Integrated Individualised Treatment of Colorectal Cancer

Pauline Amanda Whitehouse

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Translational Oncology Research Centre
Queen Alexandra Hospital, Portsmouth

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

In loving memory of

my mother

and

for my father

both valiant sufferers of cancer

ABSTRACT

Introduction: Advanced colorectal cancer (CRC) has a poor prognosis with a 5-year survival of only 5% despite treatment with chemotherapeutic agents. The ATP-Tumour Chemosensitivity Assay (ATP-TCA) has been used to demonstrate heterogeneity of chemosensitivity between tumours of the same tissue type, but this has been difficult to establish in colorectal cancer due to infection of cells in culture.
Methods: The *ex vivo* ATP-TCA was modified with antibiotics for use in CRC, and with immunohistochemistry and quantitative RT-PCR, has been used to assess the chemosensitivity and resistance of CRC tumour-derived cells.
Results:

(a) The addition of 2.5 μg/ml amphotericin B and 1 μg/ml metronidazole to culture media did not effect the cytotoxicity of all drugs tested on SK-MEL-28 melanoma cell lines.
(b) The metabolite of irinotecan, SN38, was found to be inactive in the ATP-TCA
(c) The ATP-TCA was performed on 71 CRC samples, 58 of which were evaluable (82%). There was considerable heterogeneity for individual samples and drugs tested.
(d) Mitomycin C + gemcitabine was the most effective combination in 78% of specimens, with all but one sample showing sensitivity. The synergistic effect between these two drugs was not found to be schedule-specific.
(e) Molecular studies determined the expression of a number of molecular targets which were correlated with the ATP-TCA results. The only correlation found was between positive staining for topoisomerase I and sensitivity to irinotecan.
(f) Using qRT-PCR it was found that cyclo-oxgenase2 is up-regulated by short-term exposure to 5-fluorouracil (3-fold), but down-regulated by irinotecan (2.5-fold),

Conclusion: The results show that it is possible to perform the ATP-TCA on CRC tumour-derived cells with a high evaluability rate. The changes in gene expression after short-term drug exposure have important implications for the use of sequential therapy in the treatment of colorectal and cancers.

LIST OF CONTENTS

CONTENTS

LIST OF TABLES AND FIGURES

ABBREVIATIONS

5-FU	5-Fluorouracil
A	Adenine
α	Alpha
Ab	Antibody
ABC	ATP-binding cassette
ACF	Aberrant crypt focus
ADP	Adenosine diphosphate
AMB	Amphotericin B
AMP	Adenosine 5'-monophosphate
AMV	Avian myeoblastosis virus ??
APC	Adenomatous Polyposis Coli
ATP	Adenosine 5'-triphosphate
ATP-TCA	ATP-tumour chemosensitivity assay
AUC	Area under curve
β	Beta
BAX	BCL-2 associated X protein
BCL-2	B-cell leukaemia/lymphoma 2
BCRP	Breast cancer resistance protein
BER	Base excision repair
C	Cytosine
CA125	Carbohydrate Antigen 125
CAM	Complete assay media
CDDP	Cisplatin
cDNA	Complementary DNA
CEA	Carcino-embryonic Antigen
CI	Combination Index
CI_{50}	combination indices at 50% cell death
CI_{90}	combination indices at 90% cell death
CIN	Chromosome instability
COX-2	Cyclo-oxygenase 2
CPT-11	7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin

CRC	Colorectal Cancer
CRT	Chemoradiotherapy
Ct	Threshold cycle
DAB	Diaminobenzidine
DCC	Deleted in colon cancer
DHFU	dihydrofluorouracil
DiSC	Differential staining cytotoxicity assay
DMEM	Dulbecco'a Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DPD	Dihydropyrimidine dehydrogenase
EDR	Extreme drug resistance
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERCC-1	Excision repair cross-complementing 1
EORTC	European Organisation for Research and Treatment of Cancer
ER	Oestrogen receptor
FA	Folinic Acid / Leucovorin
FAP	Familial Adenomatous Polyposis
FCS	fœtal calf serum
FdUDP	fluorodeoxyuridine diphosphate
FdUMP	fluorodeoxyuridine monophosphate
FdUTP	fluorodeoxyuridine triphosphate
FFCD	Foundation Francaise de Cancerologie Digestive
FMCA	Fluorometric microculture cytotoxicity assay
FUDP	Fluorouridine diphosphate
FUDR	Fluorodeoxyuridine
FUMP	Fluorouridine monophosphate
FUR	fluorouridine
FUTP	Fluorouridine triphosphate
G	Guanine
GAPDH	Glyceraldehyde-3 phosphate dehydrogenase
GeM	Gemcitabine + Mitomycin C combination

GITSG	Gastrointestinal Study Group
GIVIO	Gruppo Italiano di Valutazione Interventi in Oncologia
GST	Glutathione S-transferases
GTP	Guanine triphosphate
Gy	Gray
hCE-1	Human carboxyesterase-1
hCE-2	Human carboxyesterase-2
HDFA	High dose folinic acid
HDRA	Histoculture Drug Response Assay
H&E	Haematoxylin and Eosin
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HER2	Human Epidermal Growth Factor Receptor 2
HNPCC	Hereditary Non-polyposis Colon Cancer
HRPT1	Hypoxanthine phoshoribosyl-transferase 1
HRT	Hormone replacement therapy
IC	Inhibition concentration
IC_{50}	Concentration which results in 50% tumour growth inhibition
IC_{90}	Concentration which results in 90% tumour growth inhibition
ICRF	Imperial Cancer Research Fund
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IMA	Inferior mesenteric artery
IMPACT	The International Multicentre Pooled Analysis of Colon Cancer Trials
K-RAS	Kirsten-RAS
LDFA	Low dose folinic acid
LEV	Levamisole
LFS	Li-Fraumeni Syndrome
LREC	Local Regional Ethics Committee
LRP	Lung resistance protein
MAPK	Mitogen activated protein kinase
MCC	Mutated in colon cancer
MDR	Multidrug resistance
MDR1	Multidrug resistance gene 1

MgCl	Magnesium Chloride
MI	Maximum inhibitor
MIC	Minimum Inhibition Concentration
MIN	Microsatellite instability
MLH1	mutL homologue 1
MMC	Mitomycin C
MMP	Matrix metalloproteinase
MMR	Mismatch Repair
MO	No drug inhibitor
mRNA	Messenger ribonucleic acid
MRP	Multidrug resistance-associated protein
MSH2	mutS homologue 2
MSH6	mutS homologue 6
MSI	Microsatellite Instability
MTT	(3-[4,5-dimethyl (thiazol-2-yl)-3,5-diphenyl] tetrazolium bromide)
MW	Molecular weight
NCCTG	North Central Cancer Treatment Group
NCI	National Cancer Institute
NCIC	National Cancer Institute of Canada
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NICE	National Institute of Clinical Excellence
N/R	Not recorded
NSABP	National Surgical Adjuvant Breast and Bowel Project
NSAIDs	Non-steroidal anti-inflammatory drugs
OPRT	orotate phosphoribosyltransferase
OR	Overall risk
OS	Overall survival
P-450	Cytochrome P450
PBGD	Human porphobilinigen deaminase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PETACC	Pan-European Trial for Adjuvant Treatment of Colon Cancer

