of five minor adverse events were reported by four volunteers. Those probably related to berry consumption were constipation and dark stool (Stoner et al., 2005). On the background of these published studies 25 patients, 15 with colorectal cancer and 10 with colorectal liver metastases were recruited into the clinical trial described here. Each patient consumed a seven day course of mirtocyan prior to colon/liver resection. To assess pharmacokinetics, particularly absorption, in further detail, two additional patients with colorectal liver metastases received a dose of mirtocyan intraoperatively, during a liver resection, via either a nasogastric or nasojejunal tube.

In this chapter the demographic data of patients with colorectal cancer and colorectal liver metastases who consented to participate in the clinical trial of mirtocyan are described. Furthermore adverse events encountered during the trial are presented and discussed.

5.2 PATIENT DEMOGRAPHICS

5.2.1 PATIENTS WITH PRIMARY COLORECTAL TUMOURS

Fifteen patients (10 males and 5 females) with histologically confirmed colorectal adenocarcinomas, which were amenable to surgical resection, were recruited into the trial (*Table 5.1*). The median age of patients recruited was 64 years (range of 47 to 90 years). The results of all blood tests obtained at recruitment were within normal limits, including full blood count, urea and electrolytes and liver function tests. Median ASA (American Society of Anesthesiologists) grade was 2 (9 patients) and grades of 1 and 3, were each assigned to 3 patients. Patients' drug histories included aspirin (2 patients), antihypertensives (7 patients) and simple analgesics (2 patients). None of the patients recruited received neoadjuvant radiotherapy or chemotherapy.

Of the 15 patients recruited, one operative cancellation occurred, for reasons unrelated to the consumption of mirtocyan, meaning that colorectal tissue from 14 patients was available for analysis. Two of the 14 colorectal resection specimens contained two adenocarcinomas, detected during routine histopathological assessment. The remainder of the resection specimens contained one adenocarcinoma. Considering a total of 16 adenocarcinomas, 9 were Dukes B tumours and 7 were Dukes C1 tumours. One tumour was poorly differentiated and the remainder were moderately well differentiated. Most tumours were located in the caecum/ascending colon (7) and sigmoid colon (6). Other tumours were located in the transverse colon (1), descending colon (1) and rectum (1).

Table 5.1: Demographic data from 15 patients with colorectal cancer who received oral mirtocyan

Characteristic	Patients with colorectal cancer
Age, years	
Median	64
Range	47-90
Sex	
Female	5
Male	10
Body mass index, kg/m ²	
Median	29
Range	16.7-38.6
ASA grade	
1	3
2	9
3	3
4	0
5	0
Drug history	
Aspirin	2
Antihypertensives	7
Antianginals	0
Simple analgesics ¶	2
Neoadjuvant therapy	
Radiotherapy	0
Chemotherapy	0
Tumour location*	
Caecum/ascending colon	7
Transverse colon	1
Descending colon	1
Sigmoid colon	6
Rectum	1
Tumour differentiation*	
Well	0
Moderate	15
Poor	1
Dukes stage of tumours*	
A	0
B	9
C1	7
C2	0
D	0

* A total of 16 adenocarcinomas were identified in colorectal resection specimens; ¶ Not non-steroidal anti-inflammatory drugs

5.2.2 PATIENTS WITH COLORECTAL LIVER METASTASIS

Ten patients (8 males, 2 females) with colorectal liver metastases, which were considered suitable for surgical resection, were recruited into the trial (*Table 5.2*). The median age of patients recruited was 66 years (range of 50 to 76 years). The results of all blood tests obtained at recruitment were within normal limits, including full blood count, urea and electrolytes and liver function tests. ASA grades of 1, 2 and 3 were assigned to 3, 2 and 5 patients, respectively. Patients' drug histories included aspirin (1 patient), antihypertensives (3 patients) and simple analgesics (1 patient). Eight patients had received chemotherapy prior to recruitment and no patients had received radiotherapy. The minimum time period between termination of chemotherapy and recruitment into the trial was 2 months.

Two patients had a single liver metastasis, 3 patients had 2 liver metastases and 5 patients had 5 or more metastases. The majority of patients had bilobar liver metastases (6 patients), with 4 patients having disease located only in either the right or left lobes. Peripheral blood and urine samples were collected from all trial participants, however, operative cancellations for reasons unrelated to the consumption of mirtocyan, meant liver, bile and portal blood samples from 7 patients were available for analysis.

Table 5.2: Demographic data from 10 patients with colorectal liver metastases who received oral mirtocyan

Characteristic	Patients with colorectal liver metastases
Age, years	
Median	66
Range	50-76
Sex	
Female	2
Male	8
Body mass index, kg/m ²	
Median	29
Range	24-34
ASA grade	
1	3
2	2
3	5
4	0
5	0
Drug history	
Aspirin	1
Antihypertensives	3
Antianginals	0
Simple analgesics ¶	1
Neoadjuvant therapy	
Radiotherapy	0
Chemotherapy	8
Tumour location	
Right lobe	2
Left lobe	2
Bilobar	6
Number of metastases	
1	2
2	3
3	0
4	0
5 or more	5
Original bowel resection	
Right hemicolectomy	5
Sigmoid colectomy	1
Anterior resection	4
Time from bowel resection to liver resection, months	
Median	9.5
Range	6-70

¶ Not non-steroidal anti-inflammatory drugs

5.2.3 PATIENTS RECRUITED TO RECEIVE A NASOGASTRIC OR NASOJEJUNAL DOSE OF MIRTOCYAN

Data from animal studies has suggested that anthocyanins are predominantly absorbed from the stomach (*Section 1.2.2*). To test whether the site for absorption is comparable in humans, two patients with colorectal liver metastases received a dose of mirtocyan via a nasogastric or nasojejunal tube intraoperatively, during a liver resection. Levels of anthocyanins were measured in serial peripheral and portal blood, bile and urine samples.

The patient who received nasogastric mirtocyan was a 69-year old male, with 4 metastases located in segments II and IV, which required a left hepatectomy. The patient who received nasojejunal mirtocyan was a 68-year old male, with two liver metastases located in segments V and VIII, which required a right hepatectomy. The body mass indices of the patients who received nasogastric and nasojejunal mirtocyan, were 25 and 24 kg/m², respectively. The results of blood tests obtained from both patients at recruitment were within normal limits, including full blood count, urea and electrolytes and liver function tests. The ASA grades of both patients were 1, and the only past medical history of note was mild osteoarthritis, which affected the patient who received nasogastric mirtocyan. No regular medications were consumed by either patient and both patients had received oxaliplatin based chemotherapy following their primary bowel resection.

5.3 TOLERABILITY

Consumption of up to 5.6 g of mirtocyan daily for seven days was safe and well tolerated. Patients who consumed the high dose of mirtocyan consistently reported the development of dark stool whilst on treatment, a likely consequence of high anthocyanin content. Two adverse events occurred in trial participants, both of which were considered to be unrelated to the consumption of mirtocyan. Just prior to being anaesthetized one colorectal patient, a known arteriopath, developed a supraventricular tachycardia. His operation was postponed pending cardiology review. Another trial participant suffered a myocardial infarct during a liver resection. The patient made a full recovery, and similarly this event was considered to be unrelated to the consumption of mirtocyan.

5.4 DISCUSSION

Development of dark stool, as described by participants in this trial, has frequently been reported in studies involving administration of high doses of anthocyanins over prolonged periods (Hagiwara et al., 2001; Nabae et al., 2008; Stoner et al., 2005). Quantitative analysis of faecal matter in animal studies has confirmed high levels of anthocyanins. For example, when the diet of rats was supplemented with 3.85 g/kg of either chokeberry, bilberry or grape anthocyanins for 14 weeks, faecal anthocyanin concentrations of 0.7, 1.8 and 2.0 g/kg were detected (He et al., 2005). High concentrations of anthocyanins in faecal matter is consistent with low bioavailability (*Section 1.2.2*). If one considers the potential of anthocyanins to prevent colorectal cancer this feature is appealing.

This study is pilot in design and consequently a relatively small number of patients consumed mirtocyan for a short period. Although mirtocyan was well tolerated and no severe side-effects were evident, patient numbers and duration of treatment must be borne in mind when considering the safety of any intervention. With reference to chemoprevention, these factors are particularly important as frequently agents have to be administered to healthy individuals for prolonged periods. Nevertheless short term pilot studies in a few individuals can yield valuable initial insights into the pharmacokinetics, pharmacodynamics and safety which may be used to optimise the design of trials involving a larger number of patients and longer duration of treatment. Efficacy and safety are ultimately determined in such trials.

Dietary constituents have been investigated as potential cancer chemopreventive agents, often with the expectation that such agents will be well tolerated. Unfortunately, a number of seminal cancer chemoprevention studies have disproved this expectation. The Beta-Carotene and Retinol Efficacy Trial (CARET) and the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (ATBC) involved a combined total of more than 47,000 individuals at high risk of lung carcinoma. Both trials, initiated predominantly on the basis of epidemiological data, actually revealed an increased incidence of the disease in treated groups compared to controls (Omenn et al., 1996; The Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study Group, 1994). More recently a phase III prostate cancer chemoprevention study, the Selenium and Vitamin E Cancer Prevention Trial (SELECT), which involved in excess of 35,000 men, was prematurely terminated in October 2008. Consumption of selenium

and vitamin E were associated with slightly increased risks of developing diabetes (relative risk 1.07, $p=0.16$) and prostate cancer (relative risk 1.13, $p=0.06$), respectively. The SELECT study was commenced predominantly on the basis of secondary results of two randomised control trials, the Nutritional Prevention of Cancer Study (NPC) and the ATBC study (Lippmann et al., 2008). The NPC study, which involved administration of selenium supplements with the aim of reducing recurrent non-melanoma skin cancer, and the ATBC study, in which individuals at high risk of lung cancer received alpha-tocopherol either alone or in combination with beta-carotene, revealed a reduction in prostate cancer risk of up to 63% (Clark et al., 1996; The Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study Group, 1994). Minimal data from pre-clinical and human pilot studies existed to support initiation of SELECT. Factors thought to account for the discrepancies between the SELECT, NPC and ATBC trial results, include consumption of different doses and formulations of vitamin E and selenium and significant differences between the study populations, for example men in the NPC study were selenium deplete at baseline (Lippmann et al., 2008). Experience from the CARET, ATBC and SELECT trials demonstrate significant adverse effects may be associated with administration of isolated dietary constituents. In addition, they highlight the importance of thorough evaluation in pre-clinical and human pilot studies. Such studies reduce the likelihood of negative or adverse results in large, lengthy, costly human trials.

In summary, safety issues are of critical importance in the field of chemoprevention. This pilot study has shown that consumption of up to 5.6 g of mirtocyan daily for

seven days is safe and well tolerated. Evaluation of potential chemopreventive agents in pre-clinical and human pilot studies is crucial prior to the initiation of large clinical trials.

CHAPTER 6

RESULTS

6.1 INTRODUCTION

Levels of anthocyanins obtained in human urine and plasma have been assessed in a number of pharmacokinetic studies. Levels achieved in plasma have generally been less than 100 ng/ml with less than 2% of the oral anthocyanin dose being excreted in urine (*Table 1.1*). To date no human studies have attempted to detect or quantify anthocyanins in portal blood, bile, colorectal or hepatic tissue. An Apc^{Min} mouse study revealed anthocyanin levels of 8.1 µg/g in intestinal mucosa following dietary supplementation with 0.3% mirtocyan for 12 weeks. Levels of 49 ng/g were observed in intestinal mucosa when mice received 0.3% isolated cyanidin-3-glucoside for 12 weeks (Cooke et al., 2006a). Following a single oral dose of bilberry extract to rats anthocyanins have been detected in a number of biomatrices, including the liver and bile (Ichiyanagi et al., 2006). When blackberry extract was delivered directly into the stomachs of anaesthetized rats anthocyanins were detected in plasma samples obtained from the gastric veins and aorta. Cyanidin-3-glucoside was detected in bile samples within 20 minutes (Talavera et al., 2003).

The predominant anthocyanin species identified in human plasma and urine have been unaltered glycosides and glucuronidated forms (Kay et al., 2004; Kay et al., 2005). In addition, methylated metabolites have been detected in both human urine and plasma (Kay et al., 2004; Kay et al., 2005). Aglycones and sulphate conjugates have less commonly been identified in human urine (Felgines et al., 2003; Felgines et al., 2005; Mullen et al., 2008).

In the trial described here patients with colorectal cancer and colorectal liver metastases consented to provide samples of peripheral blood, urine and colorectal/liver tissue before and after consuming mirtocyan for seven days. The aim of the work described in this chapter was to measure levels of anthocyanins and their metabolites in human biomatrices following seven days of oral mirtocyan and following administration of a single dose of mirtocyan into the stomach/small intestine. When possible portal blood samples were obtained intra-operatively and bile samples were obtained from patients undergoing a liver resection. Overall, biomatrices from 25 patients were analysed by HPLC and mass spectrometric techniques, of whom 10, 8 and 7 patients consumed 1.4, 2.8 and 5.6 g of mirtocyan daily, respectively. An additional two patients with colorectal liver metastases received a dose of mirtocyan intra-operatively during a liver resection. Serial peripheral and portal blood, urine and bile samples were obtained for detailed assessment of pharmacokinetics, particularly absorption. Mass spectrometry and analysis described in this chapter was kindly conducted by Dr Hong Cai (Department of Cancer Studies and Molecular Medicine, University of Leicester).

Representative HPLC traces are shown in the relevant sections below and all chromatograms recorded are located in *Appendix 1*. Quantitative results have been calculated on the basis of standard curves established for cyanidin-3-glucoside (C3G) and are therefore approximate, the term to describe them here is “semiquantitative”. Quantification was performed assuming that individual anthocyanins in the mirtocyan mixture possess absorption coefficients similar to that of C3G (*Section 4.3.1*).

6.2 IDENTIFICATION AND SEMIQUANTIFICATION OF ANTHOCYANINS IN HUMAN BIOMATRICES FOLLOWING ORAL CONSUMPTION OF MIRTOCYAN

6.2.1 PERIPHERAL PLASMA

Species commensurate with anthocyanins were recovered from peripheral plasma samples of all trial participants who consumed 2.8 and 5.6 g of mirtocyan daily. A prominent peak with a retention time of 10-11 minutes was noted throughout the plasma chromatograms, and this co-eluted with peonidin-3-glucoside (*Figure 6.1, Peak 11*). This peak was present in all but two plasma chromatograms, both of which were obtained from patients who consumed 1.4 g of mirtocyan daily.

Six species were discernible in every plasma sample obtained from patients who ingested 5.6 g of mirtocyan daily. These species all co-eluted with constituents of the original mirtocyan mixture, and were tentatively identified as delphinidin-3-galactoside, delphinidin-3-glucoside, delphinidin-3-arabinoside, cyanidin-3-galactoside, cyanidin-3-glucoside and peonidin-3-glucoside (*Figure 6.1*). Attempts at mass spectrometric confirmation were unsuccessful, a likely consequence of low anthocyanin concentrations in this biomatrix.

Levels of the six species consistently identified in plasma samples obtained from patients who consumed 5.6 g of mirtocyan daily were semi-quantitated (*Table 6.1*). Anthocyanins present in samples obtained from patients who consumed 2.8 and 1.4 g of mirtocyan daily exhibited high variability, and therefore quantification of

individual species was not attempted. Total anthocyanin concentrations and concentrations of the prominent species (Rt 10-11 minutes) appeared to be related to mirtocyan dose (*Table 6.2*). Most anthocyanin species in plasma samples obtained from patients who consumed 1.4 g of mirtocyan were below the limit of quantification, and calculation of a total anthocyanin concentration for this dose group was not feasible.

Controls for HPLC analysis of peripheral plasma samples were those obtained before the administration of mirtocyan (*Figure 6.1, Appendix 1*).

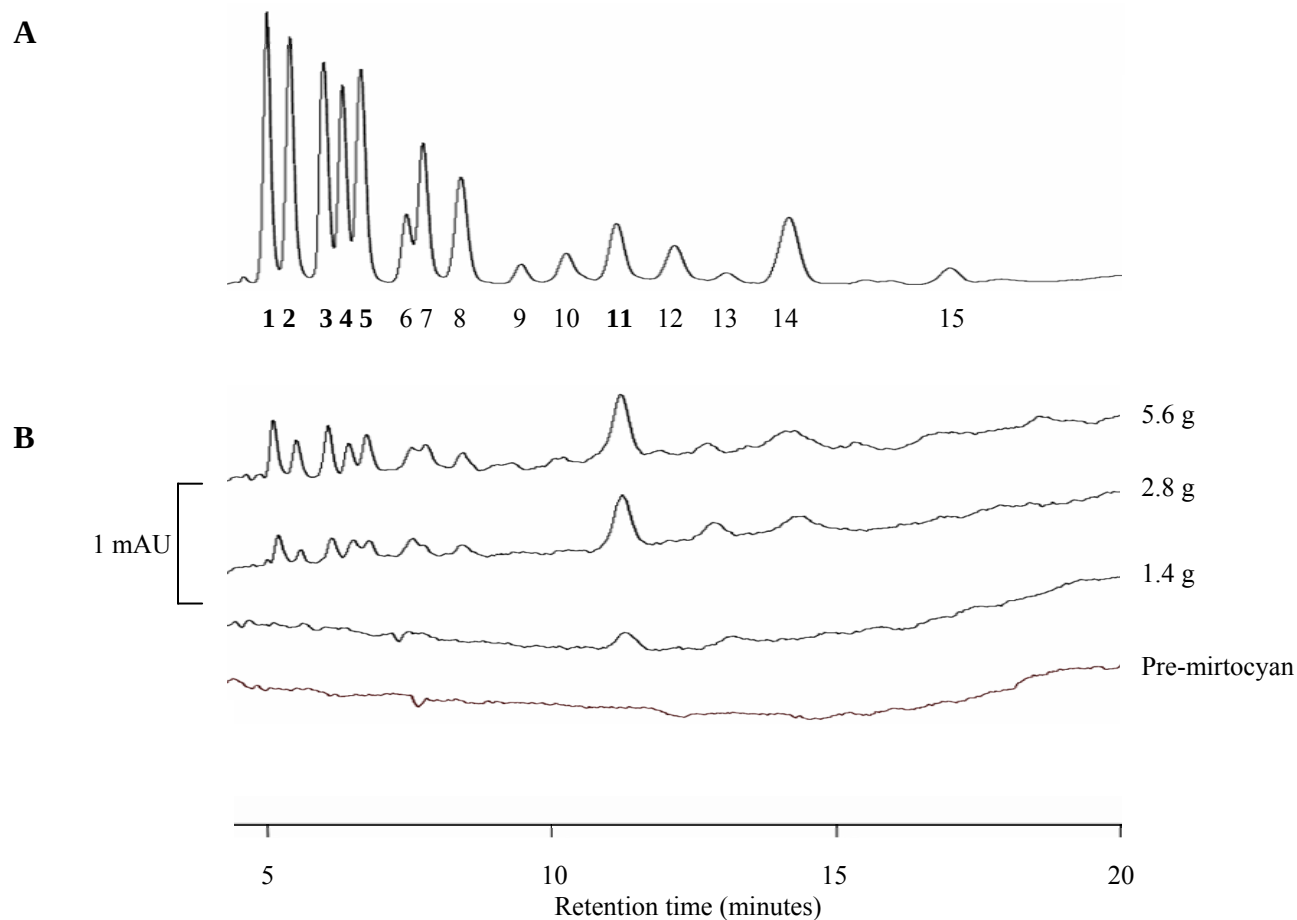


Figure 6.1: HPLC traces of mirtocyan (A) and representative pre- and post-mirtocyan peripheral plasma samples (B)

Numbers below peaks in A identify anthocyanin species present in mirtocyan. 1: Dephinidin-3-galactoside, 2: Delphinidin-3-glucoside, 3: Cyanidin-3-galactoside, 4: Delphinidin-3-arabinoside, 5: Cyanidin-3-glucoside, 6: Petunidin-3-galactoside, 7: Cyanidin-3-arabinoside, 8: Petunidin-3-glucoside, 9: Peonidin-3-galactoside, 10: Petunidin-3-arabinoside, 11: Peonidin-3-glucoside, 12: Malvidin-3-galactoside, 13: Peonidin-3-arabinoside, 14: Malvidin-3-glucoside, 15: Malvidin-3-arabinoside. Numbers in bold represent peaks identified in every plasma sample obtained from patients who consumed 5.6 g of mirtocyan daily

Traces in B are representative of samples obtained from 7-10 patients per dose group

Table 6.1: Approximate concentrations of individual anthocyanin species consistently identified in plasma samples obtained from patients who consumed 5.6 g of mirtocyan daily

Anthocyanin (number of corresponding peak in <i>Figure 6.1</i>)	Plasma concentration* (ng/ml)
Delphinidin-3-galactoside (1)	16 ($\pm$ 6)
Delphinidin-3-glucoside (2)	13 ($\pm$ 5)
Cyanidin-3-galactoside (3)	16 ($\pm$ 5)
Delphinidin-3-arabinoside (4)	12 ($\pm$ 3)
Cyanidin-3-glucoside (5)	16 ($\pm$ 5)
Peonidin-3-glucoside (11)	40 ($\pm$ 18)

* Levels were semi-quantitated using standard curves established for C3G

Table 6.2: Approximate concentrations of the prominent anthocyanin species (Rt 10-11 minutes) and total anthocyanins in plasma samples obtained following consumption of 1.4, 2.8 and 5.6 g of mirtocyan daily

Daily dose of mirtocyan (g)	Prominent anthocyanin* (peonidin-3-glucoside) (ng/ml)	Total anthocyanins* (ng/ml)
1.4	18 ($\pm$ 21)	-
2.8	23 ($\pm$ 17)	44 ($\pm$ 31)
5.6	40 ($\pm$ 18)	117 ($\pm$ 57)

* Levels were semi-quantitated using standard curves established for C3G

6.2.2 PORTAL PLASMA

HPLC analysis revealed anthocyanins above the limit of detection in 3 of 12 portal plasma samples obtained intraoperatively (*Figure 6.2*). Two of these patients had received 2.8 g of mirtocyan daily and one had received 1.4 g of mirtocyan daily. Anthocyanins were not detected in portal plasma of any patient who received 5.6 g of mirtocyan daily. When anthocyanins were above the limit of detection portal blood samples had been collected 5.7 ± 1 hours following the final dose of mirtocyan. When anthocyanins were below the limit of detection, a delay of 8.1 ± 2.1 hours had occurred between the final dose of mirtocyan and collection of portal blood ($p = 0.099$, Student's t-test).

A prominent peak was identified in portal plasma chromatograms (R_t 10-11 minutes) which was comparable to that obtained from analysis of peripheral plasma, and also co-eluted with peonidin-3-glucoside (*Figure 6.2*).

Portal blood samples were obtained intra-operatively and therefore it was not possible to obtain samples prior to the administration of mirtocyan. Peripheral plasma samples obtained prior to the administration of mirtocyan therefore served as controls (*Figure 6.1, Appendix 1*).

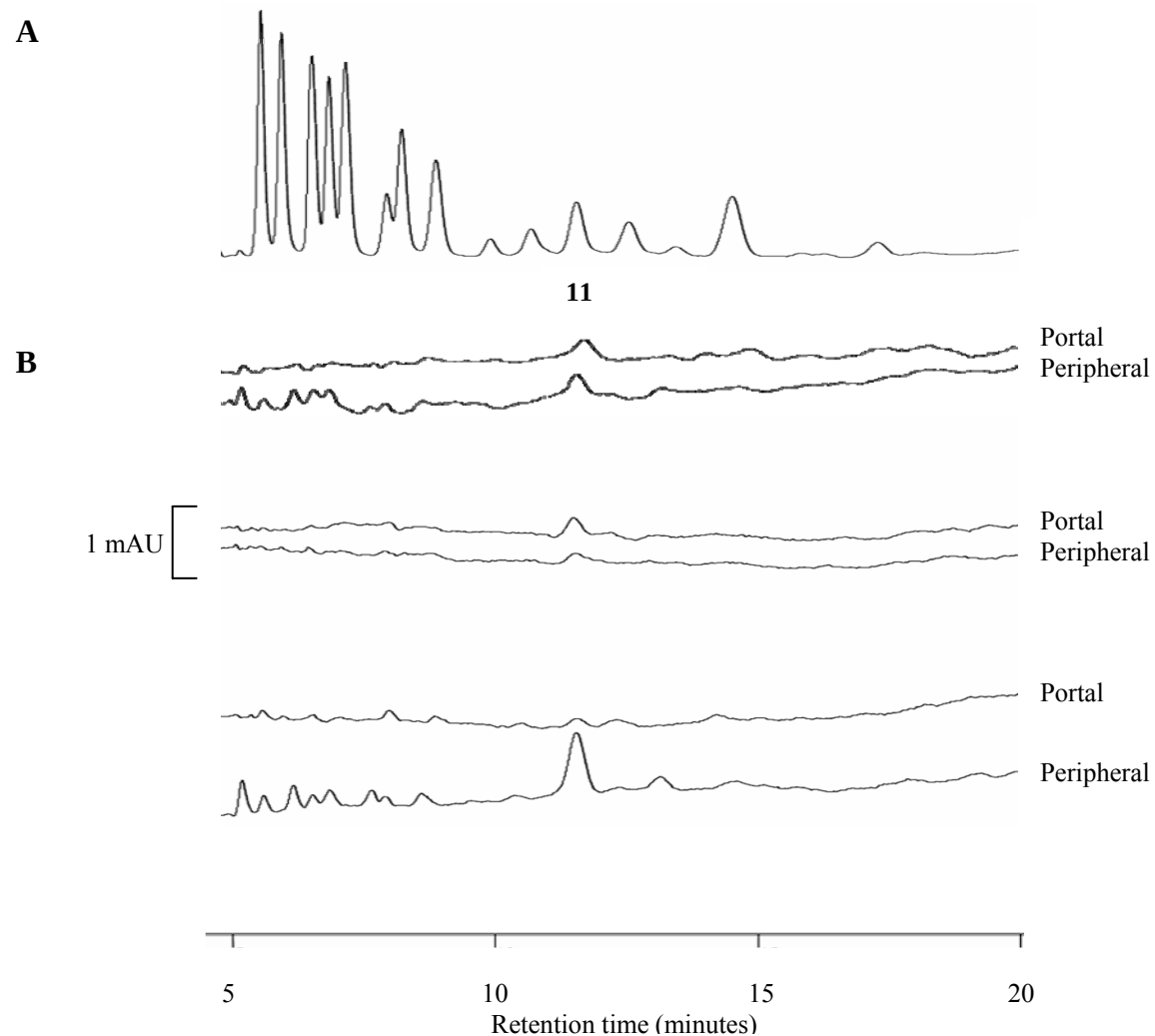


Figure 6.2: HPLC traces of mirtocyan (A) and portal plasma samples from 3 patients (on 1.4 or 2.8 g mirtocyan) in which levels of anthocyanins were above the limit of detection. Portal chromatograms are shown along with corresponding peripheral plasma traces (B)

Peak 11: Peonidin-3-glucoside

6.2.3 URINE

HPLC analysis revealed the presence of anthocyanins in every urine sample, collected for 11.5 ($\pm$ 1) hours following consumption of the penultimate mirtocyan dose (*Figure 6.3*). Akin to analysis of plasma, a prominent peak was also noted throughout HPLC traces of post-mirtocyan urine. This was characterised by LC/MS/MS on the basis of its mass transition m/z 477>301 as peonidin glucuronide or its positional isomer methylcyanidin glucuronide (*Figures 6.3 and 6.4*). In addition, mass spectrometric analysis of post-mirtocyan urine revealed the presence of cyanidin and malvidin glucuronides and confirmed the presence of constituents of the original mirtocyan mixture, with the exception of delphinidin-3-arabinoside (*Table 6.3*).