PFS	Progression free survival
PGE2	Prostaglandin E2
PGHS1	Prostaglandin H synthetase 1
PGHS2	Prostaglandin H synthetase 2
P-gp	P-glycoprotein
PTEN	Phosphatase and tensin homologue
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse transcriptase PCR
RAS	Rat sarcoma viral oncogene homolgue
RCT	Randomised Controlled Trial
RNA	Ribonucleic acid
RPM	Revolutions per minute
RR	Relative risk
RR	Ribonucleotide reductase
RT	Reverse transciptase
RT	Room temperature
RT	Radiotherapy
RT-PCR	Reverse transcriptase PCR
QA	Queen Alexandra Hospital, Portsmouth
QUASAR	Quick and Simple and Reliable (Trials)
SDH	Succinate dehydrogenase
SK-MEL-28	Skin melanoma 28 cell line
SMAD	Sma Mad related proteins
SN38	Active metabolite of Irinotecan
T	Thymine
TBP	TATA box binding protein
TBS	Tris-buffered saline
TCER	Tumour cell extraction reagent
TCF	T cell factor
TDC	Test Drug Concentration
TEM	Transanal endoscopic microsurgery
TGFβ	Transforming growth factor beta
TGFβRII	Transforming growth factor beta type 2 receptor

TK	Thymidine kinase
TME	Total mesorectal excision
TNF	Tumour necrosis factor
TNM	Tumour/Node/Metastasis staging system
TOPO I	Topoisomerase I
TOPO IIα	Topoisomerase IIα
TOPO II β	Topoisomerase II β
TP	Thymidine phosphorylase
TRAIL	TNF-related-apoptosis inducing ligand
TS	Thymidylate synthase
TSG	Tumour suppressor gene
U	Uracil
UFT	Uracil:Florafur in 4:1 molar concentration
UK	Uridine kinase
UP	Uridine phosphorylase
VEGF	Vascular endothelial growth factor
VICTOR	Vioxx® in Colorectal Cancer Therapy: definition of optimal regime
WHO	World health organisation

Symbols

%	Percentage
°C	Degree Celsius
cm	centimetre
kDa	kilodalton
M	Molar
µM	micro-molar
nM	nano-molar
g	gram
mg	milligram
ng	nanogram
l	Litre
ml	Millilitre
mg/ml	milligram per millilitre
µg/ml	microgram per millilitre
ng/ml	nanogram per millilitre
g/l	gram per litre
mg/l	milligram per litre
IU	international unit
IU/ml	international unit per millilitre

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DECLARATION

I, the undersigned, declare that no portion of this thesis, apart from the work carried out by Federica Di Nicolantonio, Louise Knight and Stuart Mercer, as stated in chapter 2.4, has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning. Apart from where stated, all experimental work was performed by myself at the Translational Oncology Research Centre, Queen Alexandra Hospital, Portsmouth, UK.

Pauline Whitehouse

CHAPTER 1

Introduction

1.1 Colorectal Cancer

1.1.1 Epidemiology

Colorectal adenocarcinoma (CRC) is the third most common cancer in men and the second most common cancer in women in the UK, with an incidence of about 30,000 cases per year (Office of National Statistics, 2002). The incidence increases with age, and it rarely occurs in the under 40s unless associated with a genetic predisposition. It is slightly more common in men than women with an odds ratio of 1.32 for proximal and 1.68 for distal cancers. There is a one in eighteen lifetime risk of developing colorectal cancer in the general population of developed countries.

Colorectal cancer is the second leading cause of cancer death, accounting for 16,000 deaths each year, in the UK. Overall five year survival varies from 30% in Eastern Europe, 42% in Western Europe and 62% in USA (Office of National Statistics, 2002), with similar mortality rates between both sexes. However, survival rapidly declines with advanced disease. 30-55% of patients present with advanced disease and half of those who do not, will progress to this stage later.

Historically colorectal cancer has been thought of as a "disease of the Western world", with the highest incidence in Western Europe and North America and the lowest incidence in underdeveloped countries (Parkin *et al.*, 1999), although a third of cases now occur outside industrialised countries. The geographic variation appears to be due to differences in exposure to dietary and environmental factors imposed on a background of genetic susceptibility. This has been confirmed by migration studies of Japanese (from a region with low incidence of CRC) to the United States (high incidence) (Haenszel and Kurihara, 1968). There has also been an anatomical shift from distal tumours to proximal tumours over several decades (Mostafa *et al.*, 2004).

1.1.2 Aetiology

Colorectal cancer occurs as a result of environmental factors in addition to an iterative genetic pathway in epithelial cells of colonic mucosa (Vogelstein *et al.*,

1988). Genetic conditions present at an early age and only account for 15% of CRC. There is still much controversy regarding environmental/dietary risk factors, but the low residue, high fat diet typical of the Western World is thought to be significant (Burkitt *et al.*, 1971). It is often difficult to distinguish risks between causative factors, for example, a high fat diet and sedentary lifestyle are often found together. Problems of accuracy also arise from using retrospective food questionnaires.

Genetic Risks

Most cases, about 75%, of colorectal cancer are of unknown aetiology, 10-15% of sufferers are found to have a positive family history, and the rest are associated with specific syndromes: Hereditary Non-Polyposis Colon Cancer (HNPCC), Familial Adenomatous Polyposis (FAP), and Li-Fraumeni Syndrome.

Family History

Close relatives of patients with CRC are at increased risk, which increases with the number of family members affected, closer family relationship and young age at diagnosis (Johns and Houlston, 2001).