Levels of anthocyanins in urine samples collected following the consumption of mirtocyan were semi-quantitated, and standardised against urinary creatinine concentration. Levels of anthocyanins in urine appeared to be related to the dose of mirtocyan consumed (*Table 6.4*). Controls for HPLC analysis of urine samples were those obtained before the administration of mirtocyan (*Figure 6.3, Appendix 1*). Small peaks with retention times consistent with anthocyanins were noted in most pre-mirtocyan urine samples obtained at recruitment, a likely reflection of normal dietary polyphenol consumption (*Appendix 1*). As patients were requested to refrain from anthocyanin rich foodstuffs whilst participating in the trial, all species present in post-intervention urine samples were assumed to be derived from mirtocyan.

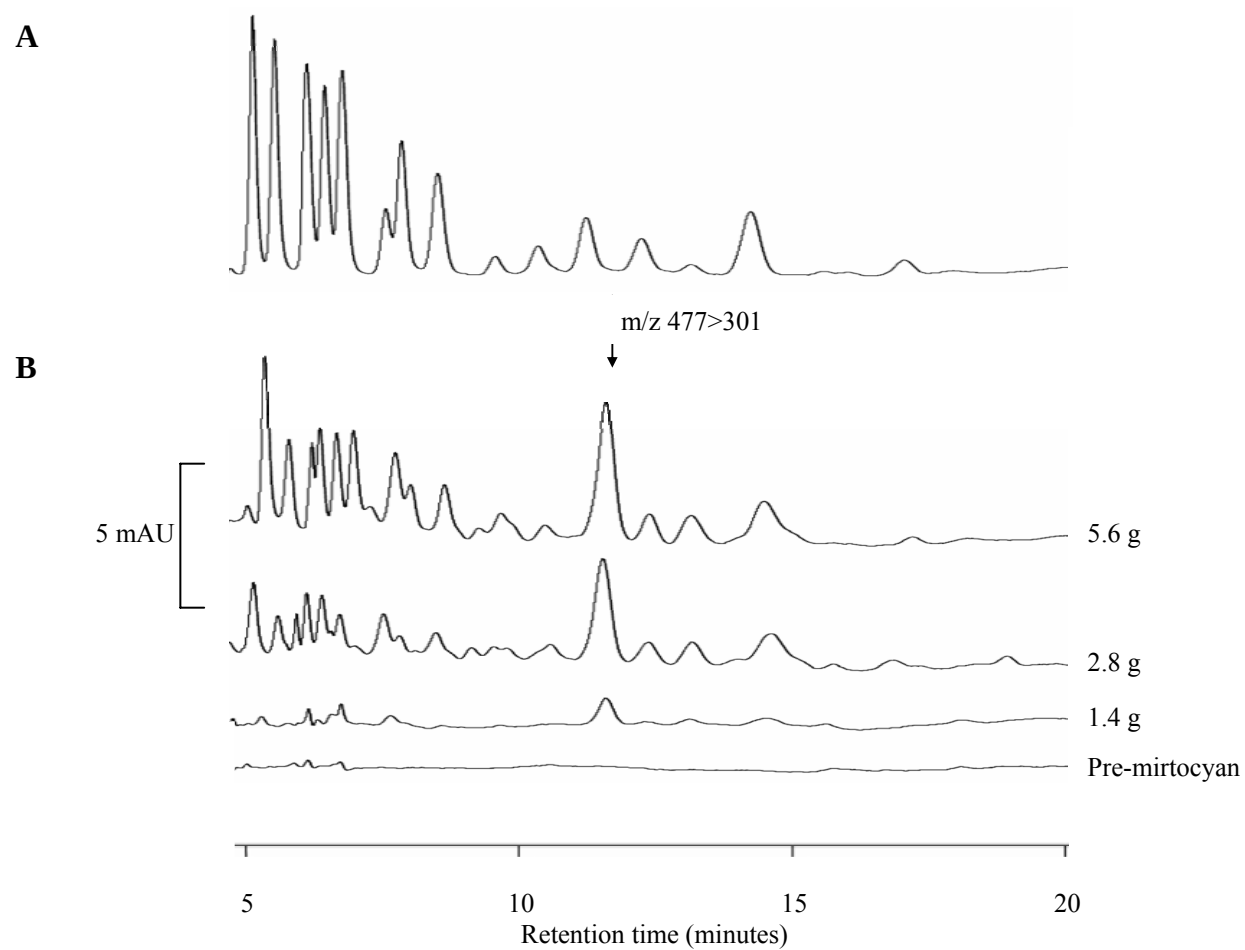


Figure 6.3: HPLC traces of mirtocyan (A) and representative pre- and post-mirtocyan urine samples (B)

Traces in B are representative of samples obtained from 7-10 patients

Arrow indicates the predominant species in urine samples which afforded the mass transition $m/z\ 477 > 301$, suggestive of peonidin glucuronide or methylcyanidin glucuronide

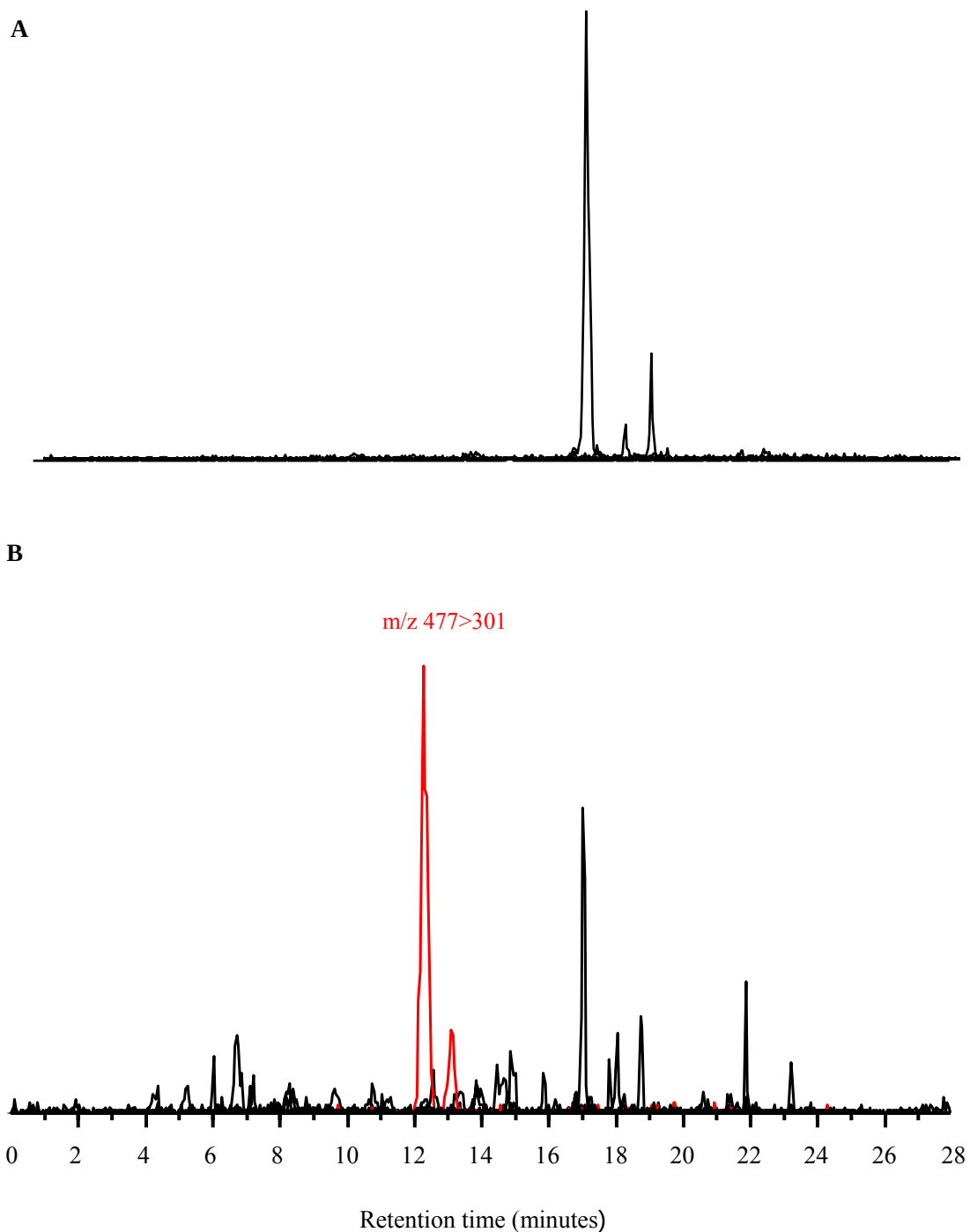


Figure 6.4: LC/MS/MS total ion chromatograms of urine samples obtained from a patient before (A) and after (B) consumption of mirtocyan (2.8 g)

Urine samples were analysed for 28 mass transitions spanning anthocyanins present in mirtocyan and possible metabolites generated by methylation, glucuronidation and sulphation. Peaks in red indicate species which afforded the mass transition m/z 477>301, suggestive of peonidin glucuronide or methylcyanidin glucuronide. Peaks in figure A are a likely reflection of normal dietary anthocyanin consumption

Table 6.3: Anthocyanins identified by LC/MS/MS in urine and malignant colorectal tissue obtained following oral consumption of mirtocyan

Anthocyanin	Retention time (minutes) [†]	Mass transition (m/z)	Urine	Malignant colorectal tissue
Delphinidin-3-galactoside*	4.9	465>303	+	+
Delphinidin-3-glucoside*	5.5	465>303	+	+
Delphinidin-3-arabinoside*	6.3	435>303		+
Cyanidin-3-galactoside*	5.9	449>287	+	+
Cyanidin-3-glucoside*	6.7	449>287	+	+
Cyanidin-3-arabinoside*	7.7	419>287	+	+
Petunidin-3-galactoside*	7.1	479>317	+	+
Petunidin-3-glucoside*	8.2	479>317	+	+
Petunidin-3-arabinoside*	10.0	449>317	+	+
Peonidin-3-galactoside*	8.9	463>301	+	+
Peonidin-3-glucoside*	10.8	463>301	+	+
Peonidin-3-arabinoside*	12.1	433>301	+	
Malvidin-3-galactoside*	11.4	493>331	+	+
Malvidin-3-glucoside*	13.4	493>331	+	+
Malvidin-3-arabinoside*	13.8	463>331	+	+
Methylpetunidin-3-arabinoside or dimethyldelphinidin-3-arabinoside	9.0	463>331		+ [¶]
Methylcyanidin-3-galactoside	9.2	463>301		+
Methylcyanidin-3-glucoside	11.1	463>301		+
Cyanidin glucuronide	6.4	463>287	+	
Peonidin glucuronide or methylcyanidin glucuronide	11.2, 12.6	477>301	+ [¶]	
Malvidin glucuronide	13.8	507>331	+	

* A constituent of the original mirtocyan mixture, [¶] Predominant species in respective biomatrix, [†] Retention times quoted for LC/MS/MS analysis may vary from those presented in HPLC traces in this chapter, reflecting differing chromatographic conditions (column length, detector)

Table 6.4: Approximate levels of total anthocyanins in urine samples collected for 11.5 ($\pm$ 1) hours following consumption of the penultimate dose of mirtocyan, standardised to urinary creatinine

Daily dose of mirtoselect (g)	Concentration of anthocyanins* ($\mu\text{g/ml}$)	Concentration of creatinine (mmol/L)	Anthocyanins (μg)*/ creatinine (mmol)
1.4	0.9 ($\pm$ 0.6)	9 ($\pm$ 9)	181 ($\pm$ 137)
2.8	1 ($\pm$ 0.8)	9 ($\pm$ 6)	264 ($\pm$ 221)
5.6	3 ($\pm$ 1)	6 ($\pm$ 2)	514 ($\pm$ 284)

* Levels were semi-quantitated using standard curves established for C3G

6.2.4 COLORECTAL TISSUE

Preliminary HPLC analysis of normal and malignant colorectal tissue samples revealed species likely to be anthocyanins near the limit of detection. Colorectal tissue samples were therefore pooled within individual dose groups to allow more accurate evaluation of the anthocyanin species present and an approximation of anthocyanin levels.

HPLC analysis of pooled tissue samples revealed anthocyanin species above the limit of quantification in normal and malignant colorectal tissue obtained from patients who consumed 5.6 g of mirtocyan daily. Approximate levels of anthocyanins are shown in *Table 6.5*. Most anthocyanin species were below the limit of quantification in pooled tissue samples obtained from patients who consumed 1.4 and 2.8 g of mirtocyan daily and therefore calculation of total anthocyanin levels for these dose groups was not feasible.

A prominent peak was identified in HPLC traces of normal and malignant colorectal tissue (Rt 14-15 minutes) (*Figure 6.5, Appendix 1*). LC/MS/MS analysis revealed a mass transition m/z 463>331, suggestive of methylpetunidin-3-arabinoside or its positional isomer dimethyldelphinidin-3-arabinoside. In addition, LC/MS/MS analysis of malignant colorectal tissue confirmed the presence of all constituents of the original mirtocyan mixture, with the exception of peonidin-3-arabinoside. No glucuronide or sulphate conjugates were detected in colorectal tissue (*Table 6.3*).

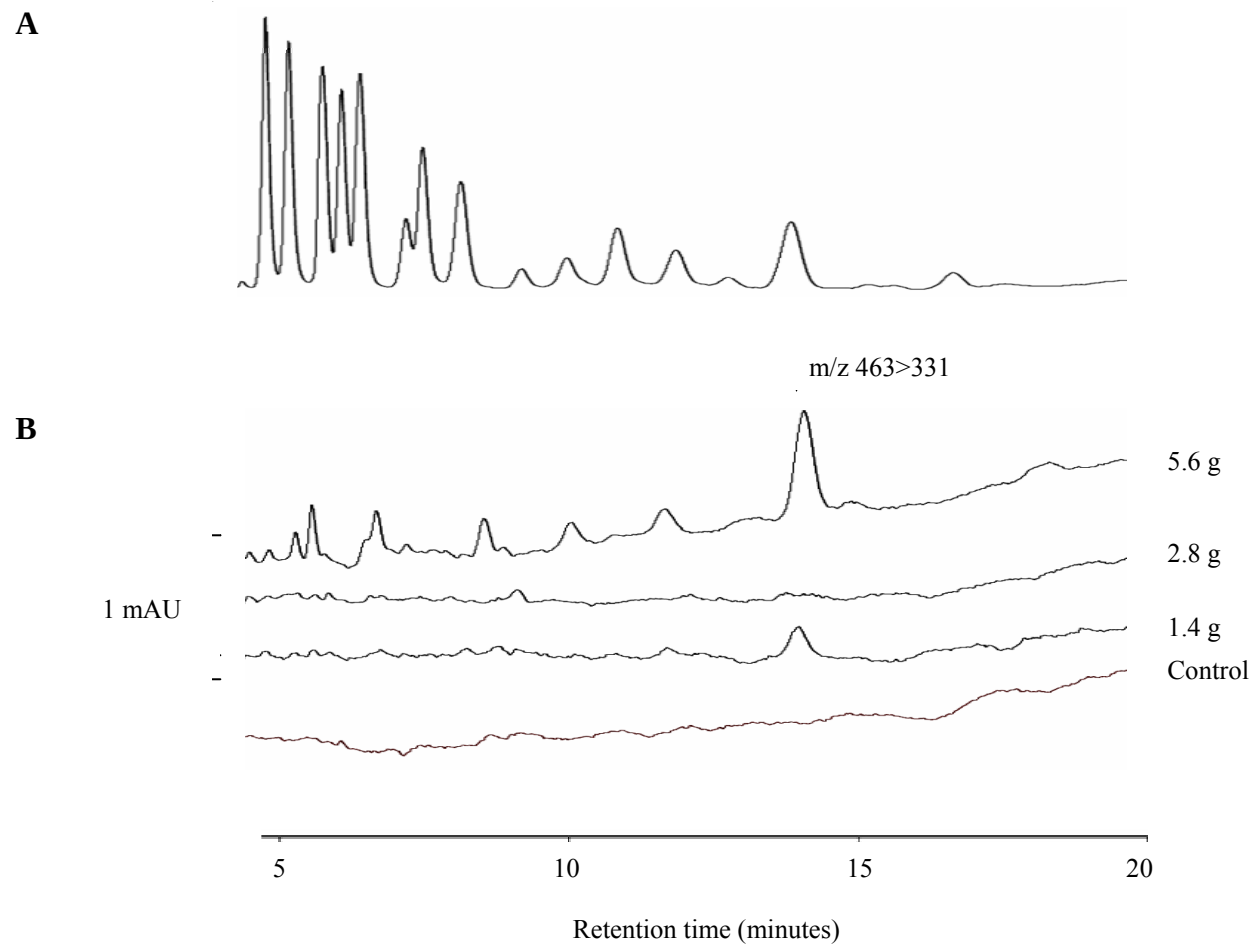


Figure 6.5: HPLC traces of mirtocyan (A) and pooled malignant colorectal tissue samples from individual dose groups (B)

Arrow indicates the major species present in colorectal samples. Its mass transition $m/z\ 463>331$ was suggestive of methylpetunidin-3-arabinoside or dimethyldelphinidin-3-arabinoside

Traces in B represent pooled samples obtained from 4-5 patients. 'Control' colorectal biopsies were obtained during diagnostic colonoscopy

Table 6.5: Approximate levels of anthocyanins in normal and malignant colorectal tissue samples obtained following consumption of 5.6 g of mirtocyan daily

Nature of tissue	Total anthocyanins* (ng/g)
Tumour	179
Normal (Proximal to tumour)	96
Normal (Distal to tumour)	123

* Levels were measured in samples pooled from 5 patients and semi-quantitated using standard curves established for C3G

Biopsies obtained during routine colonoscopy (pre-mirtocyan) served as control tissue (*Figure 6.5, Appendix 1*). Biopsies, of both normal and tumour tissue, were pooled. This allowed extraction from a quantity of tissue within the range used for post-mirtocyan samples obtained from resection specimens (*Section 4.3.1*).

6.2.5 HEPATIC TISSUE

HPLC and mass spectrometric analysis failed to detect anthocyanin species in any hepatic tissue sample following the consumption of mirtocyan (HPLC traces in *Appendix 1*). Analysis of pooled samples also failed to reveal the presence of anthocyanins.

Controls for HPLC analysis of hepatic tissue were biopsies obtained before administration of mirtocyan (*Appendix 1*). Biopsies were pooled to allow extraction

from a quantity of tissue within the range used for post-mirtocyan samples obtained from resection specimens (*Section 4.3.1*).

6.2.6 BILE

HPLC analysis of bile revealed the presence of species likely to be anthocyanins (*Figure 6.6*). These species exhibited mass transitions commensurate with anthocyanidin glucuronides on LC/MS/MS analysis (*Figure 6.7*). However, retention times were longer than that which would be expected for mono- glycoside glucuronides. These species were therefore possibly generated from the breakdown of larger molecules.

Bile samples were obtained intra-operatively and therefore could not be obtained before the consumption of mirtocyan. A limitation of this analysis is the lack of a control.

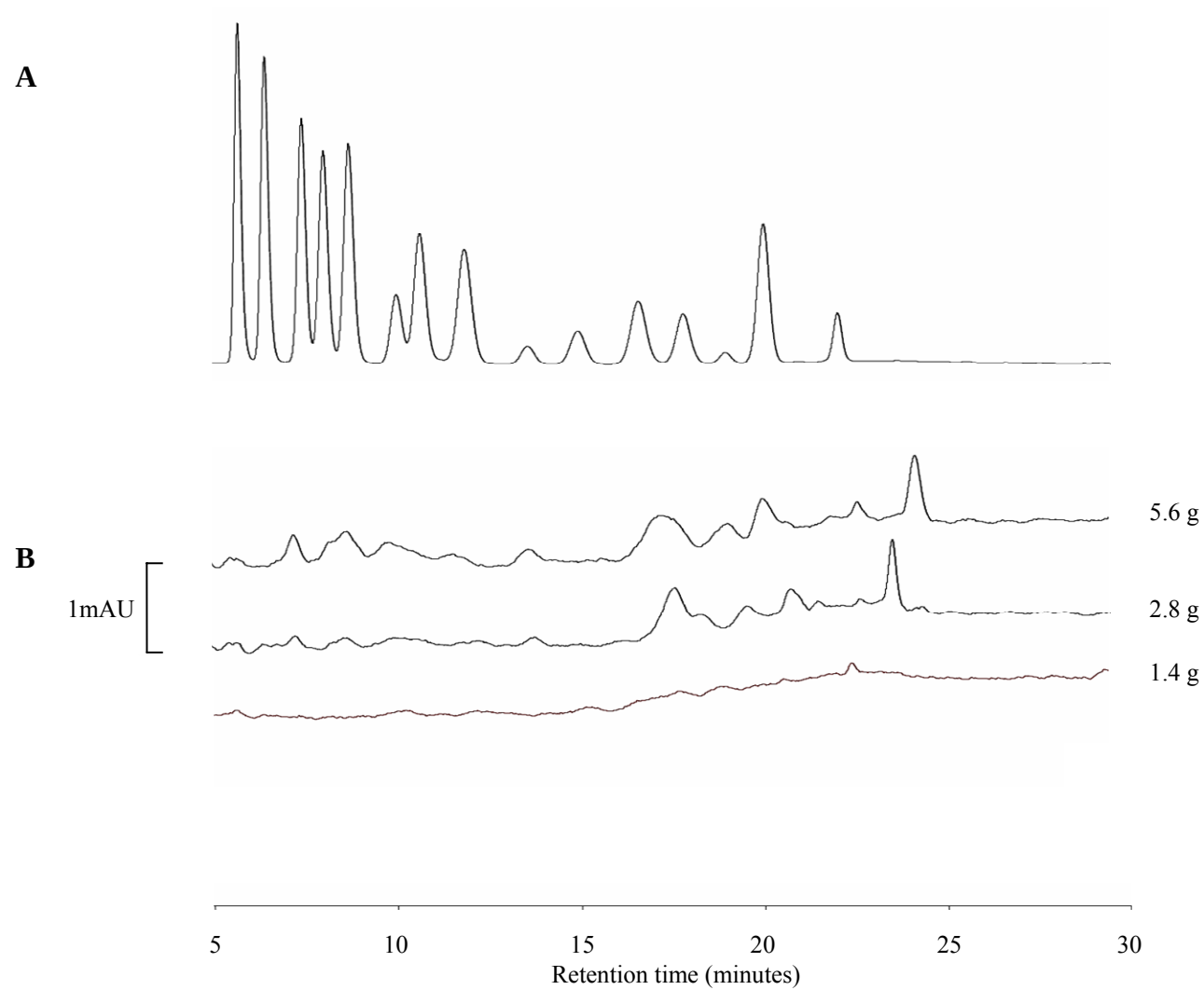
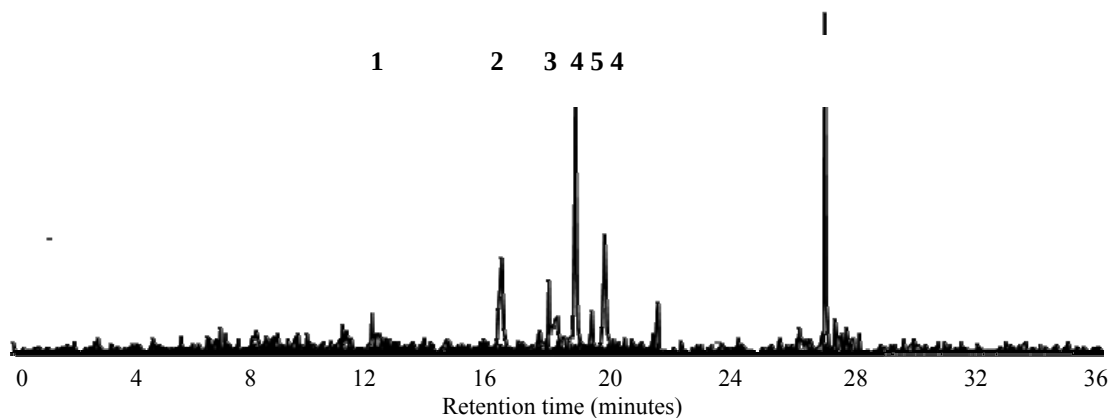


Figure 6.6: HPLC traces of mirtocyan (A) and bile obtained following the consumption of mirtocyan (B)

Traces in B are representative of samples obtained from up to 3 patients



1. Delphindin glucuronide	479>303
2. Cyanidin glucuronide	463>287
3. Petunidin glucuronide	493>317
4. Peonidin glucuronide	477>301
5. Malvidin glucuronide	507>331

Figure 6.7: LC/MS/MS total ion chromatogram of bile following consumption of 5.6 g of mirtocyan daily

Numbered peaks produced mass transitions compatible with anthocyanidin glucuronides, however, exhibited retention times longer than those expected for mono-glycoside glucuronides

6.3 ANTHOCYANIN LEVELS IN PATIENTS FOLLOWING NASOGASTRIC AND NASOJEJUNAL ADMINISTRATION OF MIRTOCYAN

Data from animal studies has suggested that anthocyanins are predominantly absorbed from the stomach (*Section 1.2.2*). To test whether the site for absorption is comparable in humans, two patients with colorectal liver metastases received a dose of mirtocyan via a nasogastric or nasojejunal tube intraoperatively, during a liver resection. Patients received 1880 mg of mirtocyan, the same quantity as that consumed by individuals in the high dose group, three times daily. Levels of anthocyanins were measured in serial peripheral and portal blood, bile and urine samples.

6.3.1 PERIPHERAL AND PORTAL PLASMA

Anthocyanins were detected in all peripheral and portal plasma samples, collected 15-120 minutes following administration of mirtocyan into the stomach and jejunum (*Figures 6.8 and 6.9*). Over the time course studied, concentrations of anthocyanins achieved in the peripheral and portal circulations were up to 14 and 22 fold higher, respectively, when mirtocyan was administered into the stomach, compared to the jejunum. Anthocyanin concentrations were higher in portal than corresponding peripheral plasma samples for approximately 30 minutes, beyond which time the relationship was variable. $C_{\max}$ was observed in both peripheral and portal plasma samples collected 15 minutes after jejunal administration, however, $C_{\max}$ was not definitely reached in either circulation over the time course studied following gastric administration of mirtocyan.

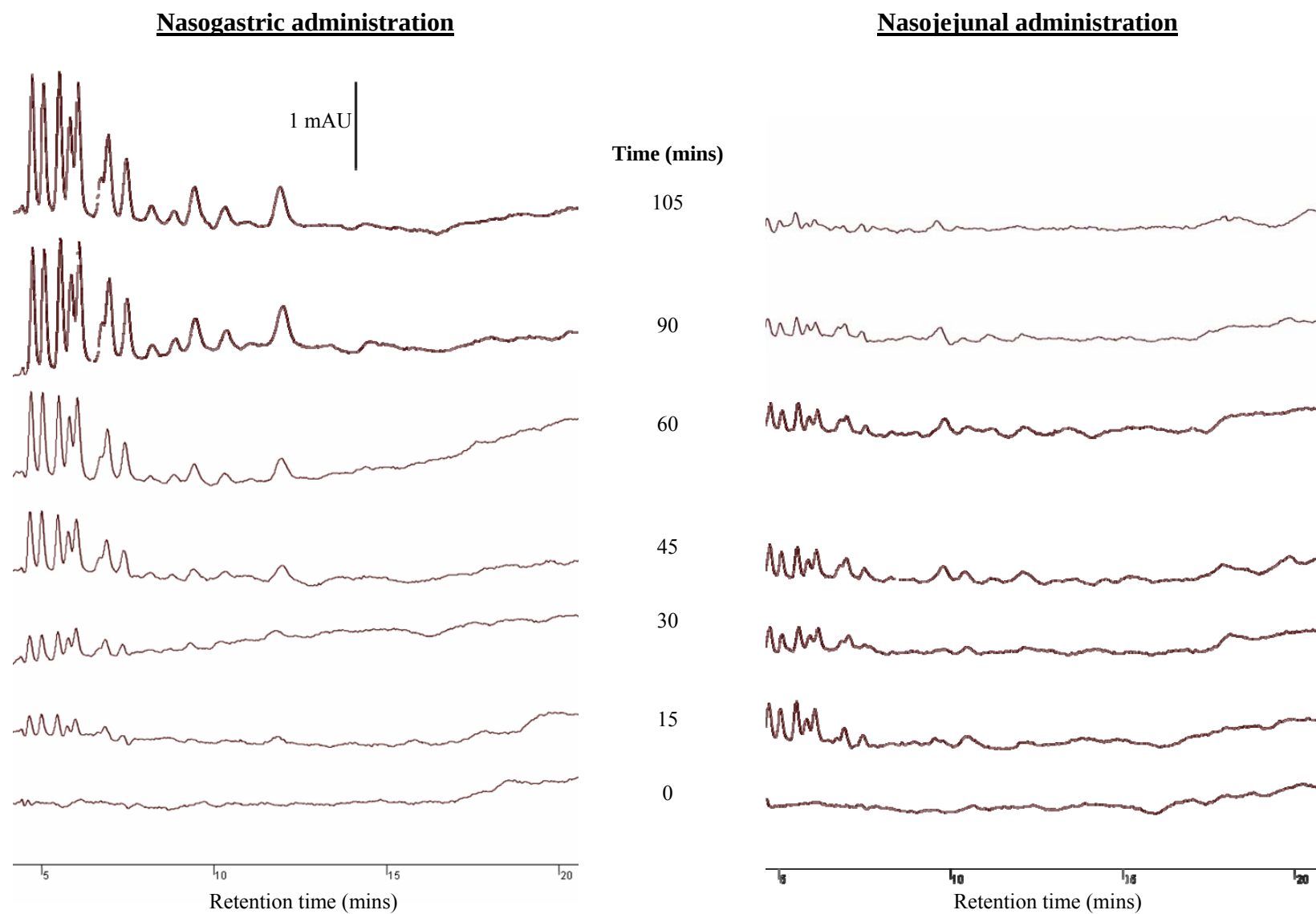


Figure 6.8: HPLC traces of serial peripheral plasma samples obtained from one patient each following administration of a single dose of mirtocyan into the stomach or jejunum

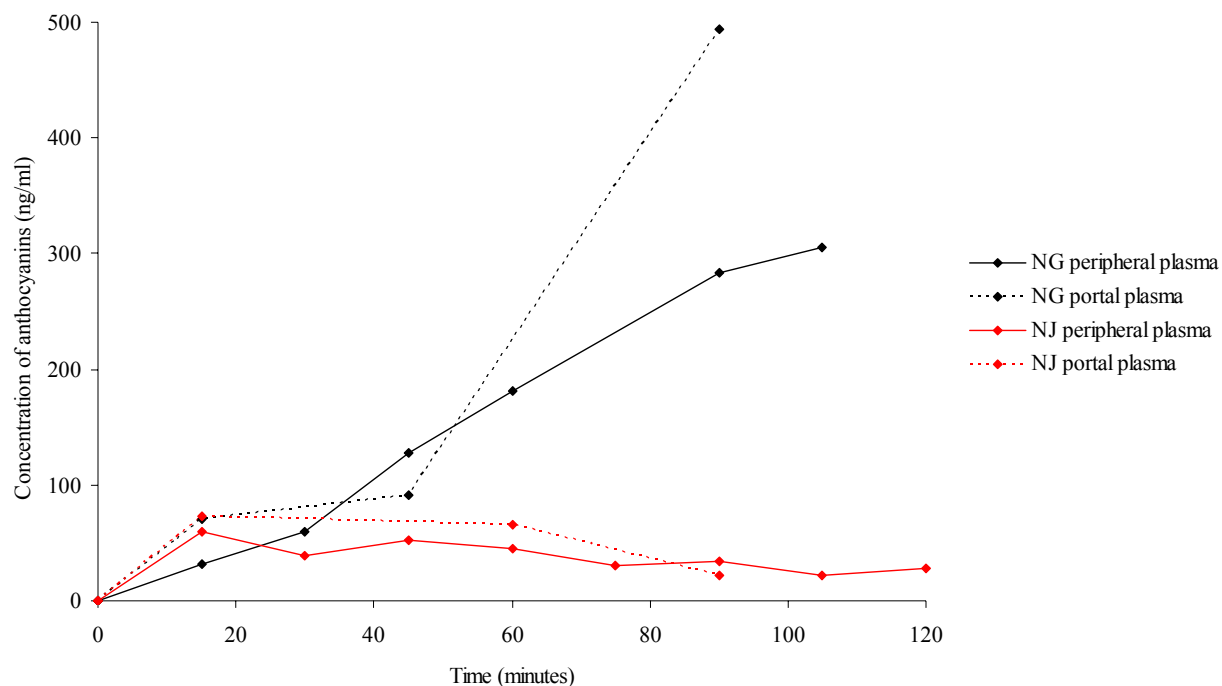


Figure 6.9: Concentrations of anthocyanins in serial peripheral and portal plasma samples obtained following administration of a single dose of mirtocyan into the stomach and jejunum

Levels were semi-quantitated using standard curves established for C3G

6.3.2 URINE

Following gastric and jejunal administration of mirtocyan the total quantity of anthocyanins excreted in urine peaked at 4-6 and 2-4 hours, respectively (*Figures 6.10 and 6.11*). The quantity of anthocyanins excreted in urine following gastric administration of mirtocyan, far exceeded that measured following jejunal delivery. For example, at 4-6 hours the quantity of anthocyanins in urine was nearly 50-fold higher in samples obtained following gastric administration of mirtocyan, compared to those collected following jejunal delivery.

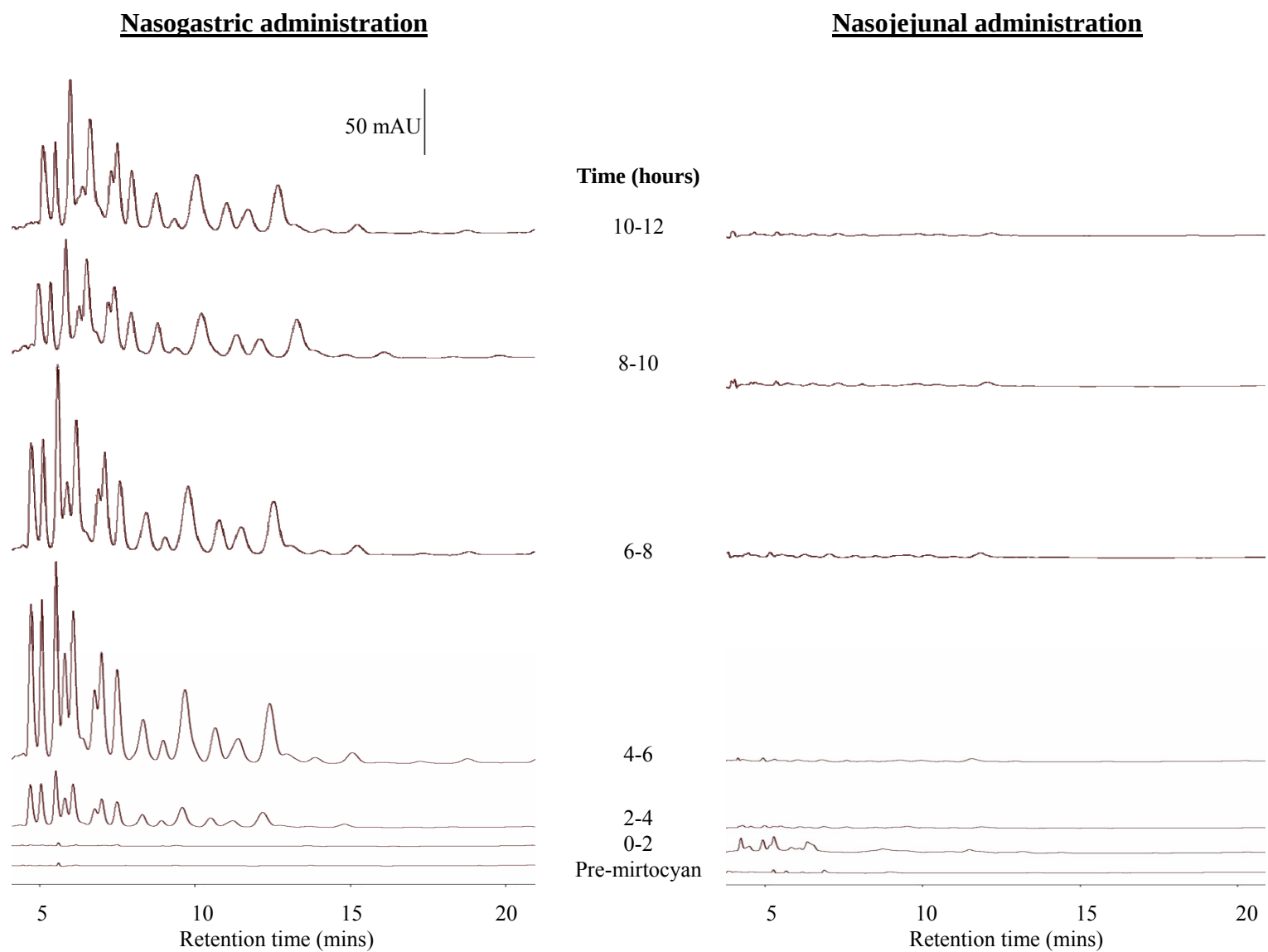


Figure 6.10: HPLC traces of serial urine samples obtained from one patient each following administration of a single dose of mirtocyan into the stomach or jejunum

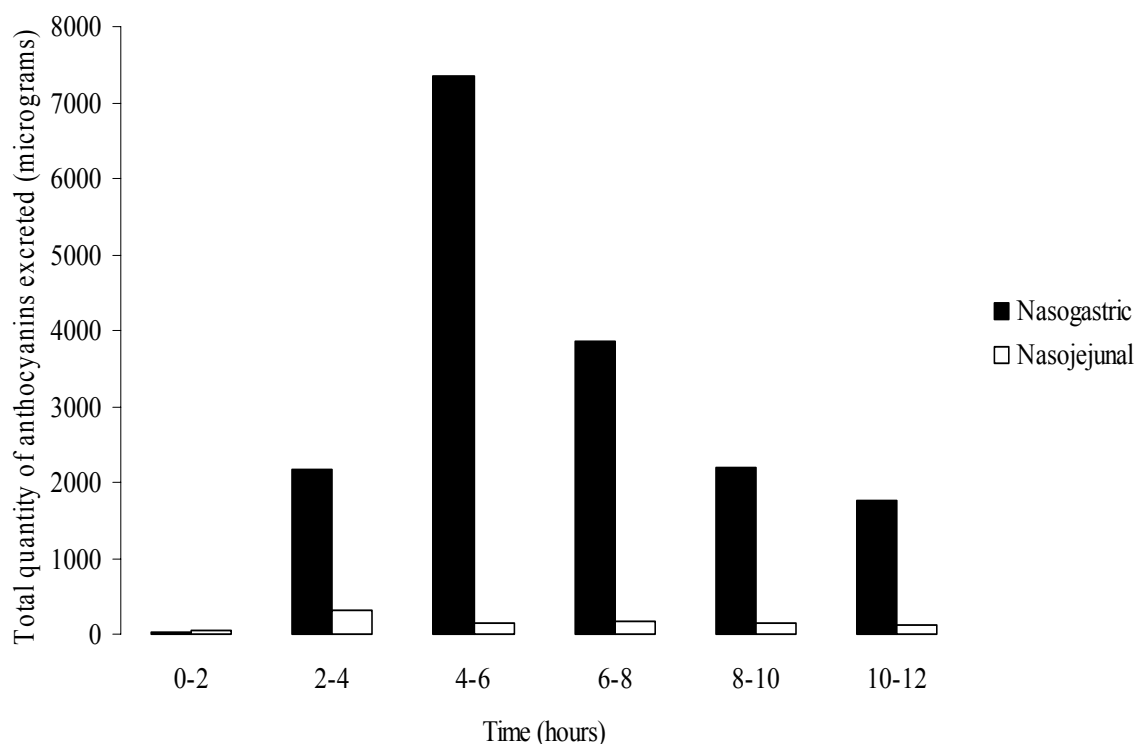


Figure 6.11: Total quantities of anthocyanins excreted in serial urine samples following administration of a single dose of mirtocyan into the stomach and jejunum

Levels were semi-quantitated using standard curves established for C3G

Given the higher quantities of anthocyanin species in urine samples following gastric, rather than oral administration of mirtocyan, LC/MS/MS analysis was repeated on these samples with the aim of identifying a full compliment of mirtocyan-derived species. Delphinidin-3-arabinoside, the only constituent of the mirtocyan mixture not observed in urine following oral consumption, was identified following gastric delivery. Other additional species to be identified included a range of methylated metabolites and the aglycone, cyanidin (*Table 6.6*).

Table 6.6: Anthocyanin species identified by LC/MS/MS in urine following gastric administration of mirtocyan, in addition to those identified following oral consumption (Table 6.3)

Anthocyanin	Retention time (mins)	Mass transition
Delphinidin-3-arabinoside*	6.3	435>303
Methyldephinidin-3-galactoside	6.0	479>317
Methyldephinidin-3-glucoside	7.2	479>317
Methyldephinidin-3-arabinoside	9.2	449>317
Methylcyanidin-3-galactoside	9.2	463>301
Methylcyanidin-3-glucoside	11.1	463>301
Methylcyanidin-3-arabinoside	13.7	433>301
Methylpetunidin-3-galactoside	12.9	493>331
Methylpetunidin-3-glucoside	14.7	493>331
Methylpetunidin-3-arabinoside	15.9	463>331
Peonidin arabinoside glucuronide	6.3	609>301
Cyanidin	18.9	287>137

* A constituent of the original mirtocyan mixture

6.3.3 BILE

A prominent peak (Rt 22-23 mins) was noted in HPLC traces of serial bile samples, however, notably this peak was also present prior to the administration of mirtocyan (Figure 6.12). Although the peak seemed to become progressively larger with time, especially following gastric administration of mirtocyan, its presence in pre-intervention samples renders its interpretation difficult.

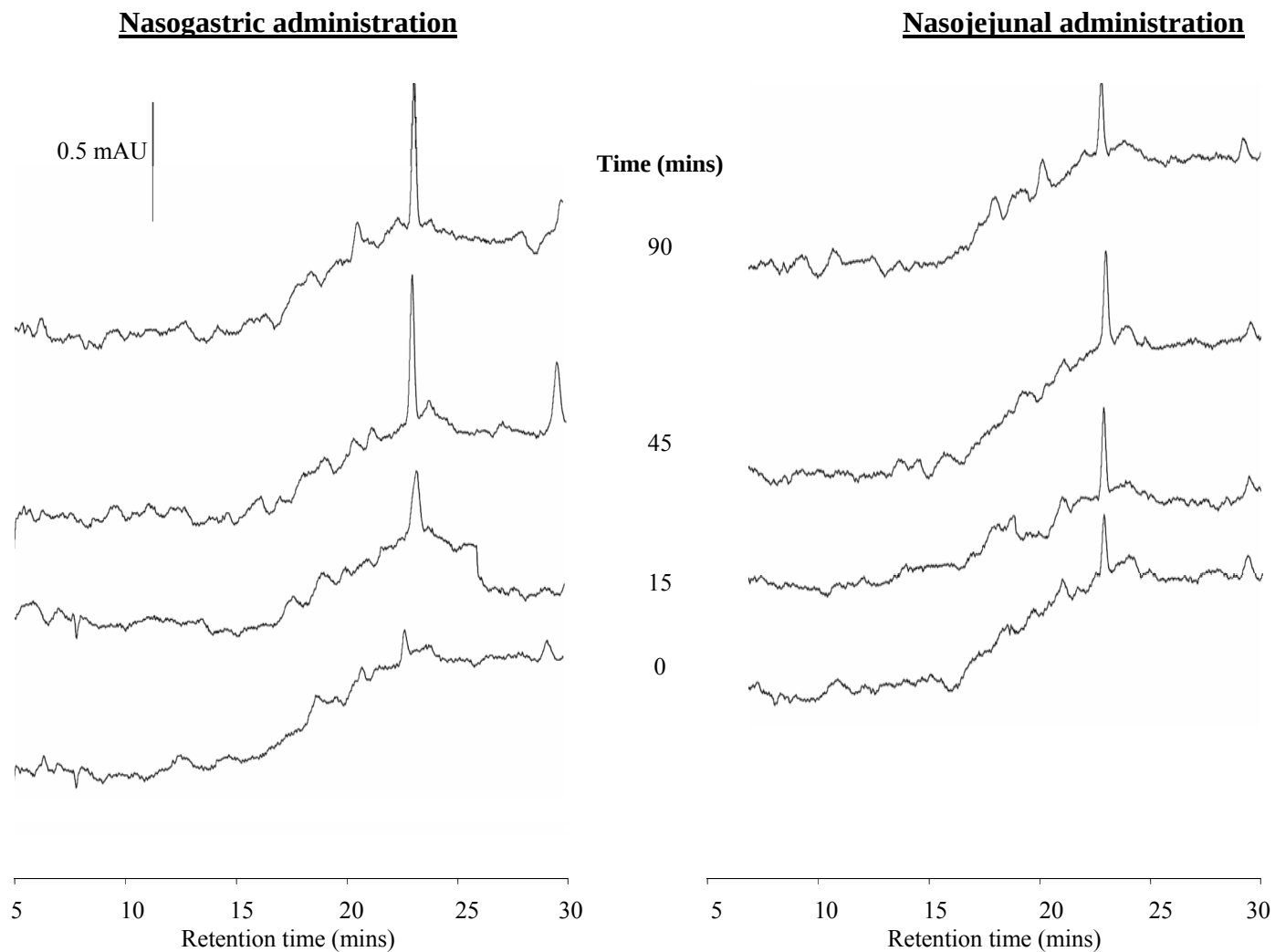


Figure 6.12: HPLC traces of serial bile samples obtained from one patient each following administration of a single dose of mirtocyan into the stomach or jejunum

6.4 DISCUSSION

A limitation of many published animal and human studies involving administration of anthocyanins has been the lack of analytical chemistry. Often compounds have been administered without determining exact anthocyanin constituents and quantities. Moreover, levels of anthocyanins achieved in biomatrices have rarely been measured in studies with a chemoprevention related rationale. For example, in a study by Kresty et al. 10 patients with Barrett's oesophagus received up to up to 45 g of lyophilised black raspberry powder for 6 months. No analytical chemistry was performed, either to analyse the anthocyanin content of the powder or levels obtained in biomatrices following its consumption (Kresty et al., 2006). Without analytical chemistry a comparison of the results of separate studies, and comparison with preclinical data, is not feasible. In view of these factors this pilot study was initiated using a fully standardised, quantified anthocyanin mixture. Anthocyanins were quantified in a range of biomatrices using HPLC and mass spectrometric techniques.

Levels of anthocyanins observed in malignant colorectal tissue following consumption of 5.6 g of mirtocyan daily were approximately 45-fold lower than pharmacologically efficacious levels measured in the intestinal mucosa of Apc^{Min} mice following dietary supplementation with the same compound at 0.3% in the diet (Cooke et al., 2006a). However, when the diet of Apc^{Min} mice was supplemented with up to 0.3% cyanidin-3-.glucoside, total anthocyanin levels of only 43 ng/g of intestinal tissue were associated with a 45% reduction in adenoma number (Cooke et al., 2006a). These anthocyanin levels were approximately 4-fold lower than that

measured in malignant colorectal tissue obtained from patients who consumed 5.6 g of mirtocyan daily. This study therefore illustrates that consumption of a modest oral dose of mirtocyan can achieve levels of anthocyanins in colorectal tissue within an order of magnitude to those associated with pharmacological effects *in vivo*. Anthocyanin species observed in the intestinal mucosa in this study were similar to those described following administration of mirtocyan to Apc^{Min} mice and comprised parent glycosides, glucuronides and methylated derivatives (Cooke et al., 2006a).

Approximate urinary anthocyanin concentrations of up to 3 µg/ml were achieved following consumption of 5.6 g of mirtocyan daily. The recovery of quantifiable amounts of anthocyanins from urine at all three dose levels suggests that it should be feasible in future intervention trials to use urinary anthocyanin concentrations as an indicator of adherence. In the light of the intense color of the anthocyanin molecule as the flavylium tautomer, development of a simple and inexpensive colorimetric method for anthocyanin determination in urine extracts seems possible.

A characteristic of anthocyanins, shared with other polyphenols, is poor systemic bioavailability (*Section 1.2*). Concentrations of anthocyanins observed in peripheral plasma in this study, of up to 117 ng/ml, are similar to those observed in previous pharmacokinetic studies (*Table 1.1*). Human studies have not previously involved analysis of liver tissue for anthocyanins. Although not directly detected in liver tissue in this study, detection in portal plasma and bile does imply presence in the liver, but levels are likely to be very low. These results do not support further development of

anthocyanins as potential chemopreventive agents for malignancies in which systemic bioavailability is important, including hepatic malignancy.

Levels of anthocyanins measured in plasma and urine samples following gastric administration of mirtocyan far exceeded levels observed following jejunal delivery. These results support the hypothesis, formulated on the basis of animal experiments (*Section 1.2.2*), that the predominant site for absorption of anthocyanins is the stomach. However, it should be emphasised that this data is preliminary. It is based on only a small number of patients (n=2) and therefore no statistical analysis is possible. Studies in humans, such as that described, in which absorption is compared in different regions of the bowel is a challenging task, and it is difficult, for example, to prove that anthocyanins were retained in the stomach and not emptied into the duodenum. In retrospect, a pre-operative gastroscopy to elicit pyloric competence may have been helpful. This element of the study was undertaken during liver resections in which the Pringle manoeuvre was utilised, however, this setting provided easy access to serial bile and portal blood samples. Although the Pringle manoeuvre may have impaired absorption when mirtocyan was administered into the stomach and jejunum, this manoeuvre was utilised in both patients. It is therefore unlikely that was solely responsible for the dramatic differences observed in plasma and urinary anthocyanin concentrations.

In summary, results of this study are consistent with previous data showing that anthocyanins possess low systemic bioavailability. However, anthocyanin levels

achieved in the colorectal mucosa are within an order of magnitude to those which have produced pharmacodynamic effects consistent with chemoprevention *in vivo*.

CHAPTER 7

RESULTS

7.1 INTRODUCTION

Numerous studies have shown that anthocyanins (anthocyanidins and anthocyanins) possess antiproliferative effects in cell lines derived from a variety of malignant tissues (Lazze et al., 2004; Olsson et al., 2004; Zhang et al., 2005). Generally, anthocyanidins have shown more potent inhibition of growth than their anthocyanin counterparts (Zhang et al., 2005), however, anthocyanidins are far more prone to chemical decomposition than the glycosides (*Section 1.4*). To date, the growth inhibitory properties of mirtocyan have not been investigated *in vitro*. HT-29 (malignant) and HCEC (non-malignant) are examples of human colorectal cell lines frequently used for *in vitro* experiments.

Anthocyanins and anthocyanidins, especially cyanidin and delphinidin, have undergone extensive mechanistic studies. These molecules engage a range of mechanisms which interfere with the process of carcinogenesis, including effects on apoptosis, COX-2 and antioxidant status (*Section 1.4*). The aim of the work described in this chapter was to assess whether concentrations of mirtocyan associated with growth inhibitory effects *in vitro*, were within an order of magnitude of anthocyanin concentrations achieved in human biomatrices following consumption of mirtocyan. Putative surrogate biomarkers of chemopreventive activity for use in the clinical trial were chosen on the basis of this *in vitro* data. The effects of consumption of mirtocyan on potential biomarkers of chemopreventive activity in colorectal tissue, plasma and urine were determined. Proliferation (Ki-67), apoptosis (cleaved caspase-3) and inflammation (COX-2) were assessed in colorectal tumour tissue before and

after mirtocyan consumption. In addition oxidative DNA damage was assessed in plasma and urine, by measurement of M₁dG and 8-oxo-dG, respectively. Effects of anthocyanins on the insulin-like growth factor system were investigated through measurement of insulin-like growth factor-1 (IGF-1), insulin-like growth factor binding protein-3 (IGFBP-3) and the ratio of IGF-1 to IGFBP-3. A number of polyphenolic compounds have been shown to affect the insulin-like growth factor axis (*Section 1.4.7*).

THE EFFECTS OF MIRTOCYAN IN VITRO

7.2 STABILITY OF MIRTOCYAN IN VITRO

Prior to assessing the effect of mirtocyan on cell growth, preliminary experiments were conducted to assess the stability of anthocyanins in mirtocyan under standard cell culture conditions (*Figure 7.1*). Within 12 hours, the concentrations of all constituent anthocyanins in mirtocyan, had declined to less than 50% of their initial concentrations in cell culture media. By 24 hours, less than 10% of anthocyanins remained. No marked differences in anthocyan stability were observed when mirtocyan was incubated in media, with and without HT-29 cells. The velocity of disappearance of delphinidin glycosides from cell culture media, was more rapid than that of all other anthocyanins. Relatively high concentrations of peonidin glycosides were detected at all time points over the 24 hour period studied.