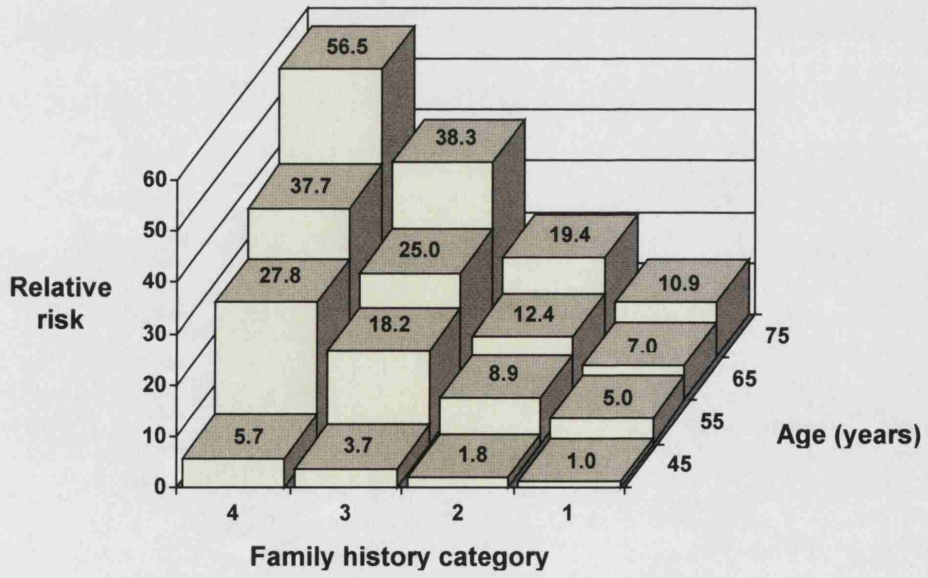


Figure 1.1. Colorectal cancer risk by age and family history. Family history category: 1 - no family, 2 - one affected first degree relative over 45 at diagnosis, 3 - one affected first degree relative under 45 at diagnosis, 4 - two affected first degree relatives.

Hereditary Non-Polyposis Colon Cancer or Lynch Syndrome

Hereditary Non-Polyposis Colorectal Cancer (HNPCC) is an inherited autosomal dominant syndrome (Lynch *et al.*, 1985) accounting for about 4-10% of all colorectal cancers. It is not easily distinguished from sporadic cancer as there is no tendency to extensive polyposis. It is characterised by early onset (<45 years) of colon cancer and other specific cancers (endometrial, small bowel, gastric, renal pelvis, ovarian, skin). The Amsterdam Criteria introduced by International Collaborative Group on HNPCC aims to define the syndrome (Vasen *et al.*, 1999).

Amsterdam Criteria

1. At least three relatives with histologically verified CRC;
   One of the relatives should be a first degree relative to the other two.
   Familial adenomatous polyposis should be excluded.
2. At least two successive generations should be affected.
3. In one of the relatives CRC should be diagnosed under 50 years of age.

HNPCC tumours are mismatch repair (MMR) deficient and thus express microsatellite instability (MSI) (see section on molecular pathways, 1.1.3). However, MSI is not specific or sensitive to HNPCC. hMLH1 and hMSH2 are the MMR genes responsible for most HNPCC.

Familial Adenomatous Polyposis

Familial Adenomatous Polyposis (FAP) is a rare autosomal dominant syndrome caused by an inherited mutation in the APC gene localized on chromosome 5q (Bodmer *et al.*, 1987; Leppert *et al.*, 1987) and accounts for only 1% of colorectal cancers. The disease is characterized by the development of many polyps, often thousands, usually at the age of 20-30 with a 100% risk of colonic cancer developing at a mean age of 44 years. Mutations in FAP families occur at different sites in the APC gene, but nearly all result in stop codons and a truncated APC protein. There are several variants of FAP including Gardener's and Turcot's syndromes.

Li-Fraumeni Syndrome

The Li-Fraumeni Syndrome (LFS) is a rare familial dominantly inherited cancer accounting for less than 1% of CRC. It is defined as the onset of sarcoma before 45 years of age with a first-degree relative with cancer before this age and another first or second degree relative with any cancer before this age or sarcoma at any age (Li *et al.*, 1988). Many different cancers are found in excess in LFS families and multiple studies report a p53 germline mutation.

Environmental/Dietary Risks

Fibre

For many years a low fibre diet has been considered a risk factor for CRC (Ghadirian *et al.*, 1997), and thus increasing the daily intake of fibre has been suggested to be protective. Fibre speeds transit and reduces exposure of gut mucosa to carcinogens. However, several studies in recent years have found no protective effect from fibre (Fuchs *et al.*, 1999) and a recent review and meta-analysis by the Cochrane Library (Asano and McLeod, 2003) has not shown that increasing dietary fibre reduces the incidence or recurrence of colorectal adenomas within a two to four year follow up period.

Red meat

A high intake of red meat, in particular processed meat, is associated with an increase in colorectal cancer risk, RR 1.35 (Norat *et al.*, 2002). This may be due to the fat content and total calorie intake, but is also linked to products from food preparation. Both heterocyclic aromatic amines and polycyclic aromatic hydrocarbons are potent mutagens resulting from cooking meat until very well done at high temperatures, especially over an open flame.

Dietary fat

Many studies have shown a relationship between total dietary fat and CRC, although it is unclear whether it is a general over consumption of food or the fat composition that is important. A high dietary intake of animal fat may increase colon cancer risk by increasing the excretion of bile acids whose products may act as carcinogens.

Animal fat carries a higher risk for CRC than vegetable fat, whilst fish oils may be protective. High cholesterol rather than saturated, monounsaturated or polyunsaturated fatty acids has been associated with increased risk (Jarvinen *et al.*, 2001).

Vitamin and Mineral intake

Calcium binds fatty acids and bile acids resulting in insoluble complexes less likely to produce hyperproliferation of colonic mucosa. Calcium supplementation has been found to moderately reduce the risk of recurrent colorectal adenomas (Baron *et al.*, 1999). Multivitamins, folate, vitamins D, C and E, and selenium are all found to be protective of CRC whereas high iron exposure is weakly associated with colorectal polyps.

Alcohol

There are conflicting reports regarding alcohol as a risk for CRC, but alcohol is known to inhibit DNA repair and a high consumption probably increases risk. In patients with at least one adenoma, alcohol has been shown to increase the risk of high-grade adenomas or cancer (OR 1.8) (Bardou *et al.*, 2002). Alcohol is probably not directly carcinogenic, but involved in early tumourigenesis by promoting growth of adenomas.

Smoking

A review of epidemiological studies has found heavy cigarette smoking increases risk of CRC 2-3 times (Giovannucci, 2001). This risk is associated with a long exposure over 3-4 decades. It is feasible that carcinogens reach the colorectal mucosa via the alimentary or circulatory systems causing damage or alteration in expression of cancer related genes.

Exogenous Hormone Use

A large RCT showed that long-term hormone replacement therapy (HRT) use reduced the risk of CRC by 37%, but the overall risk-benefit profile did not allow HRT to be initiated as intervention therapy (Anonymous, 2002). The protective effect of HRT is probably due to a decreased likelihood of silencing of the ER gene

by methylation. The recent findings of a significant increase in risk of breast cancer and the lack of cardiovascular benefit in HRT users makes it unlikely that HRT will ever be used for disease prevention (Million Women Study Collaborators, 2003).