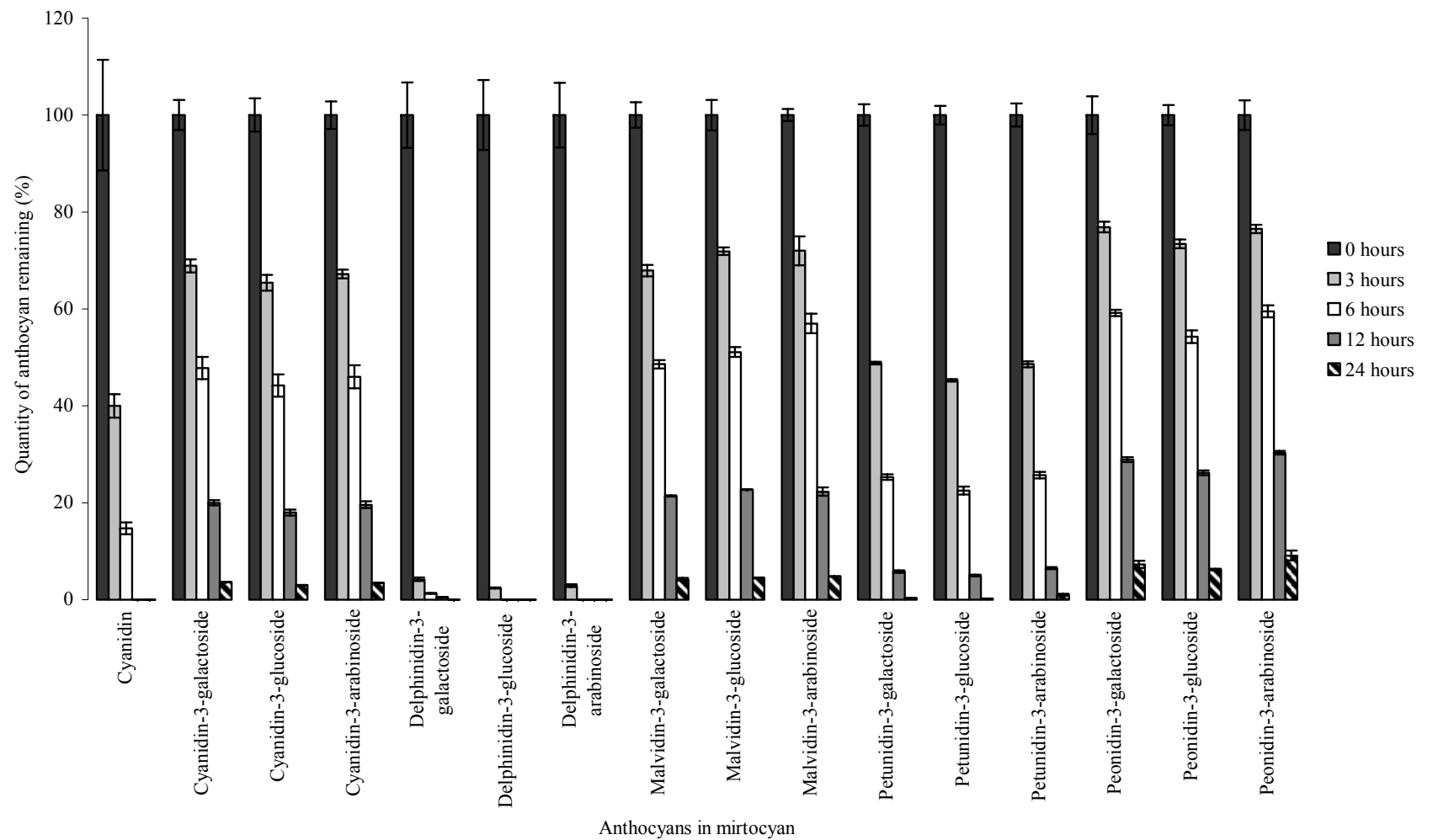


Figure 7.1: The stability of anthocyanins in mirtocyan under cell culture conditions over 24 hours

Analysis of anthocyan concentrations in cell culture media was by HPLC

7.3 THE EFFECT OF SINGLE AND DAILY DOSES OF MIRTOCYAN ON THE GROWTH OF COLON CELLS

The effect of a single dose of mirtocyan on the growth of malignant (HT-29) and non-malignant (HCEC) cells was investigated (*Figures 7.2 and 7.3*). In an attempt to tenuously replicate conditions of agent exposure in the clinical study, the experiment was repeated and cells exposed to daily doses of mirtocyan (*Figure 7.4 and 7.5*). The hypothesis was tested that exposure to daily doses of mirtocyan causes greater growth inhibitory effects than exposure to a single dose.

Mirtocyan seemed to exert greater growth inhibitory effects on the HT-29 cell line (*Figure 7.6*). Single and daily mirtocyan doses of 100 and 200 µg/ml, significantly inhibited the growth of HT-29 cells at all time points studied. Exposure of HCEC cells to single and daily doses of 200 µg/ml, significantly inhibited growth at all time points. Superior growth inhibition was observed when both cell lines were exposed to daily doses of mirtocyan, rather than a single dose. The IC₅₀ values were 154.3 and 131.2 µg/ml when HT-29 cells were exposed to single and daily doses of mirtocyan for 7 days, respectively. Corresponding IC₅₀ values for HCEC cells were 172.2 and 136.22 µg/ml.

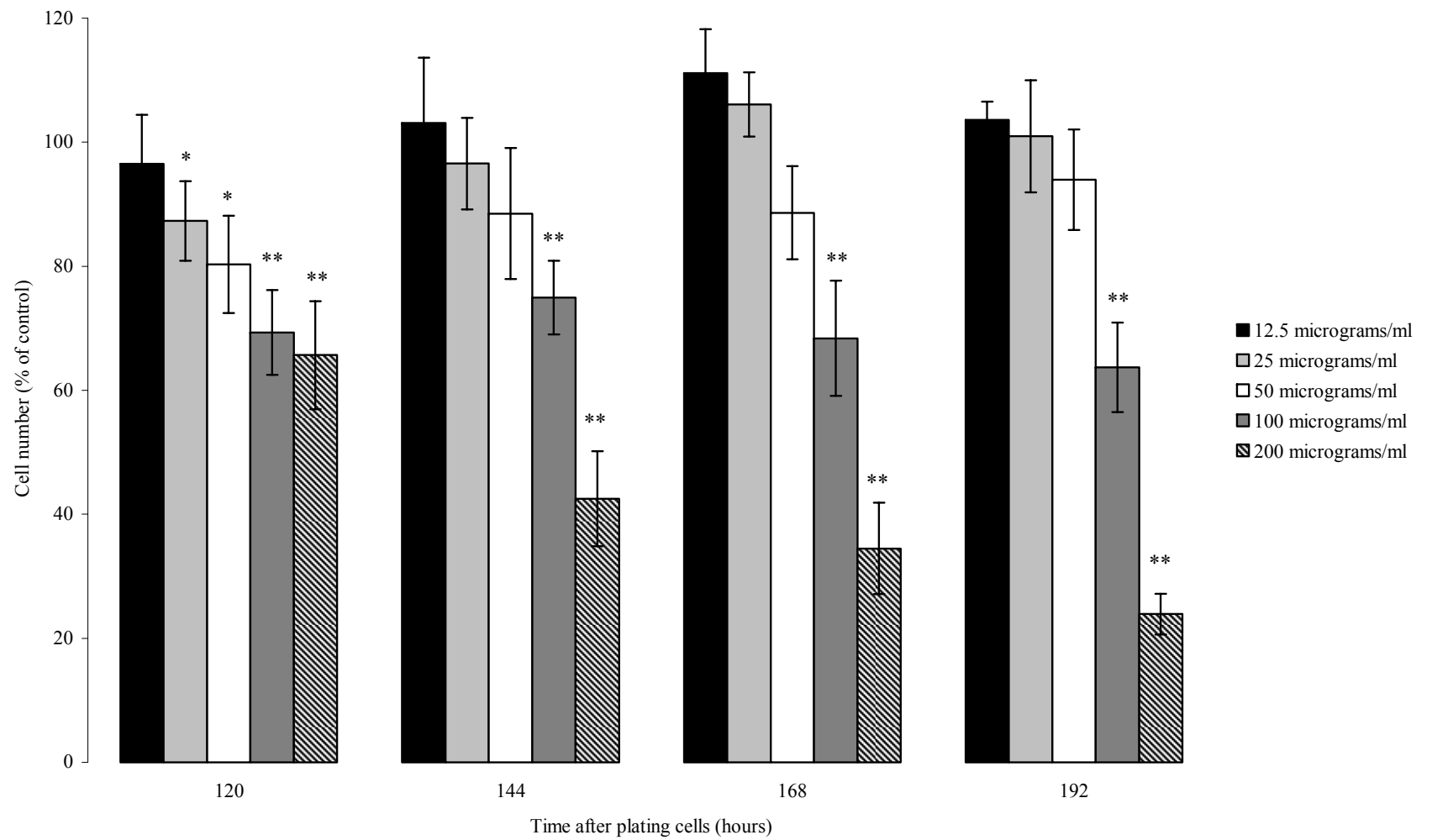


Figure 7.2: The effect of a single dose of mirtocyan on the growth of HT-29 cells

Cells were exposed to a single dose of mirtocyan in culture media and counted at time points ranging from 120 to 192 hours after plating. * $p < 0.05$, ** $p < 0.001$, compared to the relevant control

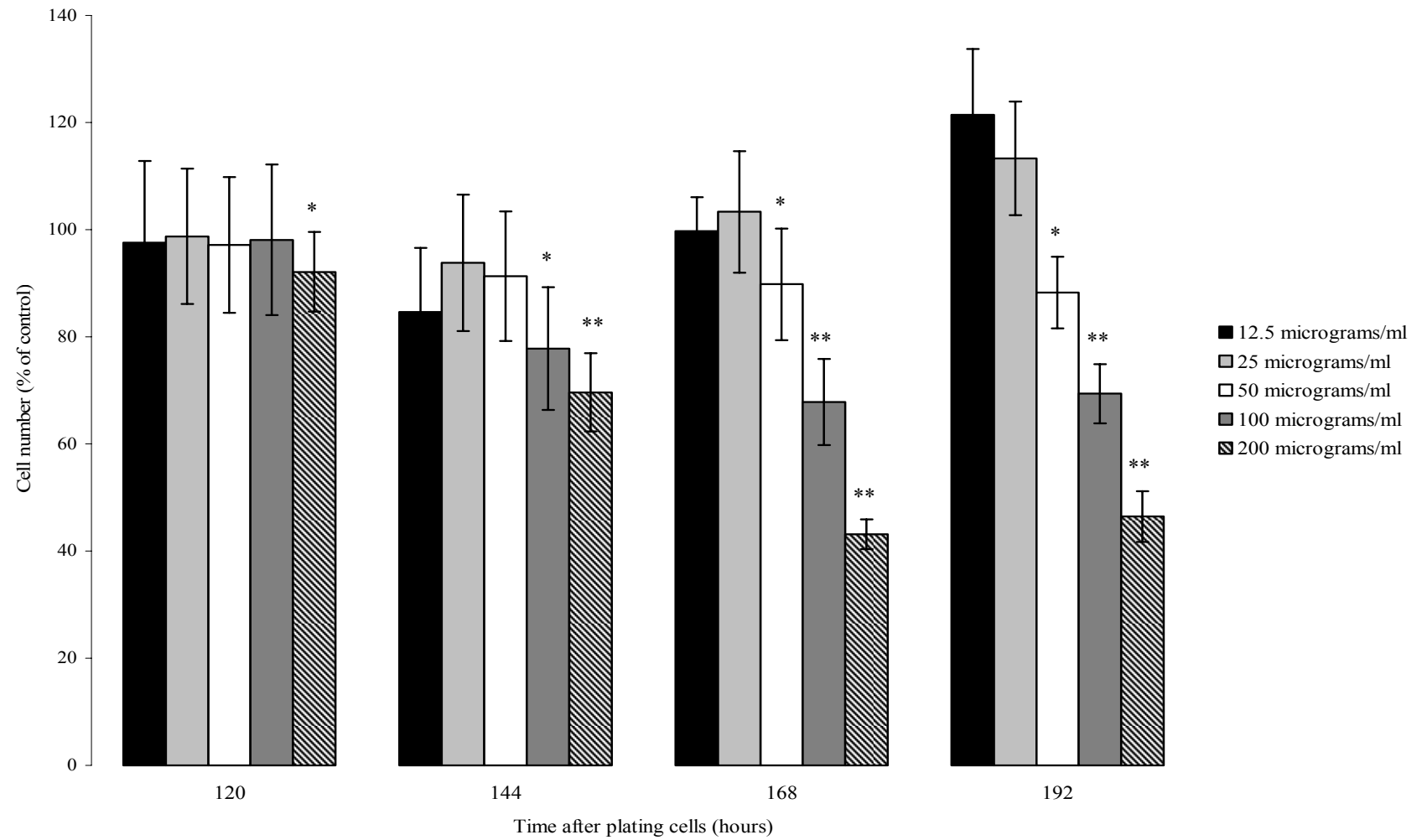


Figure 7.3: The effect of a single dose of mirtocyan on the growth of HCEC cells

Cells were exposed to a single dose of mirtocyan in culture media and counted at time points ranging from 120 to 192 hours after plating. * $p < 0.05$, ** $p < 0.001$, compared to the relevant control

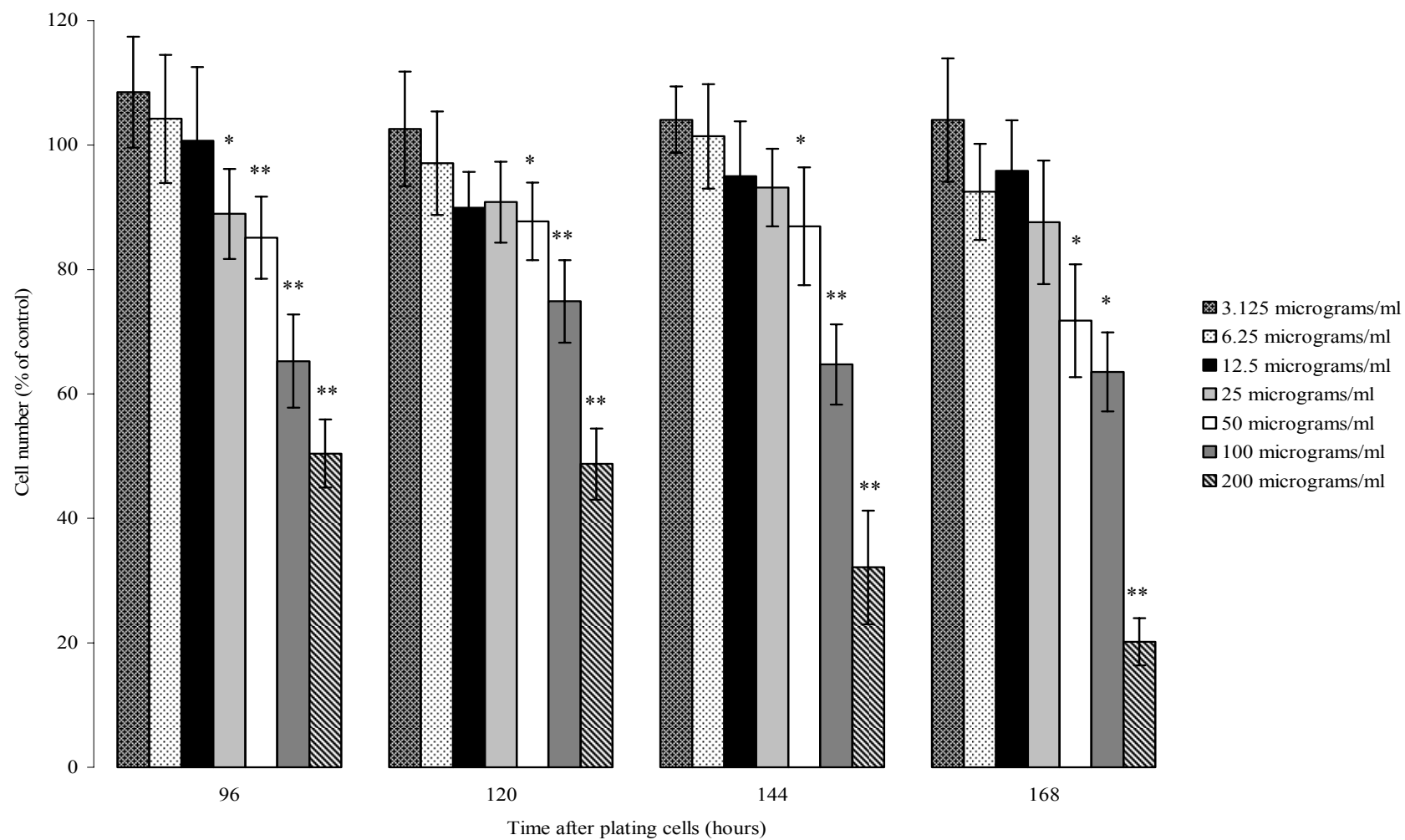


Figure 7.4: The effect of a daily dose of mirtocyan on the growth of HT-29 cells

Culture media containing mirtocyan was replaced daily and cells counted at time points ranging from 96 to 168 hours after plating. * $p < 0.05$, ** $p < 0.001$, compared to the relevant control

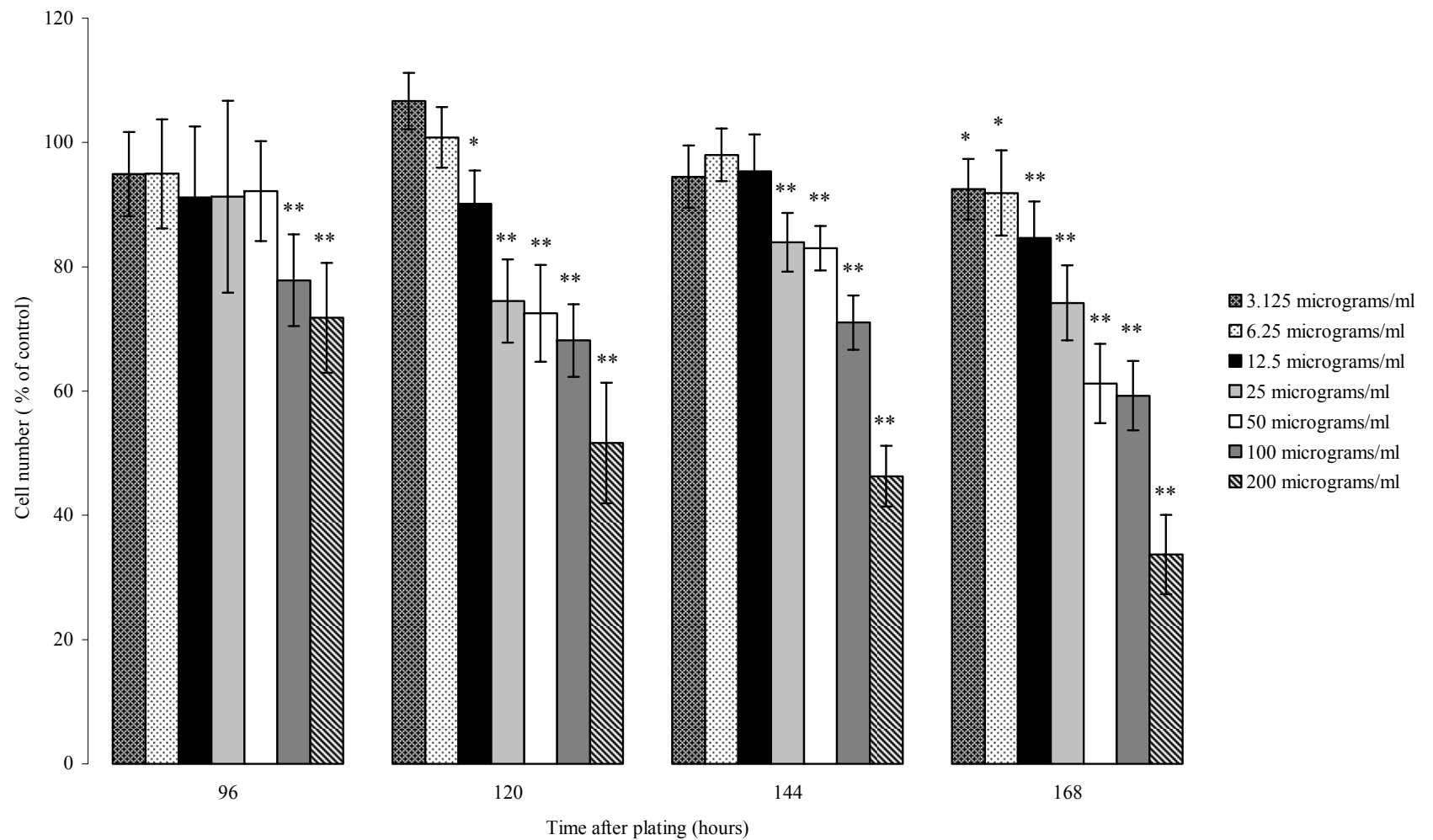


Figure 7.5: The effect of a daily dose of mirtocyan on the growth of HCEC cells

Culture media containing mirtocyan was replaced daily and cells counted at time points ranging from 96 to 168 hours after plating. * $p < 0.05$, ** $p < 0.001$, compared to the relevant control

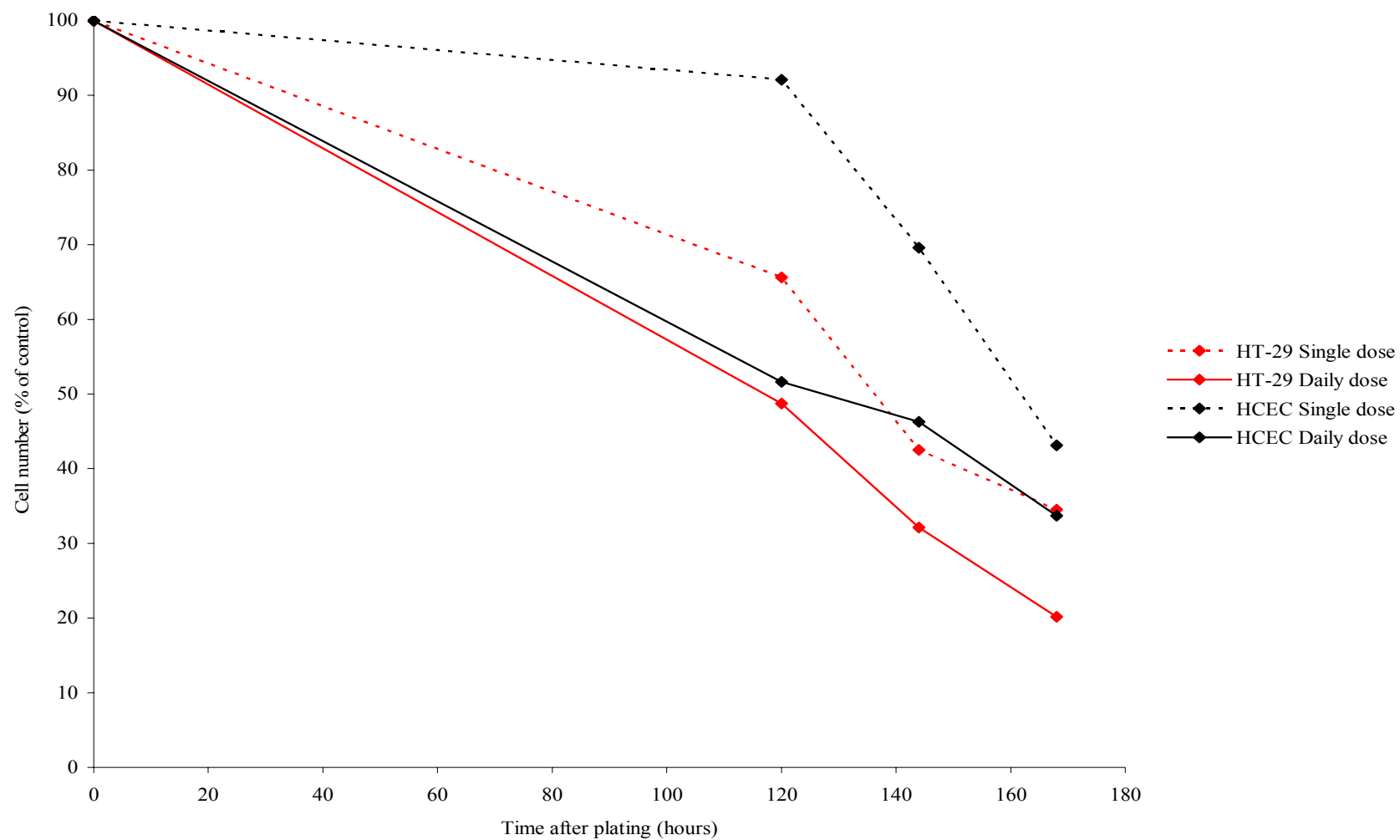


Figure 7.6: The effect of single and daily dosing with mirtocyan (200 µg/ml) on the growth of HT-29 and HCEC cells

Cells were exposed to 200 µg/ml of mirtocyan in cell culture media, either as a single dose or daily doses. Cell numbers were counted at time points ranging from 120 to 168 hours after plating

7.4 THE EFFECT OF MIRTOCYAN ON THE CELL CYCLE

Effects of incubation with mirtocyan on the cell cycle were investigated in HT-29 and HCEC cell lines. No significant effects were observed when HT-29 cells were incubated with concentrations of mirtocyan ranging from 12.5 to 200 µg/ml, for 24 and 48 hours. However, when HCEC cells were incubated with 100 and 200 µg/ml of mirtocyan for 48 hours, arrest in the G₂/M phase was observed (*Figure 7.7*).

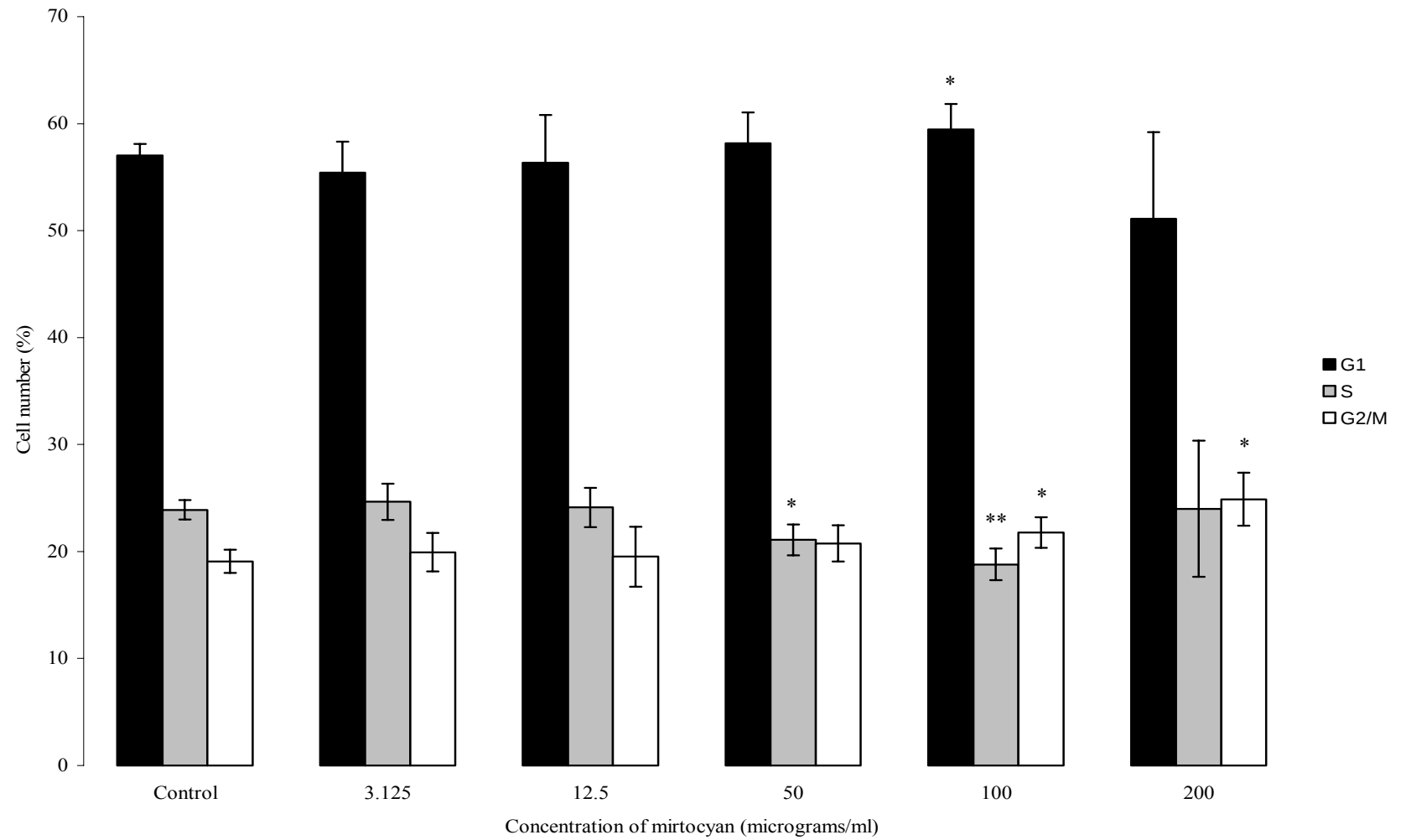


Figure 7.7: The effect of incubation with mirtocyan for 48 hours on the cell cycle of HCEC cells

Cells were exposed to a single dose of mirtocyan in cell culture media for 48 hours and cell cycle analysis undertaken using flow cytometry. * $p < 0.05$, ** $p < 0.001$, compared to the relevant control

7.5 THE EFFECT OF MIRTOCYAN ON BIOMARKERS IN BLOOD

The hypotheses were tested that consumption of mirtocyan leads to increases in IGFBP-3 levels, and suppresses levels of IGF-1, in addition to the molar ratio of IGF-1 to IGFBP-3 in plasma. Levels of the malondialdehyde-DNA adduct M₁dG were measured in whole blood samples to test the hypothesis that mirtocyan suppresses levels of this marker of oxidative DNA damage. Pre-treatment blood samples were obtained at recruitment and post-treatment blood samples were obtained an hour after the penultimate dose of mirtocyan.

7.5.1 INSULIN-LIKE GROWTH FACTOR SYSTEM

INSULIN-LIKE GROWTH FACTOR-1

Levels of IGF-1 were 239 ± 54 ng/ml in pre-treatment plasma samples and 234 ± 53 ng/ml in plasma samples obtained following the consumption of mirtocyan ($p=0.168$) (*Table 7.1 and Figure 7.8*). Considering individual dose groups in isolation, decreases in IGF-1 levels of 4.5 and 10.6 ng/ml were evident in groups that consumed 1.4 and 2.8 g of mirtocyan daily, respectively. The reduction in IGF-1 levels associated with consumption of 1.4 g of mirtocyan daily approached statistical significance ($p=0.060$). A slight, but not significant, increase in IGF-1 levels of 0.4 ng/ml was observed in patients who consumed 5.6 g of mirtocyan daily ($p=0.956$).

Table 7.1: IGF-1 levels in peripheral plasma samples before and after the consumption of mirtocyan

Daily dose of mirtocyan (g)	IGF-1 in pre-treatment plasma samples (ng/ml)	IGF-1 in post-treatment plasma samples (ng/ml)	p-value
1.4 (n = 10 patients)	217.0 ($\pm$ 57.2)	212.5 ($\pm$ 57.3)	0.060
2.8 (n = 7 patients)	271.7 ($\pm$ 63.2)	261.1 ($\pm$ 62.0)	0.337
5.6 (n = 7 patients)	238.1 ($\pm$ 14.1)	238.5 ($\pm$ 113.9)	0.956
All groups combined (n = 24 patients)	239.1 ($\pm$ 54.0)	234.3 ($\pm$ 52.6)	0.168

p-values were calculated using the Paired student's *t* test

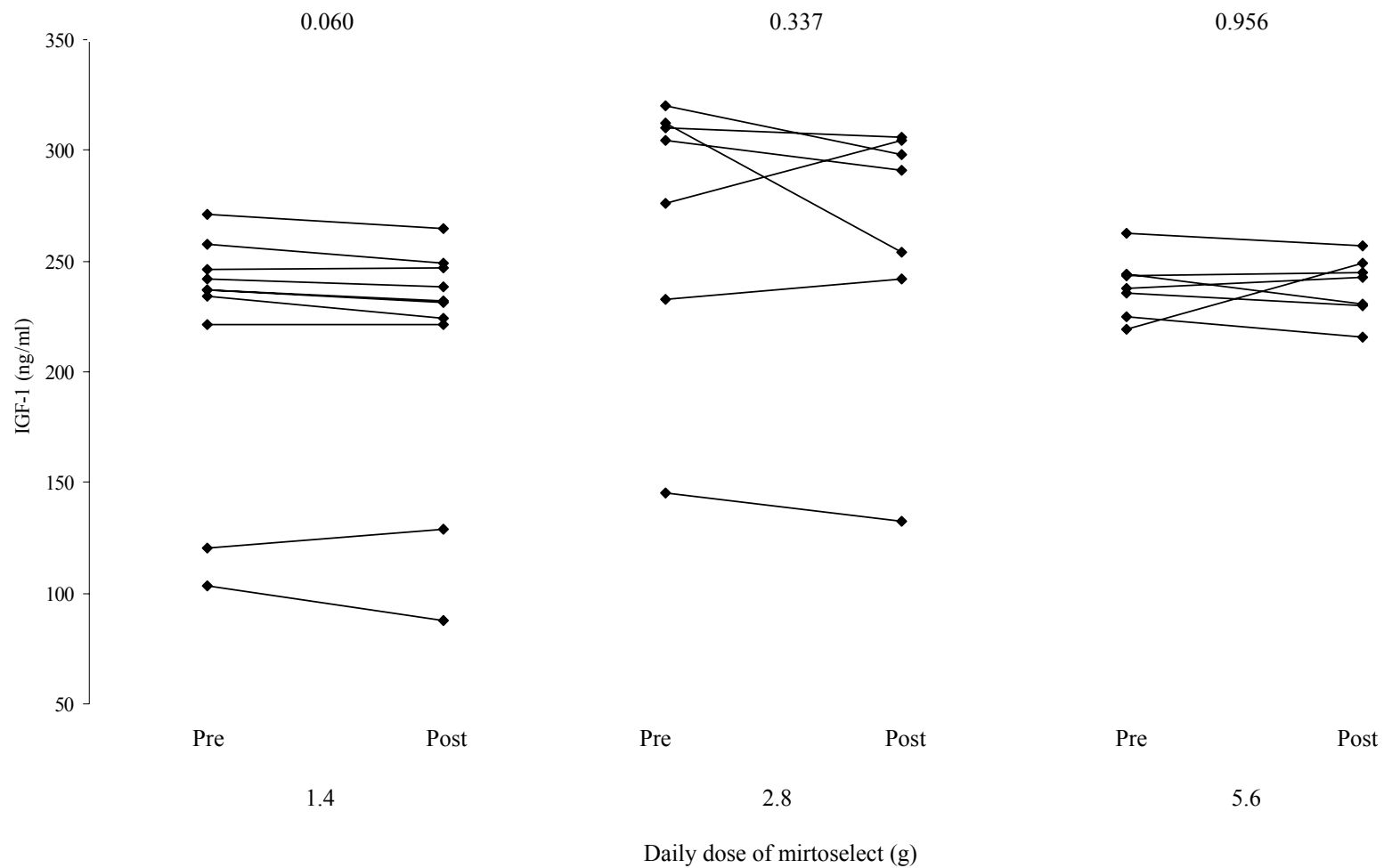


Figure 7.8: Individual changes in plasma IGF-1 levels before and after the consumption of mirtocyan

Analysis of IGF-1 was undertaken using ELISA kits. The assay was validated and performed according to the instructions of the manufacturer (R&D systems). p-values are shown above the relevant graphs which have been calculated using the Paired student's *t* test

INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-3

Levels of IGFBP-3 were unaffected by treatment with mirtocyan. Levels of IGFBP-3 were 2327 ± 279 ng/ml prior to treatment with mirtocyan and 2301 ± 326 ng/ml following treatment ($p=0.733$). Analysis of individual dose groups did not reveal any changes which were statistically significant, or which approached significance (*Figure 7.9*).

INSULIN-LIKE GROWTH FACTOR-1: INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-3

The molar ratios of IGF-1 to IGFBP-3 were calculated for individual pre- and post-treatment plasma samples as detailed in *Section 4.3.5*. The ratios of IGF-1 to IGFBP-3 were 0.3890 ± 0.0685 and 0.3894 ± 0.0782 before and after treatment with mirtocyan, respectively ($p=0.974$). When individual dose groups were analyzed in isolation, no effects on IGF-1 to IGFBP-3 ratios were evident (*Figure 7.10*).

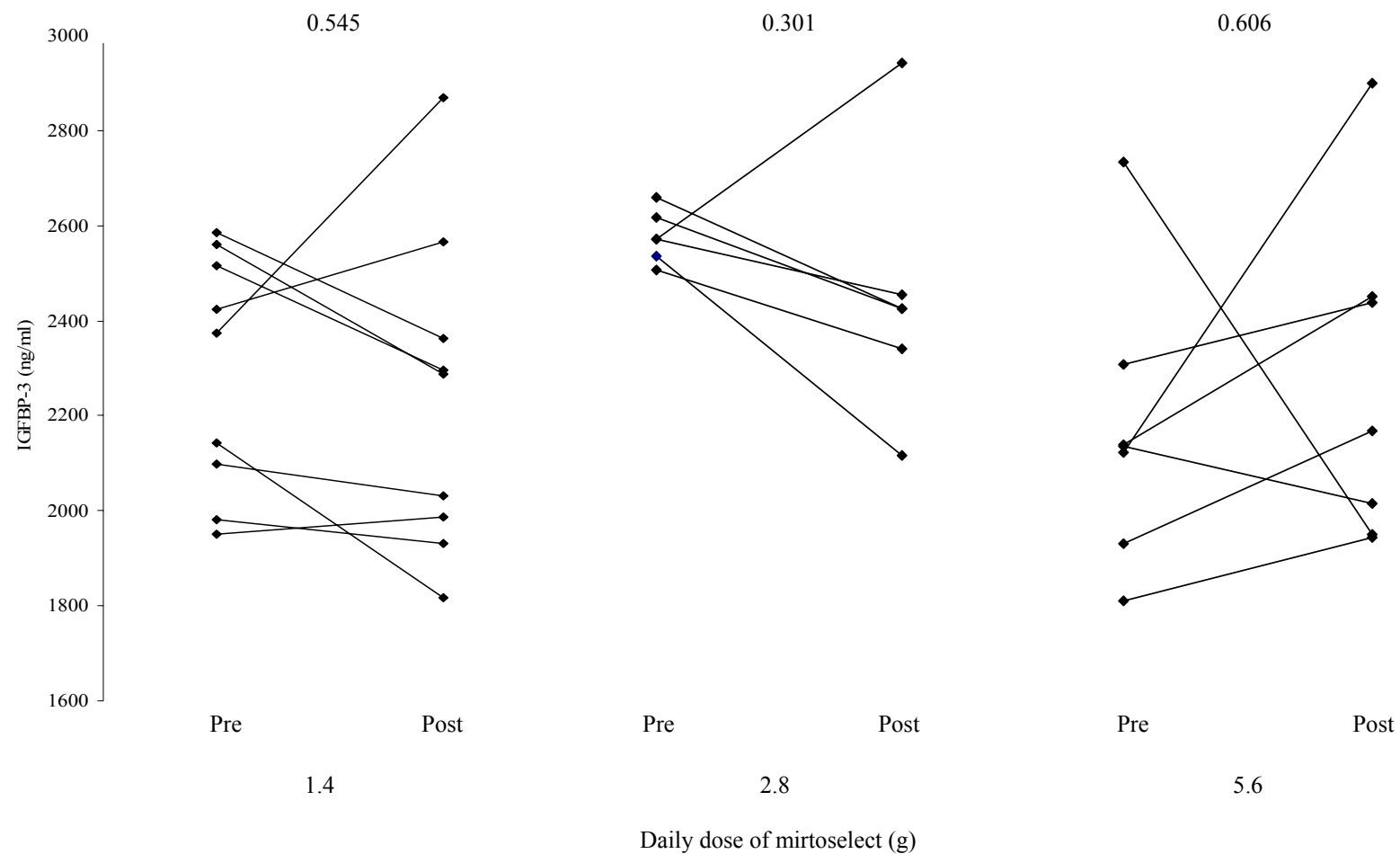


Figure 7.9: Individual changes in plasma IGFBP-3 levels before and after the consumption of mirtocyan

Analysis of IGFBP-3 was undertaken using ELISA kits. The assay was validated and performed according to the instructions of the manufacturer (R&D systems). p-values are shown above the relevant graphs which have been calculated using the Paired student's t test.

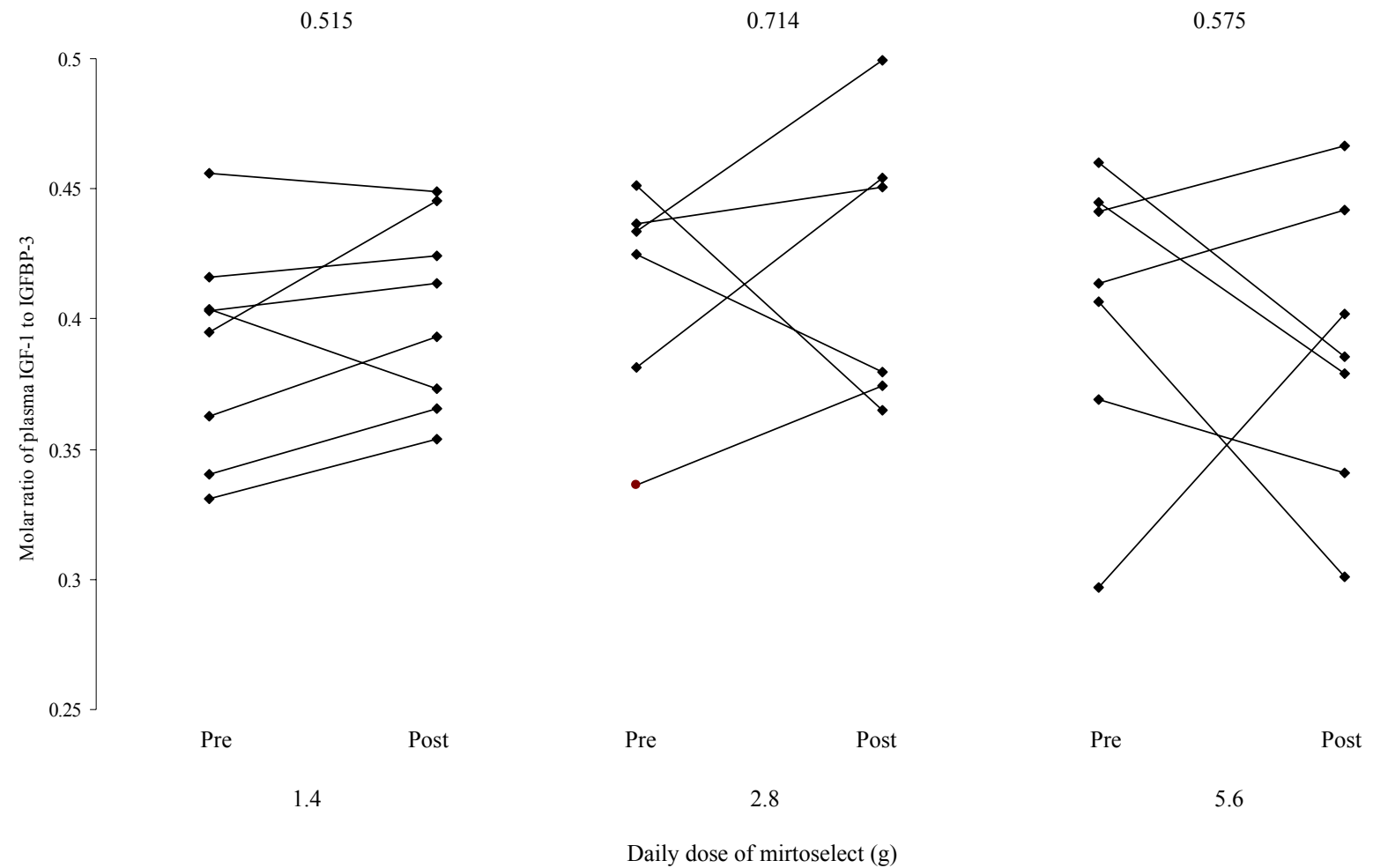


Figure 7.10: Individual changes in the molar ratios of plasma IGF-1 to IGFBP-3 before and after the consumption of mirtocyan

Analysis of IGF-1 and IGFBP-3 was undertaken using ELISA kits. The assays were validated and performed according to the instructions of the manufacturer (R&D systems). IGF-1:IGFBP-3 was calculated as described in *Section 4.3.5*. p-values are shown above the relevant graphs which have been calculated using the Paired student's t test.

7.5.2 MALONDIALDEHYDE-DNA ADDUCT

Contrary to the hypothesis that consumption of mirtocyan suppresses levels of M₁dG in whole blood, a trend towards an increase in levels was observed (*Figure 7.11*). Levels of M₁dG were 42 ± 20 and 46 ± 23 adducts per 10^7 nucleotides, before and after treatment with mirtocyan, respectively. However, this increase was not statistically significant ($p=0.119$). When individual dose groups were analyzed in isolation, increases in M₁dG levels of 2, 9 and 2 adducts per 10^7 nucleotides, were observed in patients who consumed 1.4, 2.8 and 5.6 g of mirtocyan daily, respectively. The only increase which was statistically significant occurred in the group that consumed 2.8 g of mirtocyan daily ($p=0.018$).

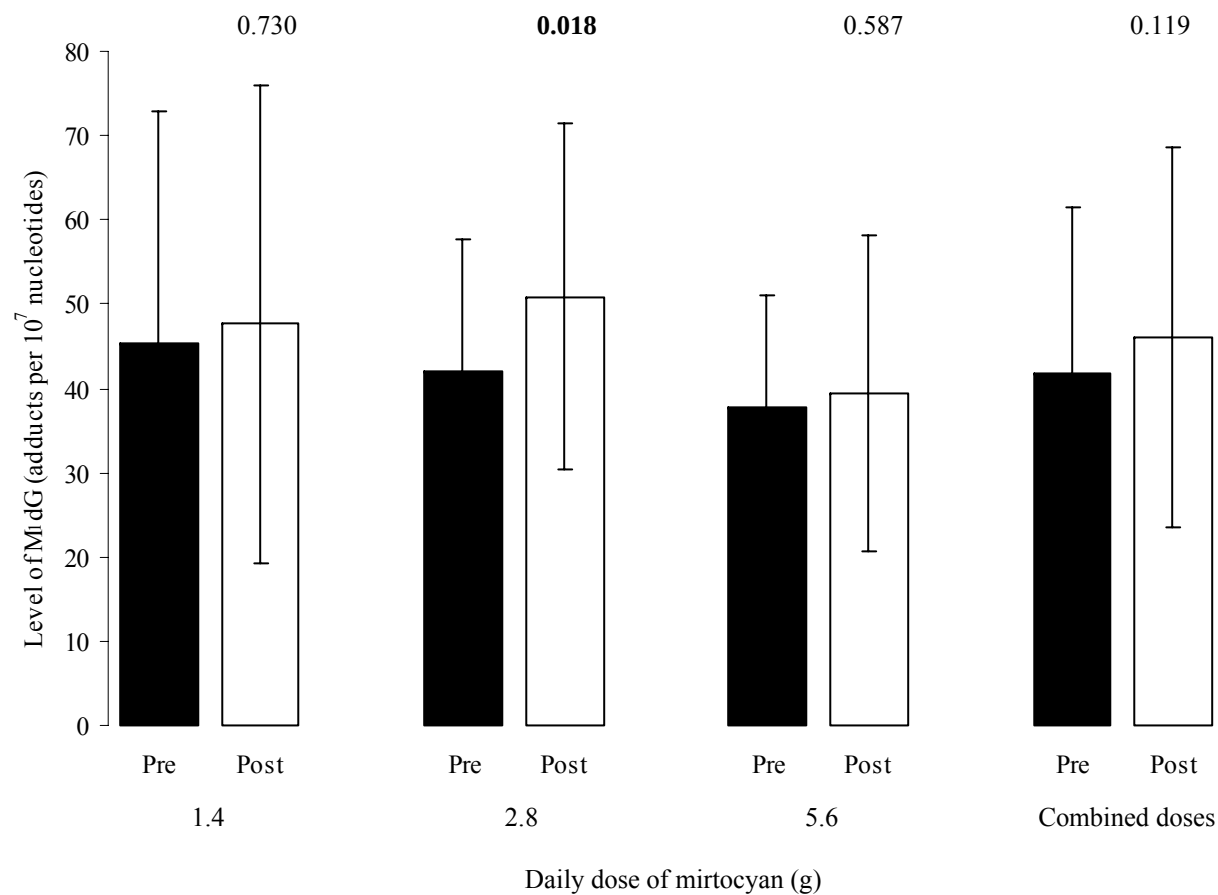


Figure 7.11: Levels of M₁dG in peripheral blood samples before and after the consumption of mirtocyan

Levels of M₁dG were measured by immunoslot blot. p-values are shown above the relevant columns which have been calculated using the Paired student's *t* test. p-values in bold are statistically significant

7.6 THE EFFECT OF MIRTOCYAN ON BIOMARKERS IN URINE

The hypothesis was tested that consumption of mirtocyan suppresses levels of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG) in urine. Pre-treatment urine samples were obtained at recruitment and post-treatment urine samples were collected for 11.5 ± 1 hours following consumption of the penultimate dose of mirtocyan.

7.6.1 8-OXO-7,8-DIHYDRO-2'-DEOXYGUANOSINE

Levels of urinary 8-oxo-dG were unaffected by the consumption mirtocyan (*Figure 7.12*). Levels in urine before and after treatment with mirtocyan were 1.37 ± 0.56 and 1.42 ± 0.60 pmol per μmol of urinary creatinine, respectively ($p=0.696$). Analysis of individual dose groups failed to reveal any changes in urinary 8-oxo-dG levels which were statistically significant, or which approached significance.

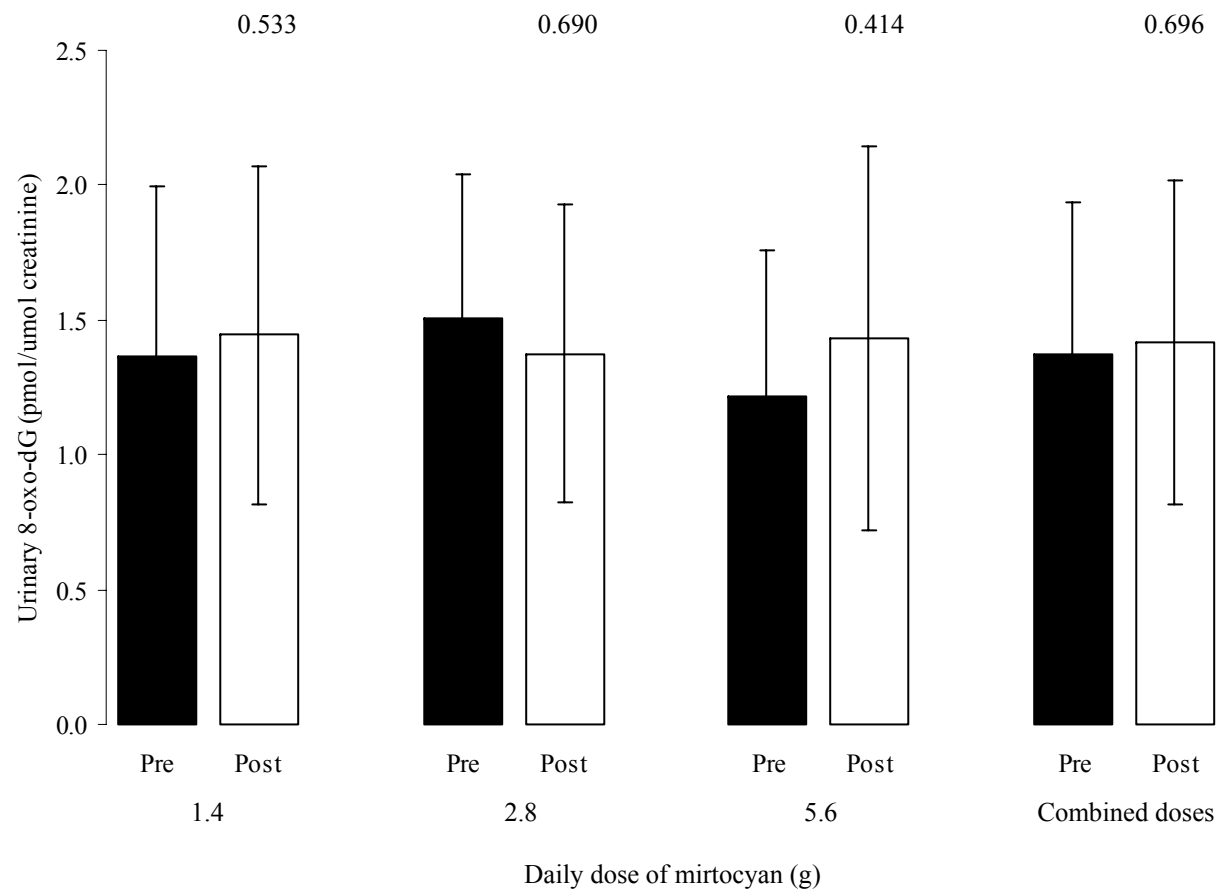


Figure 7.12: Levels of urinary 8-oxo-dG before and after the consumption of mirtocyan

Levels of urinary 8-oxo-dG were measured by LC-MS. p-values are shown above the relevant columns which have been calculated using the Paired student's *t* test

7.7 THE EFFECT OF MIRTOCYAN ON BIOMARKERS IN COLORECTAL TUMOUR TISSUE

Pre-clinical data suggest that anthocyanins exert effects on a range of molecular mechanisms which are fundamental to the process of carcinogenesis, including cell proliferation, apoptosis and inflammation (*Section 1.4*). Immunohistochemical methods were utilized to test the hypotheses that consumption of mirtocyan inhibits cell proliferation (Ki-67), induces apoptosis (cleaved caspase-3) and suppresses inflammation (COX-2) in colorectal tumour tissue.