Physical Activity/Obesity

It has been postulated that physical activity stimulates bowel activity and peristalsis, thus reducing the time colonic contents are in contact with the epithelium. High levels of physical activity are associated with lower insulin, glucose and triglyceride levels, producing a less favourable environment for the growth of cancers. There are subgroups of the population where obesity, physical inactivity, smoking, alcohol, high meat and low vegetable intake are all present and may cumulatively increase the risk of CRC (Martinez *et al.*, 1999).

Nonsteroidal Anti-Inflammatory Drugs

Nonsteroidal Anti-Inflammatory Drugs (NSAIDs), including aspirin, have been found to reduce the number of polyps in FAP (Steinbach *et al.*, 2000), and are associated with a reduced risk of colorectal cancer (Thun *et al.*, 1991). NSAIDS inhibit prostaglandin synthesis, as well as modulate the formation of aberrant crypt foci and oncogene (myc, ras, p53) expression. Specific cyclo-oxygenase-2 inhibitors were under investigation for chemoprevention and as maintenance therapy (e.g. VICTOR trial) but have been withdrawn for safety reasons.

Predisposing medical conditions

Previous CRC or polyps

Metachronous CRC occurs in 0.5-3% of cases, with an average interval of 8.7 years between the first and second malignancies, and are four times more common in HNPCC. Risk from polyps is stratified according to size (>1cm), number (>3) and histology (villous) (Atkin and Saunders, 2002).

Inflammatory Bowel Disease

In ulcerative colitis, CRC risk is related to the duration and extent of disease and dysplasia. The risk is 5-10% at 20 years after diagnosis, and 12-20% at 30 years, with a 14.8 fold relative risk increase in pancolitis. Synchronous cancers occur in 10-

20%. In Crohn's disease there is no increased risk of CRC in the absence of colonic involvement. The relative risk of CRC with colonic involvement is 5.6.

Bile acids and Cholecystectomy

The presence of bile acids correlates with fat consumption, which in itself is a risk factor for CRC. Bile acids induce intestinal mucosal hyperproliferation and activate AP-1, a transcription factor associated with promotion of neoplastic transformation in colonic cells. Cholecystectomy can result in high levels of bile acids in the caecum and ascending colon, which may increase the risk of right-sided CRC (RR 1.86).

Pelvic Irradiation

Patients who have received pelvic irradiation are at higher risk of rectosigmoid carcinoma.

1.1.3 Colorectal Carcinogenesis

Colorectal cancer (CRC) is unique in the study of cancer development because it has a distinct precursor lesion, the adenoma. The current model for colon carcinogenesis demonstrates the multiple and stepwise (but not linear) progression of CRC development, and the role of both oncogenes and tumour suppressor genes (figure 1.2). The progressive accumulation of genetic alterations is described by Vogelstein *et al.* (1988) who found that nine per cent of grade I adenomas possess more than one genetic alteration, whereas two or more alterations are found in 24% of grade II adenomas, 43% of grade III adenomas and 90% of carcinomas. At least four sequential changes must occur before colorectal cancer develops (Fodde *et al.*, 2001), which accounts for the long lead-time of many years.

(i) The adenoma-carcinoma sequence

There is little direct evidence that CRC develops from polyps as it is unethical to leave a polyp *in situ* in order to study its natural history. However, there is a lot of information supporting this theory. Cancers and adenomatous polyps have the same anatomical distribution; cancers rarely arise in the absence of polyps; the onset of polyps precedes cancer by several years; patients with one or more large polyps are at increased risk of developing cancer; patients with FAP have 100% risk of developing CRC; detecting and removing polyps reduces the incidence of CRC. Only about 5% of all polyps develop into cancer which occurs slowly over 10-15 years, and is termed "polyp dwell time".

There are two types of CRC distinct by their carcinogenic process, displaying either chromosomal or microsatellite instability. (1) Chromosomal instability (CIN) or loss of heterozygosity (LOH) positive tumours possess defects in chromosome segregation leading to qualitative and quantitative variations in chromosome number mainly involving chromosome 18q and 17p. This mechanism is responsible for more than two thirds of spontaneous CRC. (2) Microsatellite instability (MSI or MIN) positive tumours are due to mutations in the DNA mismatch repair system resulting in a mutator phenotype. This occurs in 15% of sporadic colorectal cancers.