Colorectal tumour sections, obtained before and after the consumption of mirtocyan, were stained for Ki-67 and cleaved caspase-3, and numbers of positive epithelial cells in six representative, adjacent high power fields (x 400) were counted in each sample (*Section 4.3.8*). Pre-treatment colorectal tumour samples obtained from two patients contained insufficient tissue to provide six adjacent fields, and therefore these patients were excluded from the analysis. Analysis of Ki-67 and caspase-3 were conducted on samples obtained from a total of 12 patients, of whom 4 patients each received 1.4, 2.8 or 5.6 g of mirtocyan daily.

7.7.1 CELL PROLIFERATION (Ki-67)

Ki-67 is a ubiquitous human nuclear protein which is expressed in the G₁-, S- and G₂-phases of the cell cycle, and is widely used to measure the growth fraction of cells in human tumours. An average of 1161 ± 312 and 1330 ± 465 epithelial cells were counted in each pre- and post-treatment colorectal sample stained for Ki-67, respectively.

The proportion of epithelial cells which stained positively for Ki-67 decreased from a pre-treatment value of 84 ± 10 % to 77 ± 12 % following consumption of mirtocyan ($p=0.003$) (*Figures 7.13 and 7.14*). Although a decrease in Ki-67 expression was observed in all dose groups, the extent of this decrease was not proportional to the dose of mirtocyan consumed. Average decreases in Ki-67 expression were 9, 4 and 7 %, in patients who consumed 1.4, 2.8 and 5.6 g of mirtocyan daily, respectively. Considering individual dose groups, the decrease in Ki-67 expression was only statistically significant in the group that consumed 1.4 g of mirtocyan daily ($p=0.021$).

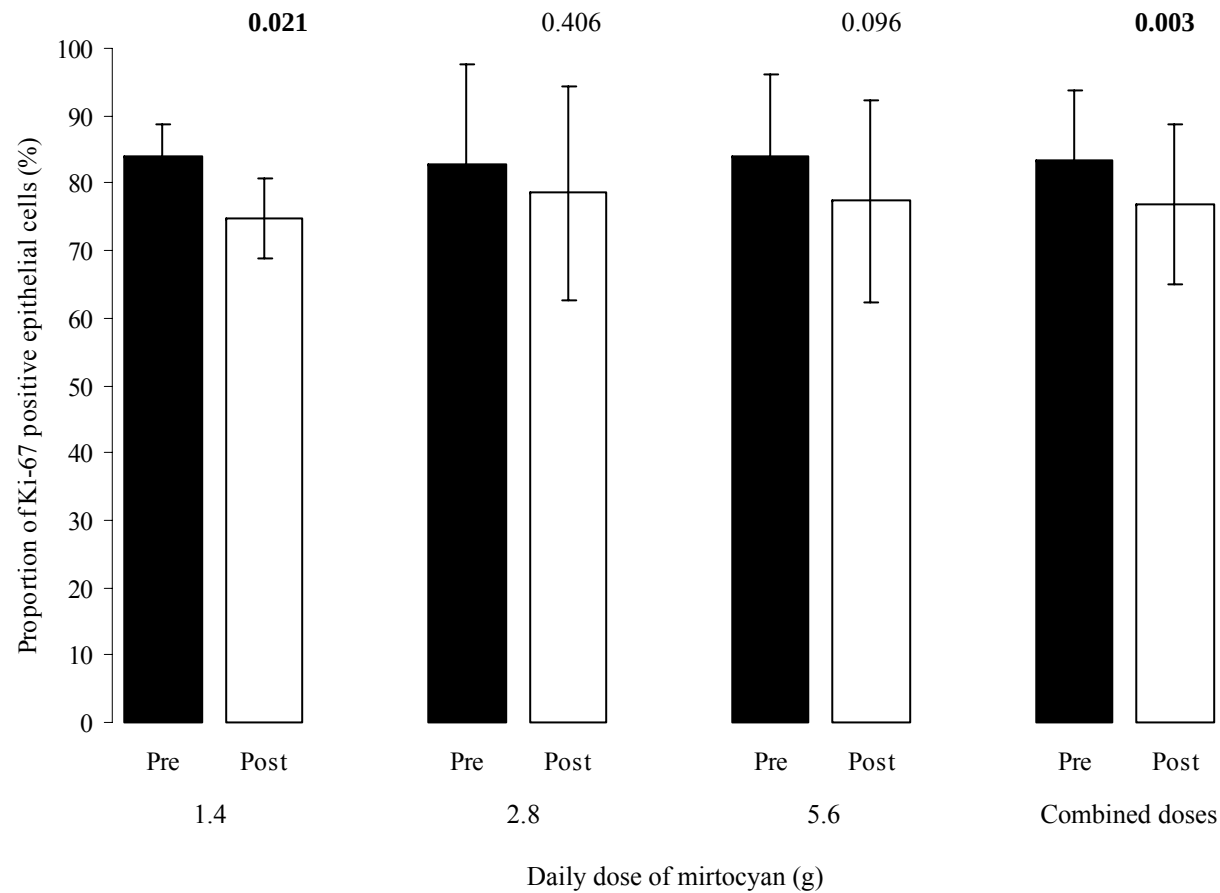
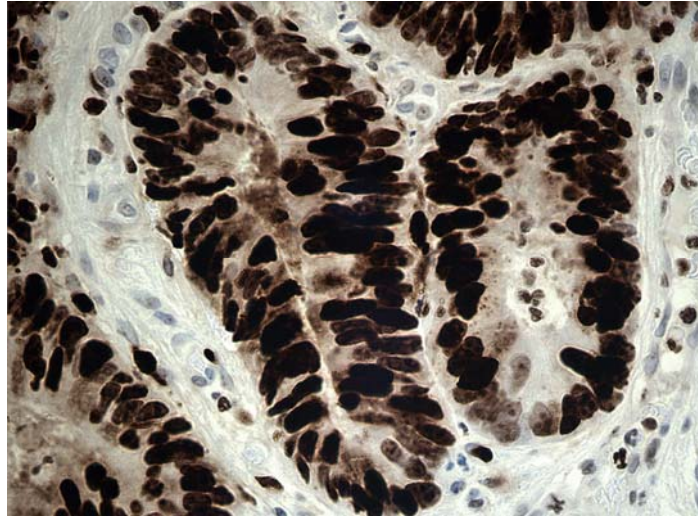


Figure 7.13: Ki-67 expression in colorectal tumour tissue before and after the consumption of mirtocyan

Samples from four patients have been analyzed in each dose group. p-values are shown above the relevant columns which have been calculated using the Paired student's *t* test. p-values in bold are statistically significant

A



B

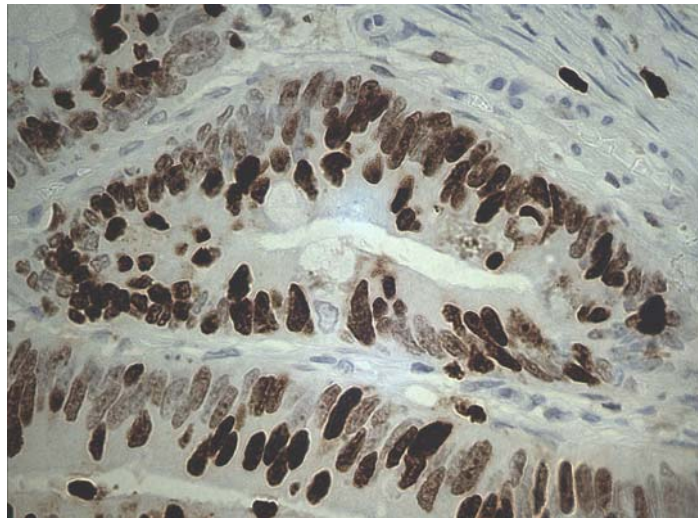


Figure 7.14: Representative immunohistochemical staining for Ki-67 in colorectal tumour tissue before (A) and after (B) the consumption of mirtocyan (x400)

The sections illustrated were obtained from a patient who consumed 1.4 g of mirtocyan daily. Ki-67 positive cells stain brown in these sections.

7.7.2 APOPTOSIS (CLEAVED-CASPASE 3)

An average of 1161 ± 403 and 1516 ± 703 epithelial cells were counted in each pre- and post-treatment colorectal sample stained for caspase-3, respectively. Prior to the consumption of mirtocyan 3.5 ± 1.9 % of colorectal tumour epithelial cells stained positively for caspase-3, and following treatment this number rose to 5.2 ± 2.9 % ($p=0.044$) (*Figure 7.15 and 7.16*). Average increases in caspase-3 expression of 3.2, 1.6 and 0.4 % were observed in patients who consumed 1.4, 2.8 and 5.6 g of mirtocyan daily, respectively. When individual dose groups were evaluated in isolation no statistically significant increases in caspase-3 were measured. However, a trend towards significance was observed, which was inversely related to the dose of mirtocyan consumed. p-values were 0.130, 0.299 and 0.730 in groups that consumed 1.4, 2.8 and 5.6 g of mirtocyan daily, respectively.

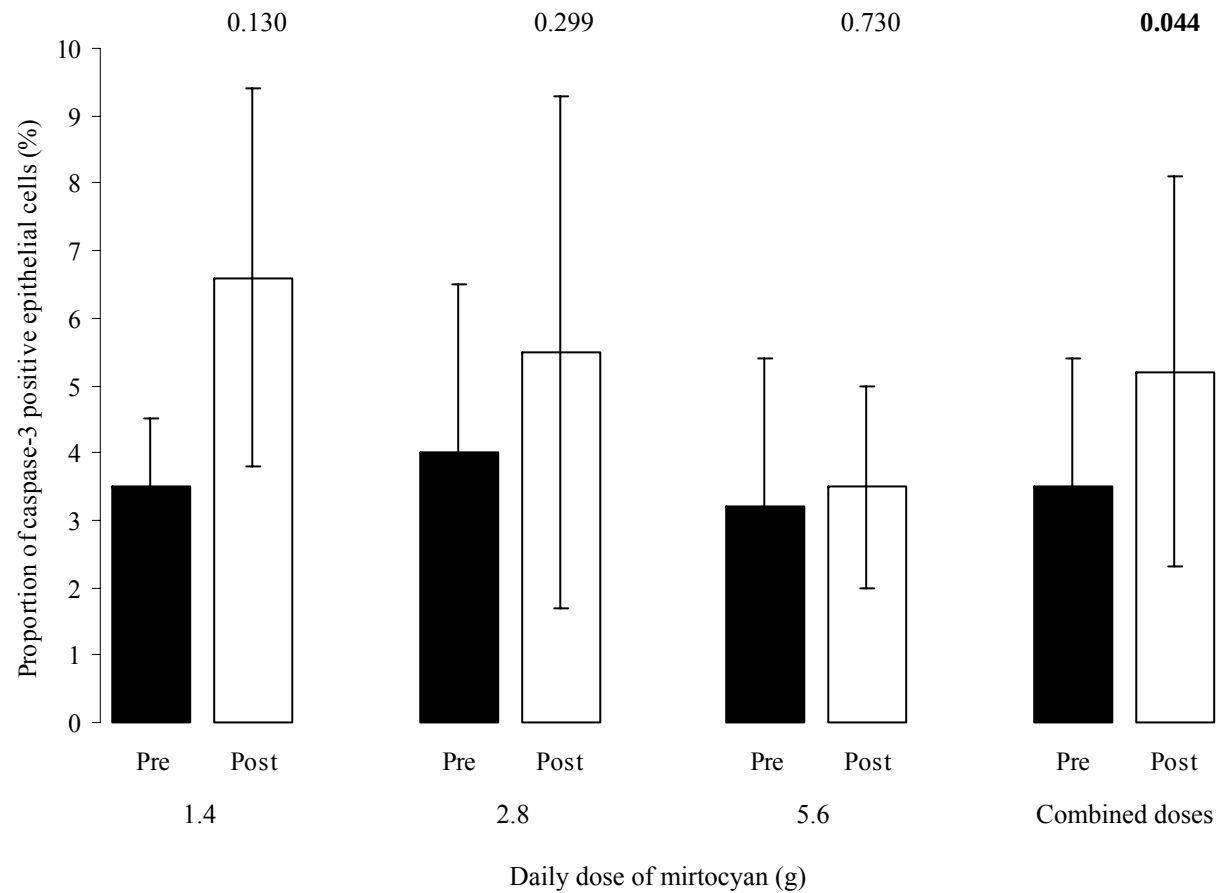
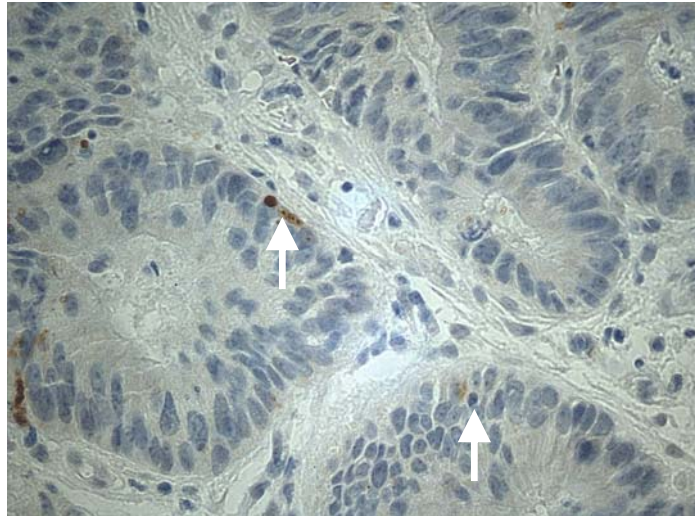


Figure 7.15: Cleaved caspase-3 expression in colorectal tumour tissue before and after the consumption of mirtocyan

Samples from four patients have been analyzed in each dose group. p-values are shown above the relevant columns which have been calculated using the Paired student's *t* test. p-values in bold are statistically significant

A



B

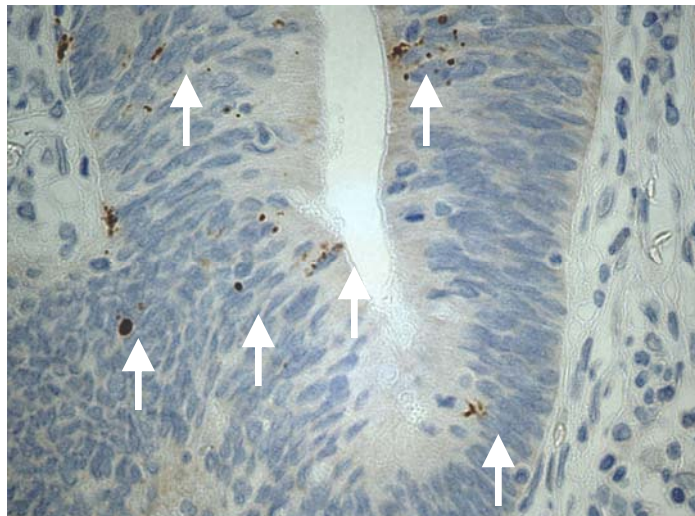


Figure 7.16: Representative immunohistochemical staining for cleaved caspase-3 in colorectal tumour tissue before (A) and after (B) the consumption of mirtocyan (x400)

The sections illustrated were obtained from a patient who consumed 1.4 g of mirtocyan daily. Cleaved caspase-3 positive cells stain brown in these sections (examples indicated by white arrows).

7.7.3 INFLAMMATION (COX-2)

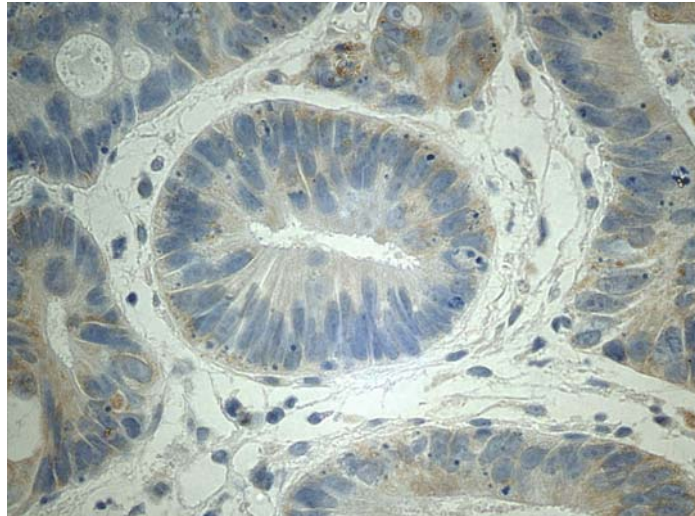
In view of the cytoplasmic location of COX-2 and the generality of staining, sections of colorectal tumour tissue were scored for COX-2 expression using the protocol described in *Section 4.3.8*. Analysis of COX-2 expression was conducted in samples obtained from 14 patients, of whom 5 patients received either 2.8 or 5.6 g of mirtocyan daily, and 4 patients received 1.4 g of mirtocyan daily.

Contrary to the hypothesis that mirtocyan would suppress COX-2 expression, levels of COX-2 were actually significantly greater in colorectal samples obtained following the consumption of mirtocyan, compared to pre-treatment samples ($p=0.025$) (*Table 7.2 and Figure 7.17*). Of the 14 patients evaluated, scores assigned to indicate COX-2 expression increased between pre- and post-treatment samples in 5 patients, and remained static in the remainder. Considering the 5 patients in whom COX-2 expression increased, 1 patient received 1.4 g of mirtocyan daily, another received 2.8 g daily and 3 patients consumed 5.6 g of mirtocyan daily.

Table 7.2: Intensity of COX-2 staining in colorectal tumour tissue obtained from 14 patients before and after treatment with mirtocyan

COX-2 intensity	Negative	Weakly positive	Positive	Strongly positive
Number of patients pre-treatment	0	8	5	1
Number of patients post-treatment	0	3	10	1

A



B

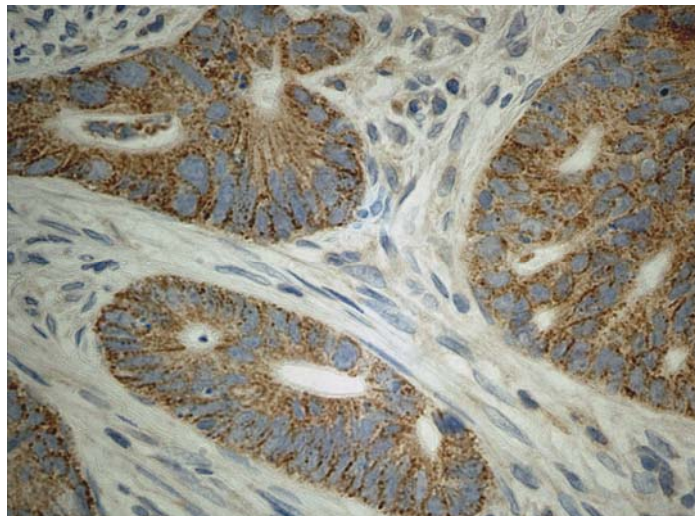


Figure 7.17: Representative immunohistochemical staining for COX-2 in malignant colorectal tumour tissue before (A) and after (B) the consumption of mirtocyan (x400)

The sections illustrated were obtained from a patient who consumed 1.4 g of mirtocyan daily. COX-2 expression is indicated by the brown cytoplasmic stain. The intensity of staining was scored as weakly positive in photograph A and positive in photograph B.

7.8 DISCUSSION

The results presented above provide evidence, for the first time, that the specific mixture of anthocyanins utilised in this study (mirtocyan), inhibits growth of malignant and non-malignant colon cell lines *in vitro*. *In vitro* stability data obtained for mirtocyan concurs with the results of a recent study in which degradation of aglycons was investigated under standard cell culture conditions (Kern et al., 2007). The velocity of disappearance of delphinidin from cell culture medium was more rapid than that of other anthocyanidins, a likely consequence of the substitution pattern on the B-ring (*Figure 1.2*) (Kern et al., 2007). When HT-29 cells were exposed to a daily dose of mirtocyan for 7 days an IC₅₀ value of 131.2 µg/ml was observed. Considering mirtocyan is composed of 36% anthocyanins, the IC₅₀ for HT-29 cells was approximately 260-fold higher than anthocyanin levels in colorectal tumour tissue following consumption of 5.6 g of mirtocyan daily (179 ng/g). Despite this discrepancy a number of pharmacodynamic effects were evident in colorectal tissue, including a reduction in Ki-67 and induction of apoptosis. In addition, anthocyanin levels measured in colorectal tissue were within an order of magnitude to those described, and associated with pharmacodynamic effects, *in vivo* (*Section 6.4*). A possible explanation for the discrepancy between anthocyanin levels which have been associated with chemopreventive effects *in vitro* and *in vivo* is underestimation by current analytical techniques. Current methods involve acidification to transform anthocyanins into the coloured flavylum cation (*Section 1.2*). It may be that a significant proportion of anthocyanin derivatives *in vivo* have undergone metabolic conversion impeding their conversion back to the flavylum cation. An alternative

possibility is that different metabolites are generated *in vitro* and *in vivo*. These may differ in terms of their potency on mechanisms germane to cancer chemoprevention.

Contrary to the hypothesis that mirtocyan suppresses markers of oxidative DNA damage, urinary levels of 8-oxo-dG and levels of M₁dG in peripheral blood samples were unaffected by treatment. In fact, an increase in levels of M₁dG was observed in patients who consumed 2.8 g of mirtocyan daily. Multiple dietary compounds, which have been investigated as potential cancer chemopreventive agents, are known to possess ‘dual activity’ with respect to their antioxidant properties (Cemeli et al., 2008). For example, green tea extract (GTE) and (-)-epigallocatechin-3-gallate (ECGC) have been shown to induce oxidative stress and DNA damage in murine macrophage RAW 264.7 and human leukaemic HL60 cell lines (Elbling et al., 2005). Vitamin A has induced oxidative stress and oxidative DNA damage in rodent lung V79 cells (Klamt et al., 2003). Several *in vitro* studies have also shown that treatment with anthocyanins may paradoxically lead to the generation of reactive oxygen species, and that these may actually be capable of inducing chemopreventive effects, such as apoptosis (*Section 1.4.3*). Few data exist to explain the ‘dual activity’ of potential cancer chemopreventive agents with respect to their antioxidant properties, however, propensity to act as an antioxidant or pro-oxidant seems to depend, at least in part, on concentration (Cemeli et al., 2008).

As hypothesized, consumption of mirtocyan inhibited cell proliferation (Ki-67) and induced apoptosis (cleaved caspase-3) in colorectal tumour tissue. Although the ‘pre-

surgery' trial design integrates extremely well with patients' routine care and provides ease of access to tissue samples, a relative limitation is discrepancy in the volume of tissue obtained before and after treatment. To reduce any possible effects afforded by discrepancy in tissue volume, care was taken to establish a standardized method for selecting histological fields, and counting cells, which was approved by an experienced Consultant Gastrointestinal Pathologist (*Section 4.3.8*). When insufficient biopsy material was present in pre-intervention samples, to satisfy the aforementioned method, patients were excluded from analysis (n=2).

An increase in COX-2 expression was observed in colorectal tumour tissue in this study. Considerable pre-clinical data exist to show that anthocyanins suppress COX-2 expression and activity (*Section 1.4.2*), and it therefore seems unlikely that administration of mirtocyan was related to the observed increase in COX-2 expression. Induction of COX-2 has been demonstrated in animal models of cerebral and cardiac ischemia (Abbate et al., 2007; Collaco-Moraes et al., 1996). For example, when anesthetized cats were subjected to 15 minutes of total cerebral ischaemia, levels of prostanoids rose significantly, and were reduced by administration of a cyclooxygenase inhibitor (Stevens & Yaksh, 1988). It is conceivable that the rise in COX-2 expression observed in this study occurred secondary to the effects of ischaemia. The effect of ischaemia would be expected to be more pronounced in samples obtained from colorectal resection specimens, when the vascular supply has been clamped intraoperatively, than in samples which were obtained at endoscopy. Further support for this hypothesis is provided by a clinical study, which also utilized

a 'pre-surgery' design, in which the chemopreventive effects of curcumin were investigated in patients with colorectal cancer (Garcea et al., 2005). Despite convincing pre-clinical evidence that curcumin causes a reduction in COX-2 levels, increased COX-2 activity was observed in post-treatment samples obtained from resection specimens, compared to pre-treatment samples obtained during endoscopy.

In summary, consumption of up to 5.6 g of mirtocyan for seven days was associated with a decrease in proliferation (Ki-67) and increase in apoptosis (cleaved caspase-3) in colorectal tumour tissue. A trend towards a reduction in IGF-1 in peripheral plasma was also evident. Mirtocyan did not affect levels of COX-2 or markers of oxidative DNA damage.

CHAPTER 8

FINAL DISCUSSION

Cancer chemoprevention frequently involves administering agents to healthy individuals for prolonged periods and therefore safety is of paramount importance. The potential cancer chemopreventive properties of dietary constituents have generated much interest as possible alternatives to drugs such as celecoxib and aspirin. However, administration of isolated dietary agents is not without risk, as demonstrated by the CARET, ATBC and SELECT trials (*Section 5.4*). The results of this trial show that administration of up to 5.6 g of mirtocyan for seven days is safe and well tolerated.

This study is pilot in design and consequently has involved a relatively small number of participants for a brief period. Pilot studies, such as this trial, do not aim to produce definitive data regarding efficacy and safety. Rather they aim to assess whether the agent in question is suitable for further development as a potential cancer chemopreventive agent. They also provide data to optimise future trials, should this be appropriate. A shortcoming of clinical development of a number of chemopreventive agents has been insufficient knowledge of their pharmaceutical, pharmacokinetic and pharmacodynamic properties before large, costly, lengthy human trials have been initiated. This approach has yielded some very disappointing outcomes (*Section 5.4*). Consumption of beta-carotene has been associated with an increased incidence of lung cancer (Omenn et al., 1996; The Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study Group, 1994). Consumption of vitamin E and selenium have been associated with the development of prostate cancer and diabetes, respectively (Lippmann et al., 2008). Potential chemopreventive agents should be

evaluated in a considered manner and sufficient data must exist to justify the commencement of large intervention trials. The Division of Cancer Prevention of the US National Cancer Institute adopts a meticulous approach to the development of potential chemopreventive agents. Potential agents are identified by a systematic review of epidemiological and experimental carcinogenesis literature. They are then screened using a battery of short term *in vitro* assays, followed by use of animal efficacy models. Agents demonstrating satisfactory safety and efficacy are then advanced into human studies, which are initially pilot in nature (Crowell et al., 2005). The number of potential chemopreventive agents identified is likely to continue to increase in the future (Chemoprevention Working Group, 1999). A considered approach to agent development will become increasingly important, with resources being focused towards the most promising agents.