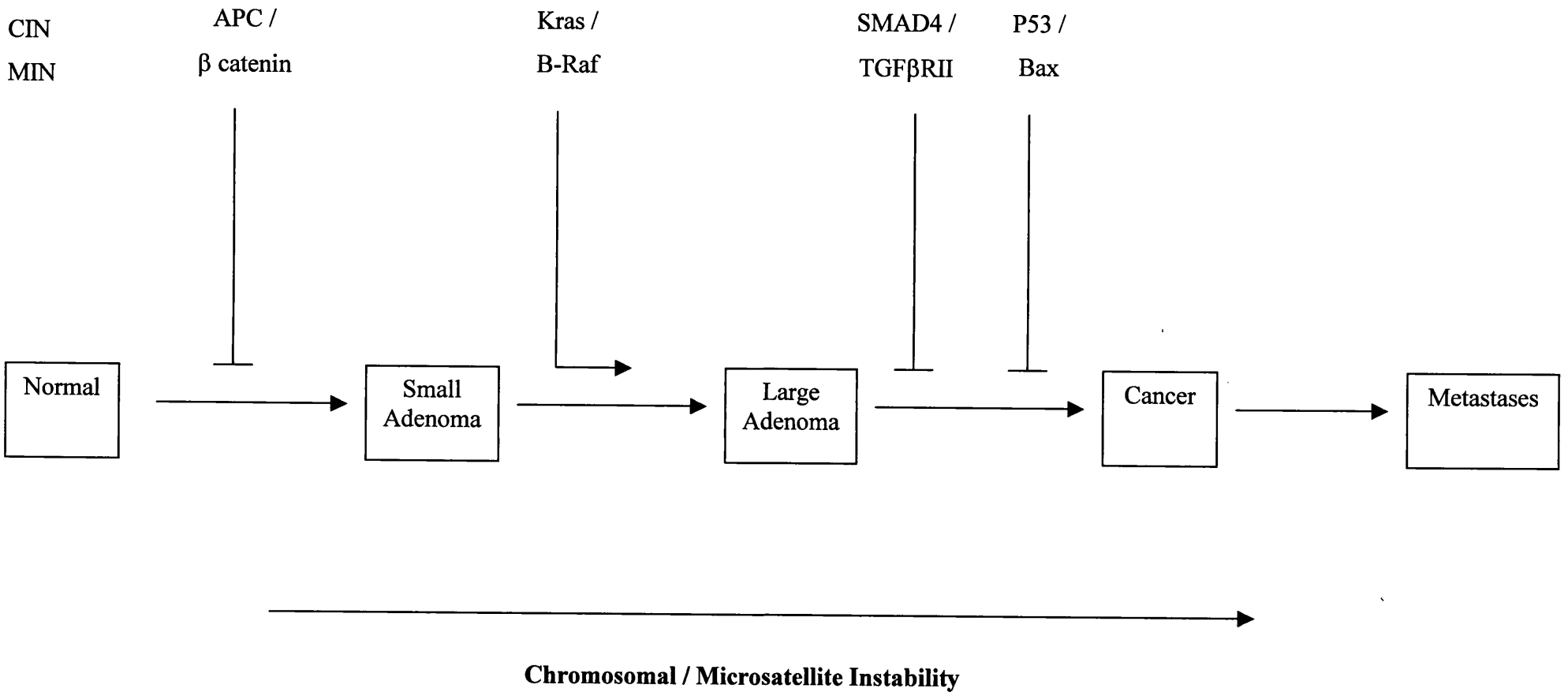


Figure 1.2. Adenoma-Carcinoma Sequence (modified from Vogelstein *et al.*, 1988)
CIN: Chromosomal Instability; MIN: Microsatellite Instability; ⊥ = Tumour Suppressor Pathway; ↓ = Oncogene Pathway

There are four main signalling pathways in the development of CRC and these are altered in different ways depending on whether the tumour is LOH-positive or MSI-positive (table 1.1)

Table 1.1. The four major signalling pathways in CRC

Pathway	**CIN**	**MIN**	**Pathology**
WNT/Wingless	APC mutation	β-catenin stabilizing	Adenoma
K-ras	K-ras mutation	B-Raf	progression to large adenoma
TGF β	Inactivate SMAD2/SMAD4	TGFβ type II receptor deleted	progression of adenoma to cancer
p53	p53 mutation	BAX inactivating mutation	adenoma-carcinoma transition

BAX: BCL-2 associated X protein

Genes responsible for cancer development

Oncogenes, which are derivatives of normal cellular gene products (proto-oncogenes), stimulate appropriate cell growth under normal conditions. Cells with mutant oncogenes continue to grow even when they are no longer receiving growth signals (eg. Ras). **Tumour-suppressor genes** (TSGs) (a) inhibit progress through the cell cycle preventing cell proliferation or (b) promote programmed cell death (apopotosis). When TSGs are mutated, control of growth is lost and the cell may become malignant (eg. p53). **Repair genes** do not control cell birth and death directly, but control the rate of mutation of all genes. Mutation of the repair genes allows cells to acquire mutations in oncogenes and TSGs at an accelerated rate (eg. mismatch-repair genes and nucleotide-excision repair genes). Knudson's Two Hit-Model postulates two inactivating "hits" (mutations) are needed to achieve loss of function in TSGs, but proto-oncogenes become active oncogenes by a single mutation (Knudson, 1985).

Table 1.2. Common gene mutations in colorectal cancer

Gene abnormality	Chromosomal location
FAP (APC)	5q21
Ras	K-ras 12p12.1, H-ras 11p15.5, N-ras 1p22
p53	17p
DCC (deleted in colon cancer)	18q
MCC (mutated in colon cancer)	5q
c-myc	8
HNPCC	2p16 (hMSH2), 3p21 (hMLH1), 2q31-33 (hPMS1), 7p22 (hPMS2)

(ii) The major signalling pathways in CRC / gene mutations

APC/β-catenin pathway

Germline mutations of the APC gene (chromosome 5q21) result in FAP, and together with somatic mutations, give rise to up to 85% of all colorectal cancers. Loss of, or mutation of, APC occurs early and is a key event in the carcinogenic process, resulting in the loss of orderly cell replication, adhesion and migration. The APC gene controls the WNT signal transduction pathway (tags β-catenin for destruction), and is also involved in cell-cell adhesion (via β-catenin and E-cadherin), stability of the microtubular cytoskelton, and apoptosis. Inactivation leads to tumour initiation (activation of WNT signal transduction pathway) and promotion (chromosomal instability) (figure 1.3) via the downstream transcriptional activator genes, c-MYC and T cell factor (Tcf). Most APC mutations involve the central region resulting in truncated APC unable to bind β-catenin, and therefore an accumulation of nuclear β-catenin and progression along the adenoma-carcinoma sequence. A change in crypt architecture leads to heaping up of a microadenoma (figure 1.4) making subsequent hits more likely.

β-catenin is an oncogene in its own right. It has two functions within the cell, a) involvement in the WNT signalling cascade, and b) in the junctions between cells, indirectly involved in linking the 'adherens junctions' to the cytoskeleton. Mutations

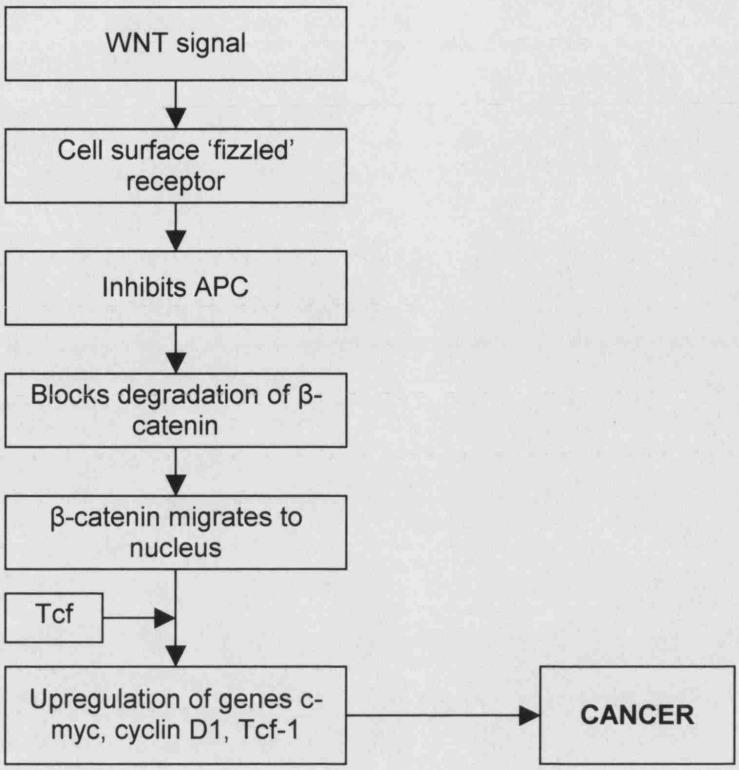


Figure 1.3. WNT signalling pathway

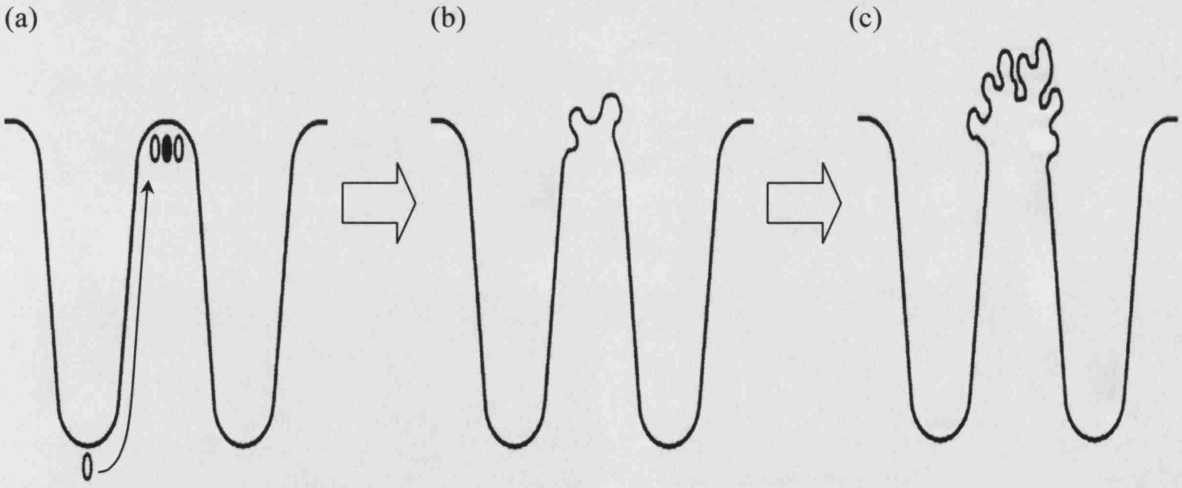


Figure 1.4. Colon microarchitecture and carcinogenesis.
(a) Stem cell mutations in crypt (blood borne carcinogen or germline mutation) results in surface cells that do not undergo apoptosis and thus may undergo further mutations and proliferate, (b) the aberrant crypt focus (ACF) increases surface in contact with faecal mutagens, (c) adenoma.

of β-catenin itself may result in increased nuclear accumulation as is in seen in other cancers such as childhood hepatoblastomas and endometrial carcinoma.

Ras/Raf pathway

Kirsten-Ras (K-ras) is a proto-oncogene which forms an integral part of intracellular signal transduction. It encodes a 21 kDa protein on the inner surface of the cell membrane which mediates signals initiated by the binding of polypeptide growth factors to their specific cell surface tyrosine kinase receptors, e.g. EGFR. The ras protein is GTP bound in the active state. GTP is hydrolysed by GTPase to GDT which renders the ras protein inactive. Mutation of K-ras, which occurs in 50-70% of colorectal cancers, results in decreased GTPase activity and a permanent state of ras activation and increased cell division. This leads to further polyp development. Mutation occurs with increasing frequency as benign adenomas become more dysplastic (i.e. it is mutated in 9% of adenomas <1cm, but in more than 50% of adenomas >1cm).

Transforming Growth Factor Beta Pathway

Transforming Growth Factor Beta (TGF-β) has a potent inhibitory effect on cell growth inducing growth arrest in late G1 phase of the cell cycle and apoptosis. TGF-β binds to the type II receptor (TGFβ-RII) leading to activation of the type I receptor (TGFβ-RI). Cytoplasmic SMAD2 and SMAD3 act as substrates for activated TGFβ-RI and are phosphorylated by it, forming a heterotrimeric complex with SMAD4, which is translocated to the nucleus to activate transcription of specific target genes (figure 1.5). TGF-β and TGFβ-RII increase in number from the proliferative compartment to the top of the crypt. Mutation of TGFβ-RII results in resistance to the growth inhibitory effects of TGF-β, and has been found in over 80% of tumours exhibiting MSI. SMAD2 and SMAD4 are located near the 'deleted in colon cancer' gene (DCC) on chromosome 18q21 and thus play a role in chromosomal instability. SMAD4, which was initially identified in juvenile polyposis syndromes, is mutated in about 15% of colorectal malignancies.

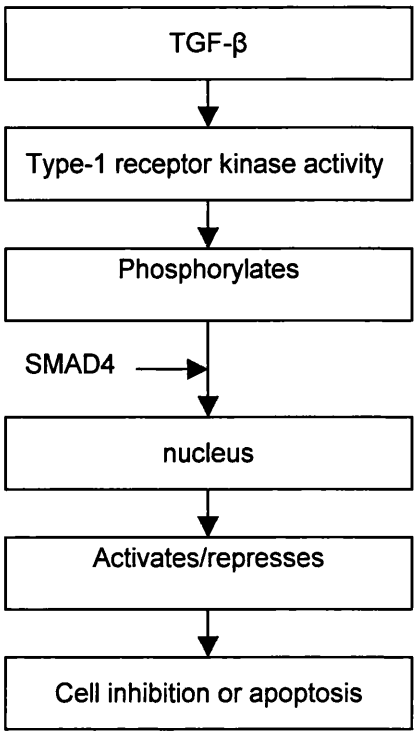


Figure 1.5. TGF-β pathway

p53 Apoptosis

p53 is a tumour suppressor gene that prevents a cell from proliferating whilst containing damaged or mutated DNA, thus reducing further carcinogenic risk. It may cause cell cycle arrest at the G1-S interface (by directly stimulating p21, an inhibitor of cyclin-dependent kinases) or commit a cell to die by apoptosis (via over expression of the Bax protein). It functions incorrectly in most human tumours, as a result of a mutation in the gene (chromosome 17p) in about 50% (Vogelstein *et al.*, 2000). p53 is normally 'off' and is activated by cell stress or damage. Activation results in inhibition of the degradation of the p53 protein, stabilizing it at a high concentration allowing it to bind to particular DNA sequences and activate the expression (transcription) of adjacent genes. The degradation process is termed ubiquitin-mediated proteolysis, which is a feedback mechanism. Mutations in p53 occur late in the adenoma-carcinoma sequence as they rare in benign lesions but are present in >75% of CRC.

Mismatch Repair Genes

Microsatellites are repeated short sequences of DNA that vary between individuals. DNA mismatch repair (MMR) genes are responsible for eliminating errors that occur in these microsatellites due to base-base mismatches and insertion/deletion loops that arise as a consequence of DNA polymerase slippage during DNA synthesis. There are five human MMR genes (hMLH1, hMSH2, hMSH6, hPMS1 and hPMS2), and mutations in these genes leads to an accumulation of errors in microsatellites, resulting in a mutator phenotype, termed microsatellite instability (MSI/MIN). Mismatched DNA induces ADP to ATP exchange leading to conformational change often affecting important growth regulatory genes (APC, kras, p53), and inducing frameshift mutations (BAX, TGFβ-RII). Microsatellite instability is present in 15% of sporadic colon tumours as well as in almost all HNPCC families. Mutation rates are 100-1000 times those in normal cells.