There is increasing interest in mixtures of chemopreventive agents, exemplified by mirtocyan. The appealing theory being that administration of combinations of low dose agents may avoid potential toxicities, with individual components acting in synergy to produce chemopreventive effects. A recent study by Meyskens et al. provides an excellent illustration of the possible benefits of administration of mixtures. Three hundred and seventy-five patients who had undergone removal of a colorectal adenoma received oral difluoromethylornithine (DFMO) 500 mg and sulindac 150 mg once daily in combination, or placebo for three years. The dose of sulindac administered was one half the usual therapeutic dose. DFMO and sulindac in combination markedly reduced the recurrence of all adenomas (70% decrease) and

advanced adenomas (92% decrease). No significant differences were identified in the incidence of cardiovascular side-effects between groups. A slight reduction in hearing measured using air conduction audiograms was found in the intervention group ($p=0.05$) (Meyskens et al., 2008). The dramatic effects on colorectal adenoma recurrence described in this trial provide considerable support for the development of mixtures of chemopreventive agents.

This is the first study in which levels of anthocyanins have been measured in human colorectal tissue. The results indicate that consumption of a modest oral dose of anthocyanins, in the form of mirtocyan, can achieve levels in colorectal tumour tissue, which are not only detectable, but that are within an order of magnitude to pharmacologically active levels observed in Apc^{Min} mice (Cooke et al., 2006a) (Section 6.4). Indeed, consumption of mirtocyan was associated with a small reduction in proliferation in target tissue (7 %) and increase in apoptosis (1.7 %). Although the biological importance of such slight changes is questionable, the fact that pharmacodynamic effects are observed after treatment for only seven days is encouraging. These findings lead to the question, would effects be more pronounced if patients were to consume mirtocyan for longer? The answer is unknown.

Intriguingly, the changes observed in Ki-67 and cleaved caspase-3, were most apparent in patients who consumed low dose mirtocyan. A decrease in IGF-1 concentrations was also observed, and this approached significance in the low dose group ($p=0.060$). Levels of anthocyanins achieved in biomatrices were approximately

related to the dose of mirtocyan consumed. The superior pharmacodynamic efficacy associated with consumption of low dose mirtocyan, could possibly reflect complex interactions between individual components of the mirtocyan mixture and their metabolites. Although mirtocyan predominantly consists of anthocyanins, it does also contain smaller quantities of other polyphenols, for example proanthocyanidins and phenolic acids. An immense number of potentially synergistic and antagonistic interactions could occur, not only between constituents of the original mirtocyan mixture, but also involving anthocyanin metabolites. Few data exist regarding differences in biological potency between individual anthocyanins, and even less is known about their metabolites. One hypothesis is that the balance between synergistic and antagonistic interactions established in biomatrices following consumption of lower doses of mirtocyan may mediate more favourable chemopreventive effects. It is also possible that an unusual dose-response relationship may exist, as has been observed for other diet-derived agents. For example, a case control study has illustrated a skewed bell-shaped curve to describe the relationship between plasma folate levels and the risk of colorectal cancer. The lowest risk was associated with the lowest levels of plasma folate (Van Guelpen et al., 2006).

Low levels of anthocyanins were detected in peripheral plasma samples. Although not directly detected in liver tissue, the presence of anthocyanins can be inferred from detection in both portal plasma and bile. It is likely that levels are very low. Poor systemic bioavailability mitigates against using anthocyanins in the prevention of malignancies distant from the gastrointestinal tract.

Quantifiable amounts of anthocyanins were recovered from the urine of all trial participants. Considering the coloured nature of anthocyanins in an acidic environment development of a simple colorimetric test may be feasible to ensure patient adherence with treatment. Detecting relatively high levels of anthocyanins in urine also supports current *in vitro* studies in which the potential role of anthocyanins in the chemoprevention of bladder cancer is being investigated in the Department of Cancer Studies and Molecular Medicine, University of Leicester.

The low dose of mirtocyan that was consumed in this study (1.4 g daily) represented an anthocyanin dose in the region of that consumed in the diet. 1.4 g of mirtocyan contains ~0.5 g anthocyanins, which would be found in ~370 g of fresh bilberries (Nyman & Kumpulainen, 2001). Normal dietary consumption of anthocyanins has been estimated at around a fifth of that amount (~100 mg) (Hertog et al., 1993).

The results of this pilot study support further development of anthocyanins as potential colorectal cancer chemopreventive agents. Future *in vitro* work may involve comparing the biological potency of the major metabolites observed in colorectal tissue, plasma and urine, to that of unaltered anthocyanins. Little is currently known regarding the biological effects of metabolites compared to parent anthocyanins. If metabolites possessed superior biological effects, such species could be chemically synthesised. In addition, development of improved analytical methods for anthocyanins obviating the need of chemical conversion to the flavylum ion forms would increase the accuracy of the determination of the pharmacokinetics of these

agents in future trials. As discussed above, methods rely on acidification to the coloured flavylium cation. Metabolic conversion which impedes this transformation therefore prevents detection of these compounds. A pilot study in which radiolabelled anthocyanins were consumed at very low doses would be extremely interesting, initially perhaps involving healthy volunteers. Comparing the results of such a study with analytical data obtained by conventional means may address the question as to whether bioavailability is being underestimated using current analytical techniques.

In terms of clinical development, a suitable next step may be a further trial in colorectal cancer patients utilising the pre-surgery model. However, such a study should include a larger number of patients, in the region of a hundred. The feasibility of a longer duration of treatment and possibility of procuring normal tissue prior to treatment should also be considered. When this study commenced patients were initially approached when attending for their endoscopy and consent was sought to obtain both normal and tumour tissue if a colorectal malignancy was identified. It was discovered that this approach was not feasible within the time constraints of this study and therefore patients were recruited following their diagnosis of colorectal cancer (*Section 4.2.3*). If a multicentre trial could be organised, the original study design, and therefore procurement of normal tissue, would be far more likely to succeed. Biomarkers against which efficacy is assessed should include the insulin-like growth factor axis in plasma and Ki-67 and cleaved caspase-3 in colorectal tissue. In retrospect, inclusion of a zero group would have aided interpretation of some of the results of this trial, and should be considered in future studies. For example, a

significant increase in COX-2 expression was observed in malignant tissue between pre-mirtocyan biopsies and samples from the post-mirtocyan resection specimens. Given the bulk of evidence which suggests that anthocyanins suppress COX-2 it is conceivable that the observed increase occurred secondary to the effects of tissue ischaemia. The effects of ischaemia being relatively more pronounced in the resection specimens (*Chapter 7.8*). If a zero dose group had been included in this trial, and COX-2 levels had also increased in this subset of patients, it could be concluded with certainty that this increase was not due to mirtocyan consumption, but instead due to an external factor, for example ischaemia. As discussed above, an intriguing finding of this trial was that changes in IGF-1, Ki-67 and caspase-3 were more pronounced in patients who consumed low dose mirtocyan (1.4 g daily). On the basis of this, future trials of mirtocyan should consider inclusion of an additional low dose treatment group, 0.7 g daily may be appropriate. It is possible in the future that clinical trials will be conducted with single anthocyanins. Such trials should be initiated only when sufficient pre-clinical data supports their commencement. Considerable data now exists on the chemopreventive effects of cyanidin-3-glucoside *in vitro* which is supported by the results of studies involving rodent models of colorectal carcinogenesis (Cooke et al., 2006a). On the basis of this data cyanidin-3-glucoside seems an obvious first choice for a pilot trial of a single anthocyanin.

The recently introduced national screening programme for colorectal cancer is also an exciting prospect from the point of view of cancer chemoprevention. The programme will identify individuals with colorectal adenomas who then undergo repeat

colonoscopies. The interval between colonoscopies could prove an excellent opportunity to undertake a chemoprevention study. It would be possible to evaluate outcomes including adenoma number and adenoma grade in patients consuming a potential chemopreventive agent compared to those receiving placebo. This design would co-ordinate extremely well with patients' routine care and no additional endoscopies would be required. The American Association of Cancer Research Intraepithelial Neoplasia Task Force has identified colorectal adenoma incidence as a suitable biomarker in individuals with a history of adenomas (Kelloff et al., 2006). Examples of trials utilising a similar methodology include the three major COX-2 colorectal cancer chemoprevention trials (*Section 1.1*) and the more recent study of DFMO and sulindac in combination, as discussed above (Meyskens et al., 2008). All of these trials successfully demonstrated effects consistent with cancer chemoprevention manifest as changes in adenoma number/grade.

In conclusion, consumption of a modest oral dose of mirtocyan furnished levels of anthocyanins in colorectal tumour tissue comparable to those capable of mediating chemopreventive effects *in vivo*. Consumption of only 1.4 g of mirtocyan daily may exert pharmacodynamic effects commensurate with colorectal cancer chemoprevention. Overall, these results support the further clinical development of anthocyanins as potential colorectal cancer chemopreventive agents.

APPENDIX 1

HPLC TRACES

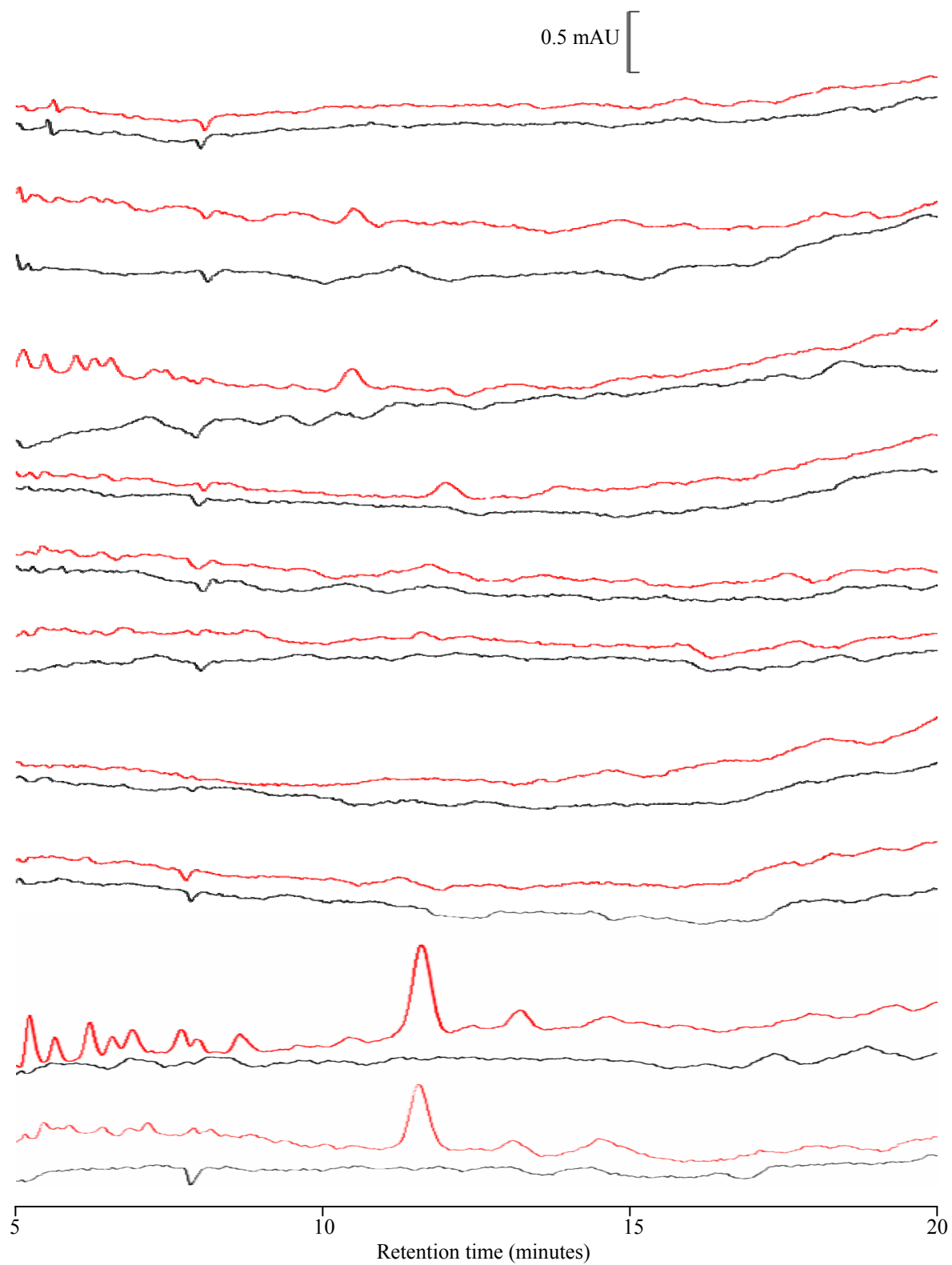


Figure 1a: HPLC traces of peripheral plasma samples before (black) and after (red) the consumption of mirtocyan Traces from 10 patients shown

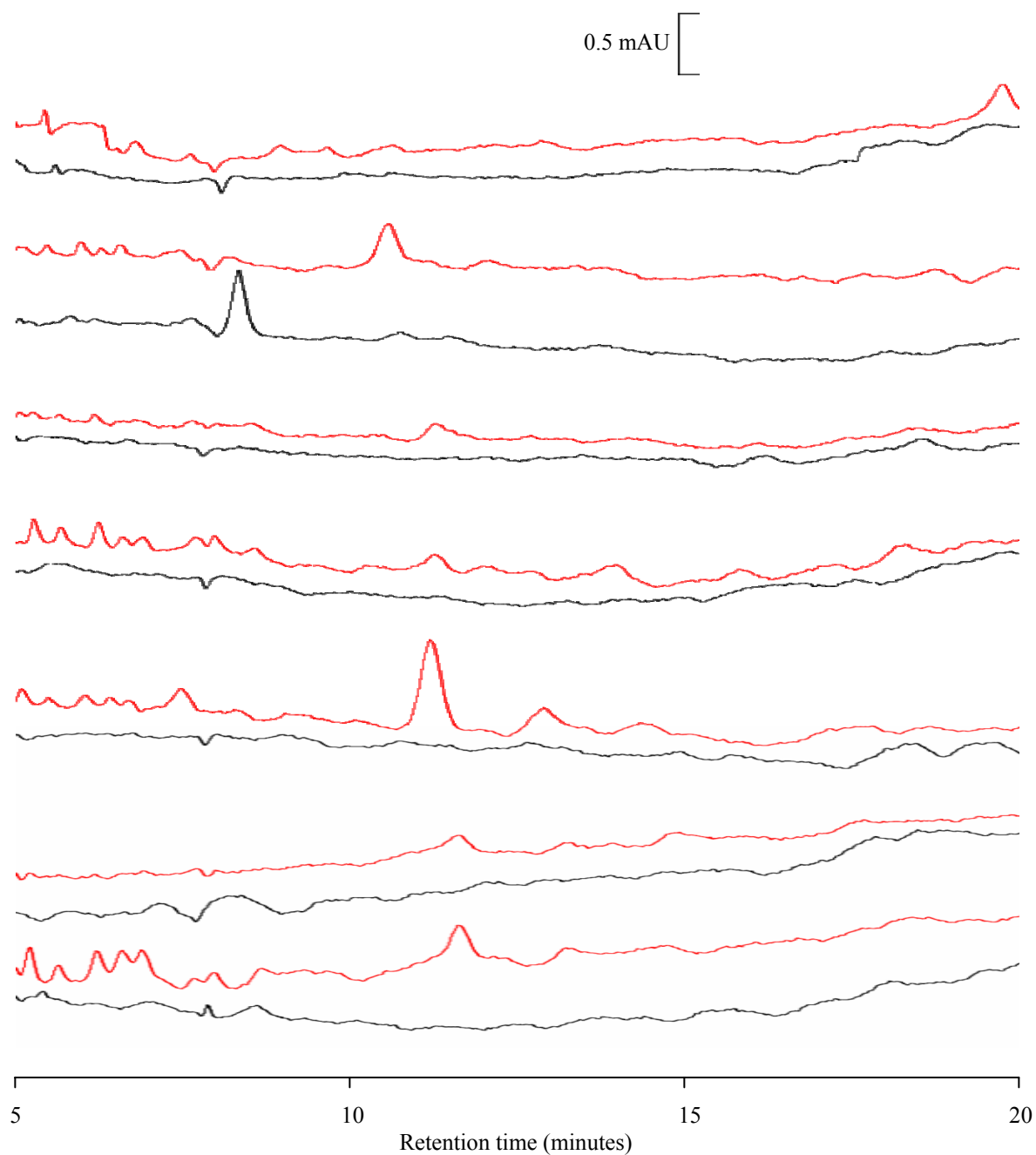


Figure 2a: HPLC traces of peripheral plasma samples obtained before (black) and after (red) the consumption of 2.8 g of mirtocyan daily

Traces from 7 patients shown

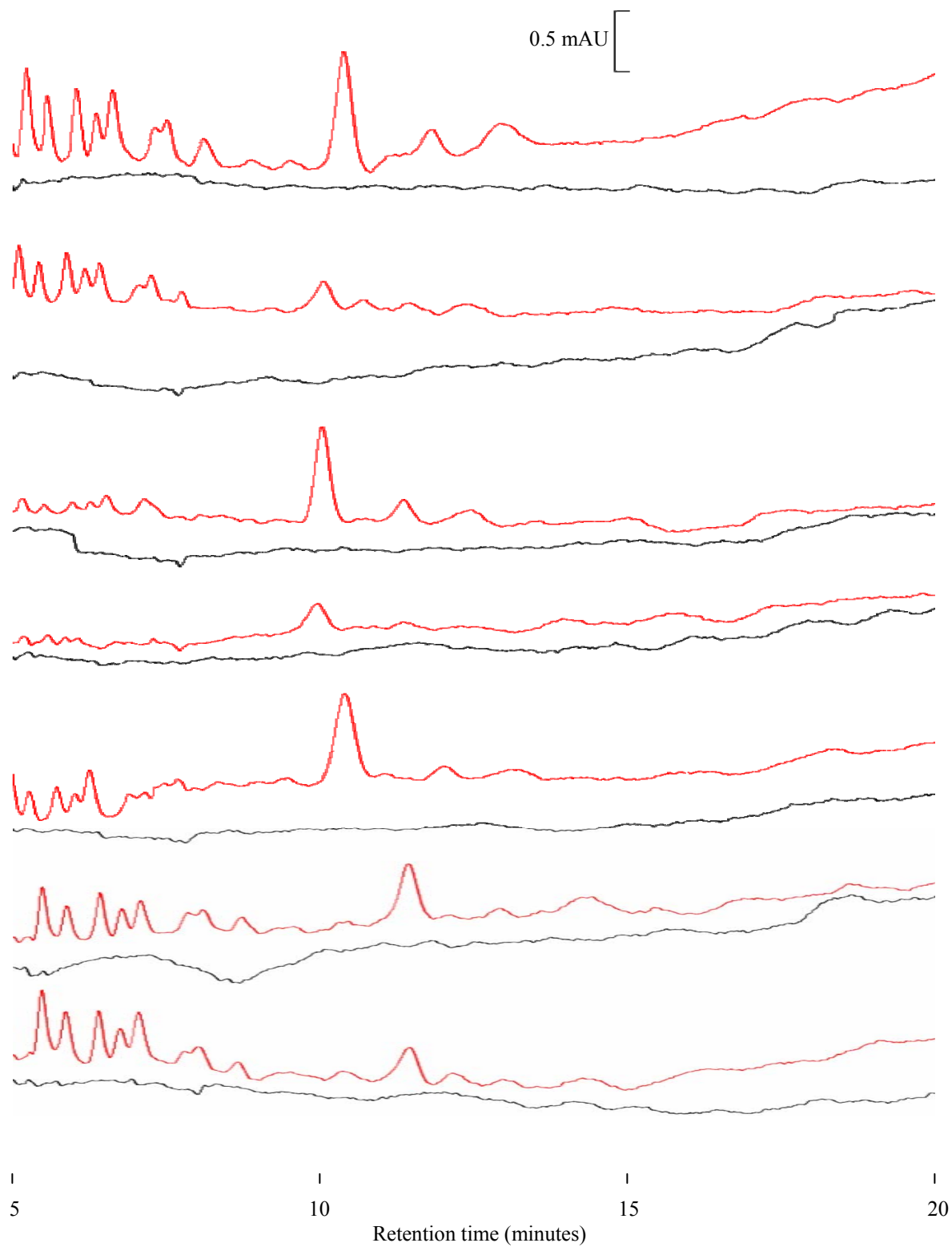


Figure 3a: HPLC traces of peripheral plasma samples obtained before (black) and after (red) the consumption of 5.6 g of mirtocyan daily Traces from 7 patients shown

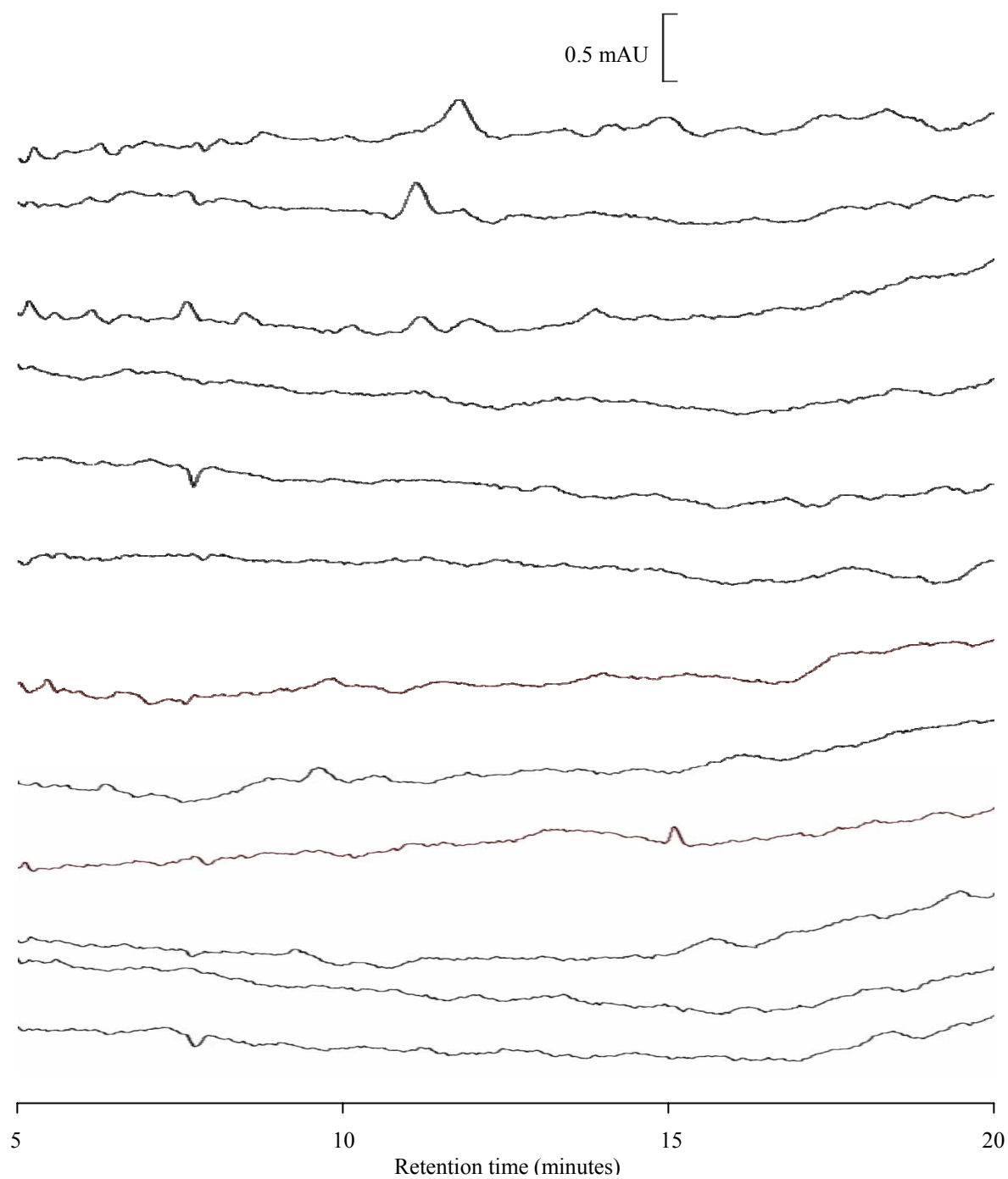


Figure 4a: HPLC traces of portal plasma samples obtained following the consumption of mirtocyan

Traces from 12 patients shown

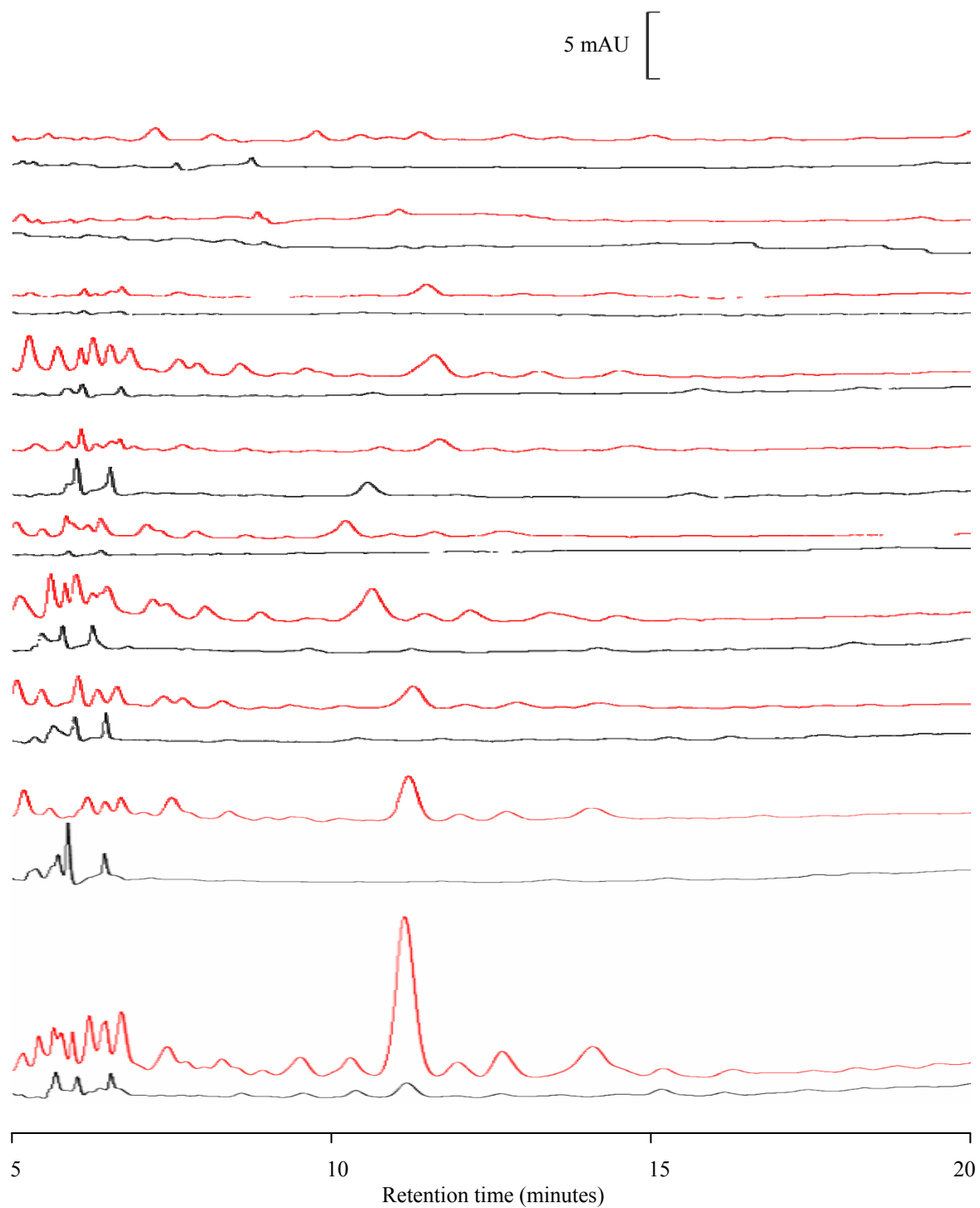


Figure 5a: HPLC traces of urine samples obtained before (black) and after (red) the consumption of 1.4 g of mirtocyan daily