Individuals with HNPCC inherit a germline mutation in the first allele of a MMR gene (first hit) and develop loss of heterozygosity or a somatic mutation in the second (second hit). Patients with MSI positive tumours but not HNPCC usually possess promoter hypermethylation of a single MMR gene (most often hMLH1). MSI positive tumours often display aggressive histopathological features (signet ring, mucinous, exophytic), with an increased incidence of metachronous and synchronous tumours, but paradoxically have a favourable outcome with a decreased metastasising potential. This is thought to be due to an enhanced immunological response as these tumours have a high level of activated intraepithelial T lymphocytes.

MMR proteins can act on DNA damage caused by alkylating agents, and therefore, MMR deficient cells are more resistant to the killing effects of these drugs. The MSI status of patients with colorectal cancer is potentially clinically important as treatment with alkylating agents may actually lead to enhanced growth of tumour cells.

(iii) Metastatic Spread

Neoplastic cells have to develop a number of attributes in order to metastasise. Initially the cell-cell homotypic interactions are disrupted, mediated by a reduction in expression of the cell surface adhesion molecule E-cadherin. Motility of the cell is due to the formation of invading pseudopodia, enhanced by chemokinetic agents. The disruption of the extracellular matrix-cell is due to altered intergrin expression and due to the action of tumour associated proteases e.g. matrix metalloproteinases (MMPs), which degrade type IV collagen in basement membranes. In order for metastatic cells to establish at a particular site, the host organ must have an appropriate environment, i.e. absence of protease inhibitors and presence of growth factors.

1.1.4 Pathology and Prognosis

More than 98% of colorectal cancers are adenocarcinomas, of which 60% are moderately differentiated. Macroscopically they may be polypoid or ulcerating, and may form a scirrhous obstructing annular lesion. They are most common on the left side of the colon with 70% occurring within 12 cm of the anal verge. 3% of patients have synchronous tumours, and 75% also have benign adenomas.

Spread

CRC may spread by a number of different mechanisms: direct, via lymphatics, blood-borne, transperitoneal and by implantation. Direct lateral spread is much more common than in the longitudinal axis. Upward spread along the superior rectal and inferior mesenteric vessels is more common than lateral or downward lymphatic spread. Blood-borne spread is usually first to the liver via the portal vein. 30-50% of patients have occult liver metastases at time of presentation. Pulmonary metastases occur in 5% of cases. Transperitoneal spread occurs in 1-10% of patients following resection. Spread to the ovaries results in Krukenberg tumours. Spread by implantation is rare, but may be important in laparoscopic resection of tumours.

Staging

Survival from colorectal carcinoma is dependent on stage of disease at presentation. Since Dukes' first staging system for rectal cancer in 1929 (table 1.3), based on the clinical classification by Lockhart-Mummery in 1927, there have been many modifications and adaptations (Dukes, 1932; Astler and Coller, 1954), making it confusing for the clinician involved in the treatment of patients with CRC (Mainprize *et al.*, 2002). This system was extended for use in colonic cancer in 1939 (Grinell, 1939). The Tumour, Node, Metastases (TNM) staging system (table 1.5) is now the system in widespread use (Denoix, 1954; Beahrs and Myers, 1983), however, this too has already undergone a number of modifications (Royal College Pathologists, 1998).

Table 1.3. Dukes' 1932 classification with 1935 modification

A	Growth limited to the wall of the rectum/colon
B	Extension of growth to extrarectal tissues but no metastases
C	Metastases in regional lymph nodes
C1	Regional lymph nodes positive
C2	Lymph nodes at the point of mesenteric blood vessel ligation involved

Survival

Over the last few decades the proportion of colorectal cancers amenable to surgery has increased and the operative mortality rates have decreased. The 5-year survival rate following surgery is 55-70%, but the overall 5-year survival rate is much lower. Pathological stage is still the most significant prognostic indicator at the present time (table 1.4).

Table 1.4. 5-year survival rates according to the 6th Classification of the American Joint Committee on Cancer (O'Connell *et al.*, 2004)

Stage	% 5 Year Survival
I	93.2
IIa	84.7
IIb	72.2
IIIa	83.2
IIIb	64.1
IIIc	44.3
IV	8.1

Table 1.5. Colorectal TNM

Primary Tumour (T)

TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma *in situ*: intraepithelial or invasion of lamina propria
T1	Tumour invades submucosa
T2	Tumour invades muscularis propria
T3	Tumour invades through the muscularis propria into the subserosa, or into nonperitonealized pericolic or perirectal tissues
T4	Tumour directly invades other organs or structures, and/or perforates visceral peritoneum

Regional Lymph Nodes (N)

NX	Regional lymph nodes cannot be assessed
N0	No regional lymph nodes
N1	Metastases in 1 to 3 regional lymph nodes
N2	Metastases in 4 or more regional lymph nodes

Distant Metastases (M)

MX	Distant metastases cannot be assessed
M0	No distant metastases
M1	Distant metastases

Stage Grouping

TNM Classification (American Joint Commission on Cancer)				**Dukes**
Stage	**T**	**N**	**M**	
Stage 0	Tis	N0	M0	-
Stage I	T1	N0	M0	A
	T2	N0	M0	
Stage IIa	T3	N0	M0	B
Stage IIb	T4	N0	M0	
Stage IIIa	T1/2	N1	M0	C1
Stage IIIb	T3/4	N1	M0	
Stage IIIc	Any T	N2	M0	C2
Stage IV	Any T	Any N	M1	D

1.1.5 Surgical Treatment of Colorectal Cancer

(i) Surgery

Rectal cancer has a higher incidence of local recurrence than colon cancer due to the difficulty in obtaining circumferential margin clearance, with most recurrences occurring outside the lumen rather than at the anastomosis (Umpleby and Williamson, 1987). The aim of surgical treatment is potential cure by complete removal of all tumour material. Sphincter-sparing anterior resection was first performed by Claude Dixon in 1930 and is the operation of choice wherever possible rather than abdomino-perineal resection. Since the advent of the circular stapling gun in the 1970s it has been possible to perform bowel anastomoses at much lower levels, thus avoiding a permanent colostomy (Fain *et al.*, 1975).

Total Mesorectal Excision (TME) by sharp dissection of the avascular plane around the mesorectum is advocated for all rectal cancers above 4cm from the anal verge (Heald *et al.*, 1982). This method has demonstrated a superior 5 year recurrence rate of 5% compared to 13-25% (MacFarlane *et al.*, 1993). A surgical teaching initiative in Stockholm reduced the local recurrence rate from 14% to 6% (Lehander Martling *et al.*, 2000). However, TME has been associated with greater morbidity; operating time is prolonged and there may be a greater need for blood transfusion. A high rate of anastomotic leaks from stapled low anastomoses (11% symptomatic and 6.4% asymptomatic) resulted in the recommendation for routine defunctioning stoma formation (Karanjia *et al.*, 1994).