Traces from 10 patients shown

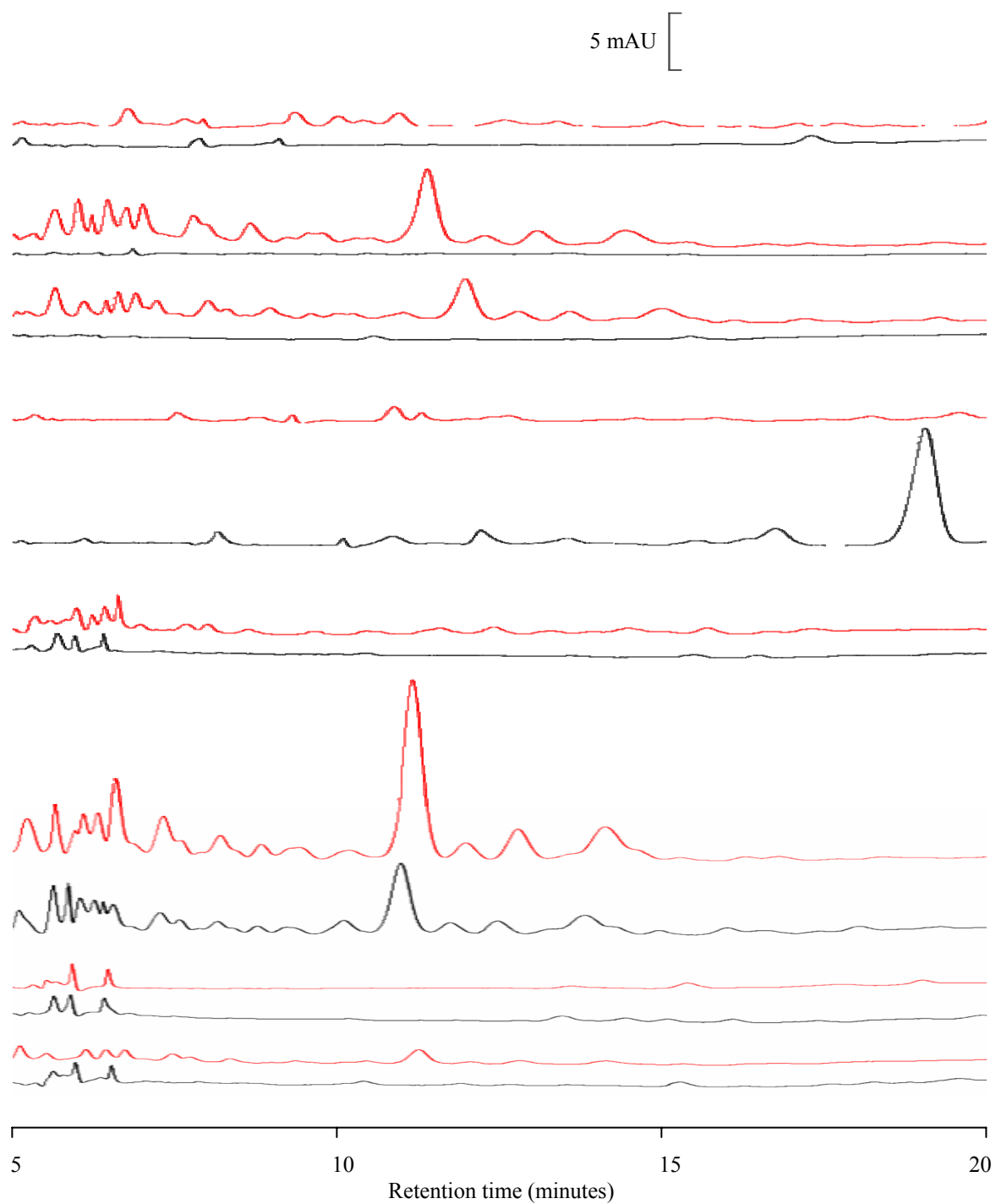


Figure 6a: HPLC traces of urine samples obtained before (black) and after (red) the consumption of 2.8 g of mirtocyan daily

Traces from 8 patients shown

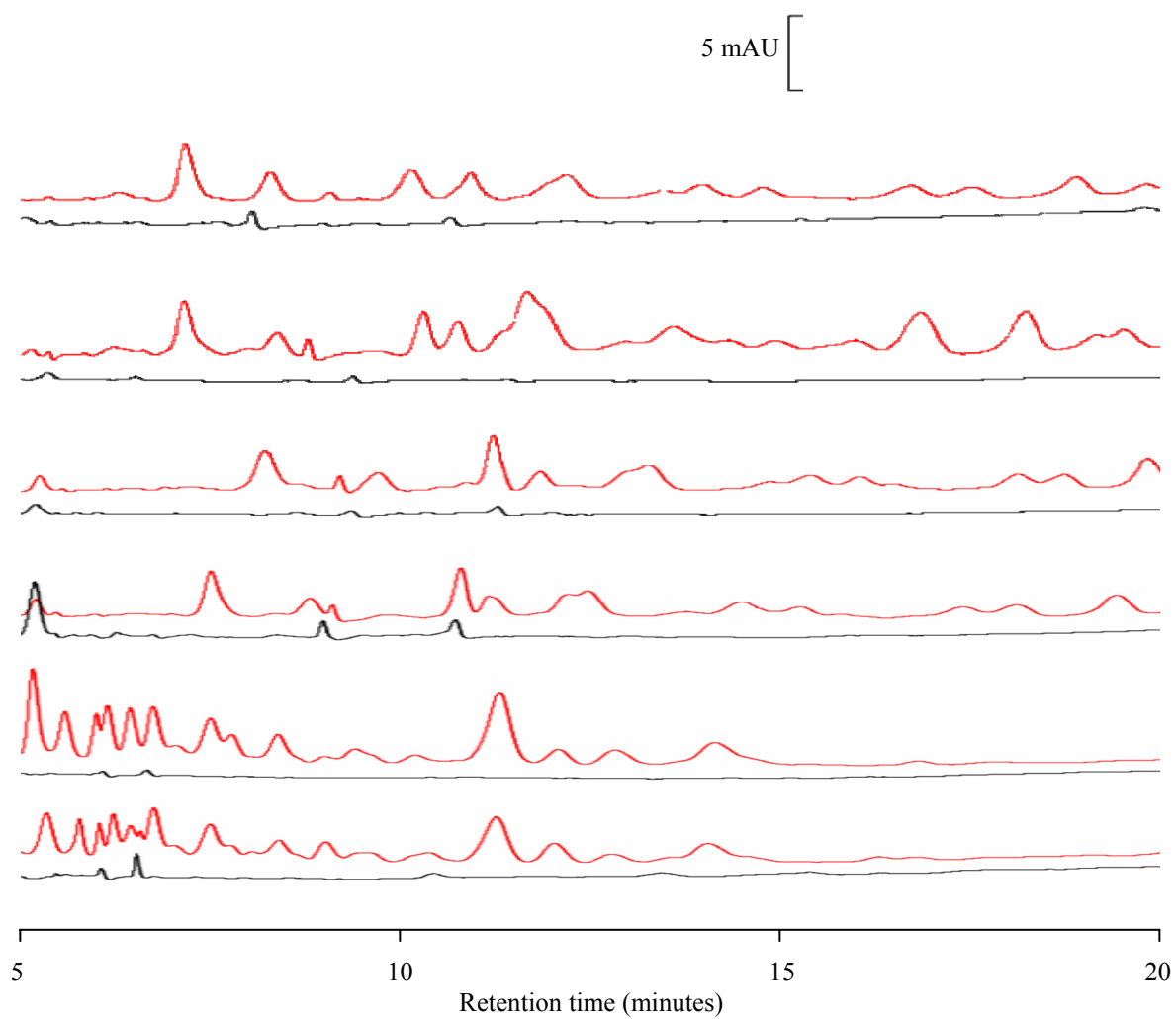


Figure 7a: HPLC traces of urine samples obtained before (black) and after (red) the consumption of 5.6 g of mirtocyan daily

Traces from 6 patients shown

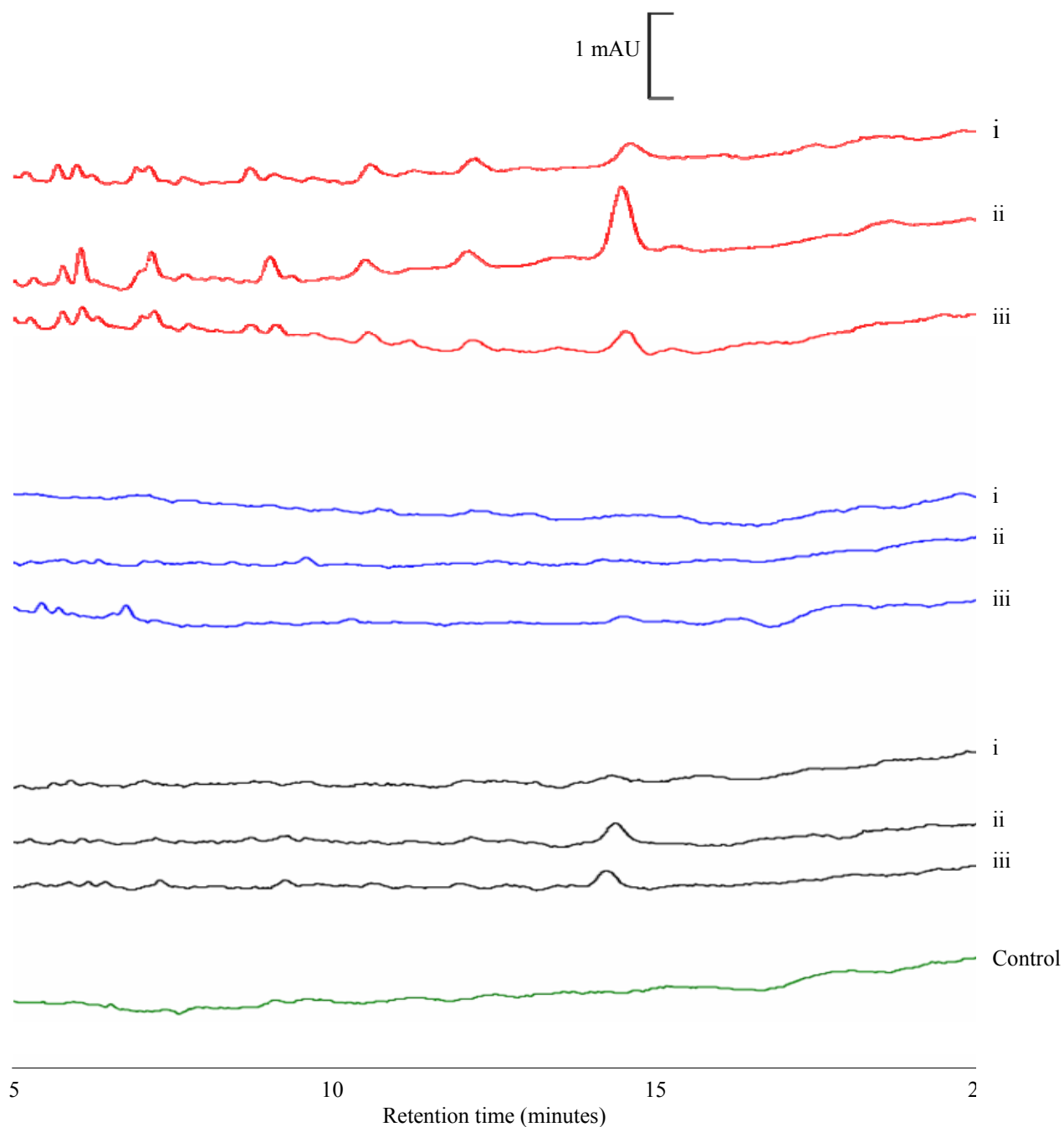


Figure 8a: HPLC traces of normal tissue, proximal (i) and distal (iii) to tumour (ii), obtained after consumption of 1.4 (black), 2.8 (blue) and 5.6 g (red) of mirtocyan daily

Traces represent samples pooled from 4/5 patients. 'Control' colorectal biopsies were obtained during diagnostic colonoscopy

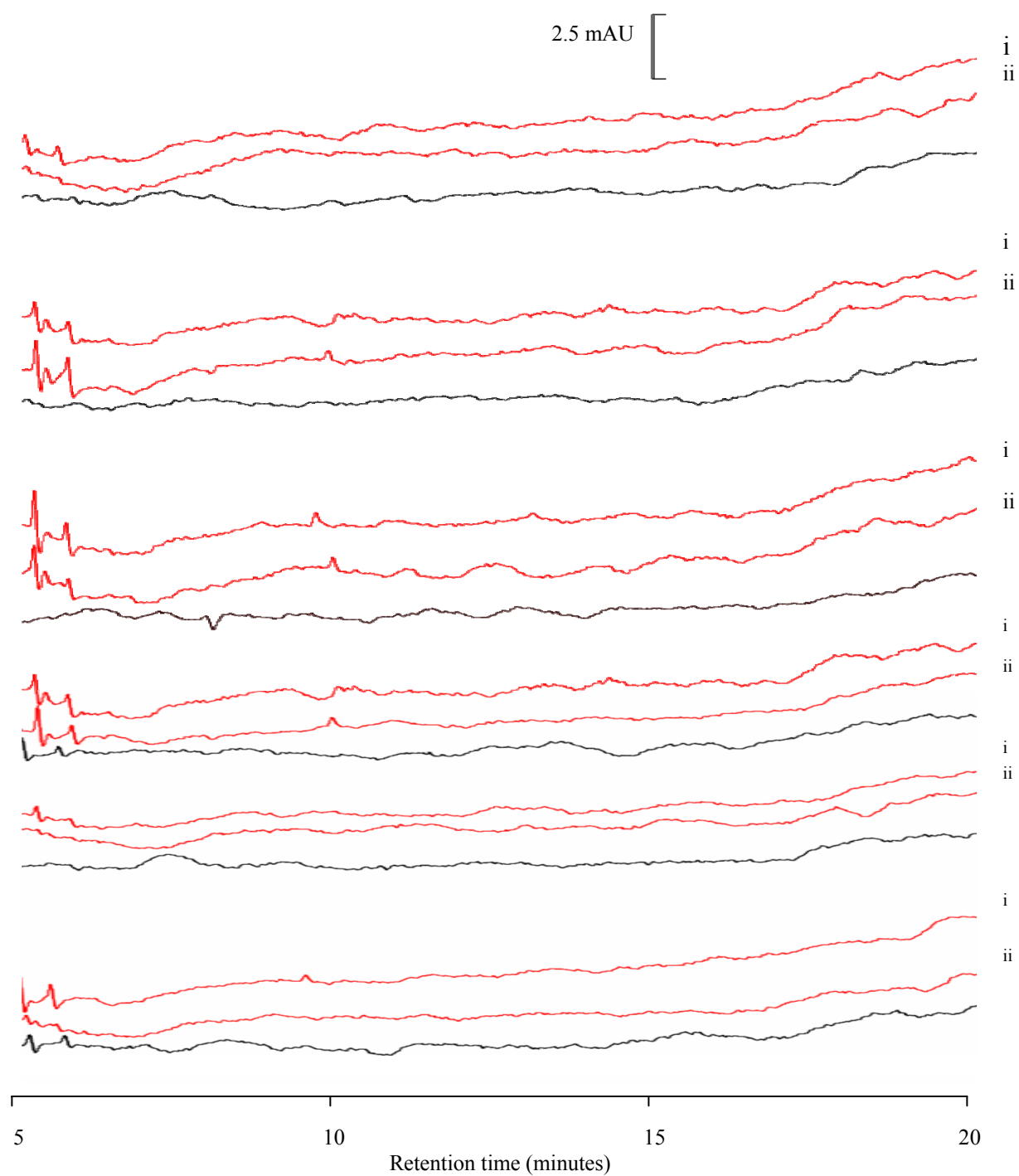


Figure 9a: HPLC traces of malignant liver tissue obtained before the consumption of mirtocyan (black) and normal (i) and malignant (ii) tissue obtained after the consumption of mirtocyan (red) Traces from 6 patients shown

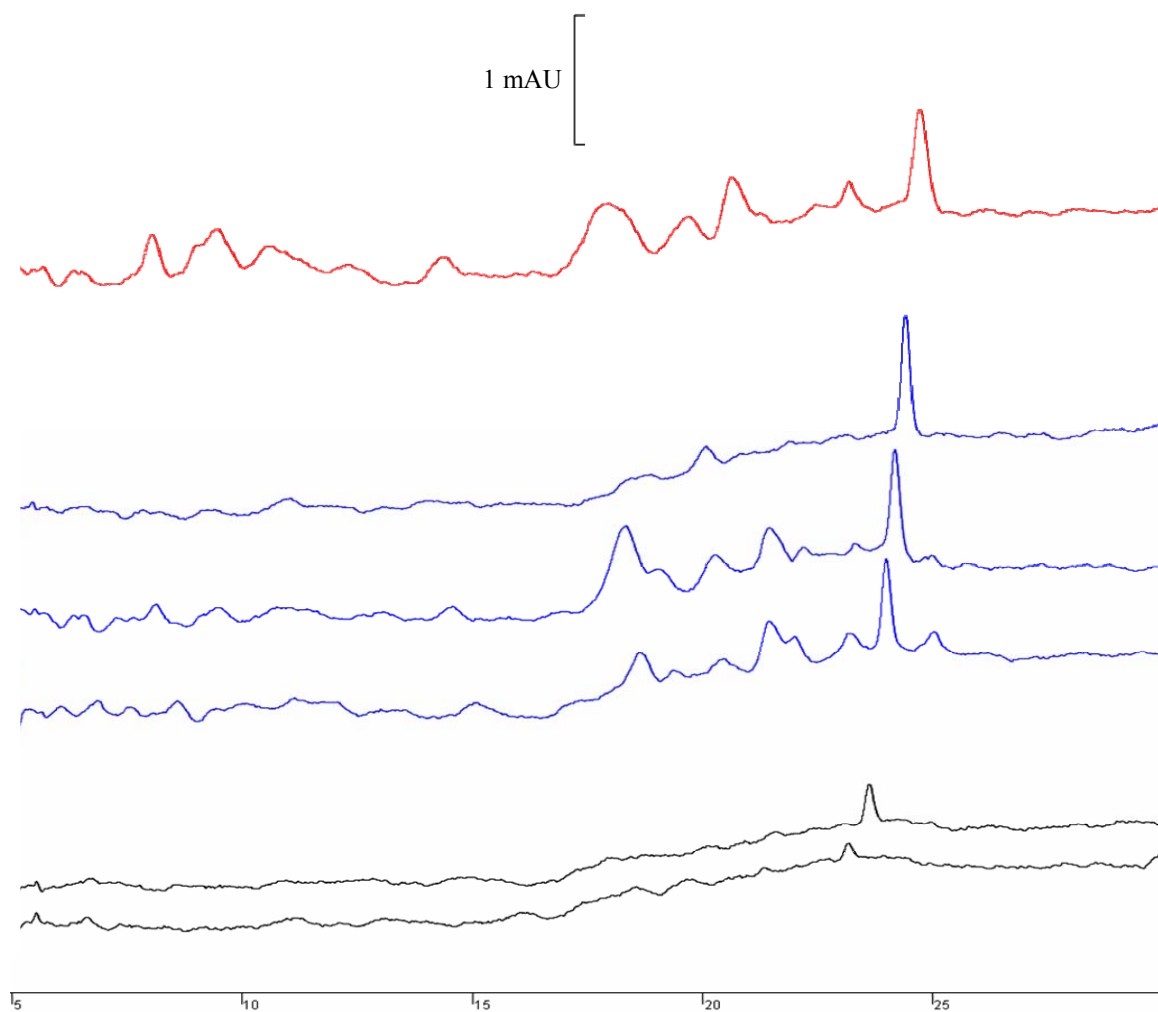


Figure 10a: HPLC traces of bile obtained following the consumption of 1.4 (black), 2.8 (blue) and 5.5 g (red) mirtocyan daily

Traces from 6 patients shown

APPENDIX 2

RELATED PRESENTATIONS AND PUBLICATIONS

Presentations related to this research

Thomasset SC, Gescher AJ, Steward WP, Marczylo T, Garcea G, Dennison AR, Berry DP. Evaluation of anthocyanin levels after consumption of bilberry extract in patients with colorectal cancer and liver metastases.

99th Annual Meeting of the American Association of Cancer Research, San Diego 2008.

Thomasset SC, Gescher AJ, Steward WP, Cai H, West K, Garcea G, Dennison AR, Berry DP. The effect of anthocyanins on surrogate biomarkers of cancer chemopreventive activity in patients with primary colorectal cancer and colorectal liver metastases.

To be presented at the Association of Upper Gastrointestinal Surgeons meeting, Liverpool 2008.

Thomasset SC, Gescher AJ, Steward WP, Cai H, Garcea G, Dennison AR, Berry DP. A pilot study to assess the chemopreventive properties of anthocyanins in patients with primary colorectal cancer and liver metastases

To be presented at the Association of Upper Gastrointestinal Surgeons meeting, Liverpool 2008.

Publications related to this research

Cooke DN, Thomasset S, Boocock DJ, Schwarz M, Winterhalter P, Steward WP, Gescher AJ, Marczylo TH. Development of analyses by high-performance chromatography/tandem mass spectrometry of bilberry (*Vaccinium myrtillus*) anthocyanins in human plasma and urine. *J Agric Food Chem.* 2006;54(19):7009-13.

Thomasset SC, Berry DP, Garcea G, Marczylo T, Steward WP, Gescher AJ. Dietary polyphenolic phytochemicals – Promising cancer chemopreventive agents in humans? A review of their clinical properties. *International Journal of Cancer.* 2007;120(3):451-8.

Submitted papers related to this research

Thomasset S, Berry DP, Cai H, West K, Marczylo TH, Marsden D, Brown K, Dennison A, Garcea G, Miller A, Hemingway D, Steward WP, Gescher AJ. Pilot study of anthocyanins for colorectal cancer chemoprevention – Agent concentration in the biophase suggests atypical dose-pharmacodynamics relationship. Submitted to *Cancer Research*.

Thomasset S, Berry DP, Cai H, Marczylo T, Garcea G, Brown K, Steward WP, Gescher AJ. Absorption of bilberry anthocyanins, putative colorectal cancer chemopreventive agents, from the gastrointestinal tract of patients with hepatic metastases. Submitted to *Nutrition and Cancer*.

1. Development of analyses by high-performance chromatography/tandem mass spectrometry of bilberry (*Vaccinium myrtillus*) anthocyanins in human plasma and urine. J Agric Food Chem. 2006;54(19):7009-13.

2. Dietary polyphenolic phytochemicals – Promising cancer chemopreventive agents in humans? A review of their clinical properties. *International Journal of Cancer*. 2007;120(3):451-8.

3. Pilot study of anthocyanins for colorectal cancer chemoprevention – Agent concentration in the biophase suggests atypical dose-pharmacodynamics relationship.

Abstract of paper submitted to Cancer Research

Pilot Study of Oral Anthocyanins for Colorectal Cancer

Chemoprevention – Agent Concentration in the Biophase Suggests

Atypical Dose–Pharmacodynamics Relationship

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Running title: Study of Berry Anthocyanins in Colorectal Cancer Patients

Key words: Anthocyanins, bilberry, chemoprevention, dose response, drug
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Abstract

Naturally occurring anthocyanins possess colorectal cancer chemopreventive properties in rodent models. We investigated whether mirtocyan, an anthocyanin-rich standardized bilberry extract, merits clinical development, specifically whether it causes pharmacodynamic changes consistent with chemoprevention and generates measurable levels of anthocyanins in blood, urine and target tissue. Twentyfive colorectal cancer patients scheduled to undergo resection of primary tumor or liver metastases received mirtocyan 1.4, 2.8 or 5.6 g (containing 0.5-2.0 g anthocyanins) daily for seven days prior to surgery. Proliferation and apoptosis were determined by immunohistochemistry of Ki-67 and cleaved caspase-3 in colorectal tumor. Concentrations of insulin-like growth factor (IGF)-1 were measured in plasma. Bilberry anthocyanins were analyzed by HPLC with visible or mass spectrometric detection. In tumor tissue from patients on mirtocyan, proliferation was decreased by 7% and apoptosis increased by 49% as compared to pre-intervention values. At the 1.4g dose, effects were more marked than at the higher doses. The low dose also caused a small reduction in circulating IGF-1 concentrations. Mirtocyan anthocyanins and methyl and glucuronide metabolites were identified in plasma, colorectal tissue and urine, but not in liver. Anthocyanin concentrations in plasma and urine were roughly dose-dependent, they reached ~179ng/g in tumor tissue at the highest dose. The results suggest that repeated administration of bilberry anthocyanins exerts pharmacodynamic effects inversely related to dose and generates concentrations of anthocyanins in humans resembling those seen

in *ApcMin* mice, a model of FAP adenomas sensitive to the chemopreventive properties of anthocyanins. Bilberry anthocyanins seem appropriate for development as colorectal cancer chemopreventive agents.

4. Absorption of bilberry anthocyanins, putative colorectal cancer chemopreventive agents, from the gastrointestinal tract of patients with hepatic metastases. Abstract of paper submitted to Nutrition and Cancer.

**Absorption of Bilberry Anthocyanins, Putative Colorectal Cancer
Chemopreventive Agents, from the Gastrointestinal Tract of Patients with
Hepatic Metastases**

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Running head: Absorption of Berry Anthocyanins in Humans

Keywords: colorectal cancer chemoprevention, anthocyanins, drug development

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Anthocyanins possess cancer chemopreventive properties in preclinical models. Their clinical pharmacology is only poorly described. Rodent experiments suggest that anthocyanins are absorbed mainly from the stomach and much less from the intestine. We investigated whether in humans there is a difference in absorbance of bilberry anthocyanins following gastric or jejunal administration. Two patients with colorectal liver metastases received a dose of 1.88g standardized bilberry extract (mirtocyan) *via* nasogastric or nasojejunal tube intra-operatively during liver resection. Anthocyanins were measured in plasma and urine by HPLC with visible spectroscopic or tandem mass spectrometric detection. Concentrations of anthocyanins in biofluids of the patient who received mirtocyan *via* the stomach were much higher than in the patient who received mirtocyan into the jejunum. The results suggest that the predominant site for anthocyanin absorption in humans is the stomach.

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