Circumferential margin tumour involvement is the main variable that influences local recurrence and survival (Quirke *et al.*, 1986; Nagtegaal *et al.*, 2002). Other surgical issues of current interest related to survival include: (a) The volume of operations and subspeciality of the surgeon (Birbeck *et al.*, 2002). (b) Luminally shed cells: cancer cells may be shed into the bowel lumen during surgery. However, most recurrences occur outside the lumen rather than at the anastomosis, and although cancer cells may be present in the distal lumen, there is no real evidence to support cytocidal irrigation of the rectal stump. (c) High division of the inferior mesenteric

artery (IMA). In 1908 Moynihan proposed that the IMA should be ligated flush with the aorta (Moynihan, 1908), supported by the demonstration that spread in the lymphatics follows the IMA to its origin at the aorta (Gordon-Watson and Dukes, 1930). High tie may downstage a Dukes C2 to a C1 if the specimen includes some uninvolved nodes. Non-randomised trials have shown that high tie in rectal cancer has no survival benefit (Surtees *et al.*, 1990).

Transanal Endoscopic Microsurgery (TEM) was introduced by Buess in 1984 as a minimally invasive technique for the local resection of early-stage rectal tumours (Buess, 1993). This technique of submucosal or full thickness excision transanally is associated with very low mortality (0-0.3%) and morbidity (4.8-8%), with completely excised margins in 92% (de Graaf *et al.*, 2002). Recurrence rates (0-4.2%) and overall survival in T1 cancers are comparable to that achieved with TME. In addition there is a significant reduction in time of hospitalisation, blood loss, operation time and opiate analgesia, making the procedure an option for medically unfit patients with a symptomatic higher stage tumour. However, patients undergoing TEM are not fully staged as the lymph nodes are not histologically examined, resulting in a lack of prognostic information, and subsequently patients may be denied systemic treatment. In addition, lymph node metastases are found in 10% of T1 tumours at radical operation (Blumberg *et al.*, 1999). Carcinoma in situ has no potential for metastatic spread, therefore local resection is justified in these cases, but up to 57% of cases are understaged preoperatively (de Graaf *et al.*, 2002).

(ii) Endoluminal Stenting

Self-expanding metal stents have been used for several years in the treatment of oesophageal, biliary and vascular obstructions. They were first used in the treatment of colorectal obstruction in 1991 (Dohmoto *et al.*, 1991). A meta-analysis has shown them to be safe and effective as palliation or as a "bridge to surgery" in obstructing colorectal cancer, with a technical success rate of 92% (Khot *et al.*, 2002).

1.1.6 Medical Treatment of Colorectal Cancer

(i) Introduction

It is now well accepted that chemotherapy improves survival and quality of life in patients with metastatic colorectal cancer (Nordic Gastrointestinal Tumour Adjuvant Therapy Group, 1992; Colorectal Cancer Collaborative Group, 2000). In recent years chemotherapy has begun to play an important role in the adjuvant setting, and may also be used neoadjuvantly to downstage liver metastases or as preoperative chemoradiation in rectal cancer. Until recently 5-fluorouracil (5-FU) was the only drug with significant activity against CRC, but newer agents and novel therapeutics are showing promising activity.

(ii) Adjuvant Chemotherapy

The aim of adjuvant chemotherapy is to target occult viable tumour cells and eradicate them before they become established and refractory to treatment, thus decreasing the recurrence rate and increasing survival. However, chemotherapy is not without side effects, some of which are potentially fatal, and therefore it is necessary to choose the patients whose benefit from chemotherapy is likely to outweigh the risks associated with the treatment. Pathological stage of disease at diagnosis is the strongest predictor of prognosis at the current time. It is generally accepted that chemotherapy is of benefit to patients with Stage III disease whose 5 year survival rate is <5%, but is not of benefit to patients with Stage I disease, >90% of whom live for more than 5 years. The 5 year survival of patients with Stage II disease is much more variable, from 30-80%, and this is the group where the benefit of chemotherapy is unclear, and where there is no international consensus.

Stage III Disease

Randomised phase III trials of adjuvant 5-FU plus folinic acid (FA) versus follow-up alone have demonstrated a 20-30% reduction in the risk of dying from CRC (IMPACT, 1995). However, there is still debate on the most effective schedule and duration of treatment. Six months treatment has been shown to be as effective as 12 months (O'Connell *et al.*, 1998), low-dose FA is as effective as high-dose, and levamisole has been shown not to contribute any additional benefit (Haller *et al.*,

Table 1.6. Studies of 5FU adjuvant therapy for colon cancer

Study	Stage of disease	Treatment Arms	Treatment duration	Patients (n)	3-5 year overall survival (%)	3-5 year disease / relapse free survival (%)	comments
IMPACT 1995	II and III	5FU/HDFA None	6 mth	1493	74 68 p=0.018	71 62 p<0.0001	10 year f/u 21% mortality reduction in stage III but only 8% in stage II
INT-0035 Moertel *et al.*, 1995	III	A) Levamisole B) 5FU/Levamisole C) None	12 mth 12 mth	929	N/R 71 55 p=0.007 (A *vs* B)	N/R 63 47 p<0.0001 (A *vs* B)	40% reduction in recurrence with 5FU/LEV vs observation. LEV alone no advantage
INT 0089 Haller *et al.*, 1998	II and III	A) 5FU/Levamisole B) 5FU/LDFA C) 5FU/HDFA D) 5FU/LDFA/Lev	12 mth 6 mth 6 mth 6 mth	3759	63 66 65 67 p=0.007 (A *vs* D)	56 59 60 60 p=0.014 (A *vs* D)	6 mth 5FU/LDFA/Lev better than 12 mth 5FU/Lev
INT 0089-46-51 O'Connell *et al.*, 1998	III and high risk II	A) 5FU/Levamisole B) 5FU/Levamisole C) 5FU/LDFA/Lev D) 5FU/LDFA/Lev	6 mth 12 mth 6 mth 12 mth	891	60 68 70 63 p<0.01 (A *vs* C)	58 63 63 57	No significant difference between 6 and 12 mths of same regimen
NSABP C-04 Wolmark *et al.*, 1999	II and III	A) 5FU/HDFA B) 5FU/Levamisole C) 5FU/HDFA/Levamisole	12 mth 12 mth 12 mth	2078	74 70 73	65 60 64 p=0.04 (A *vs* B)	Addition of Lev to 5FU/HDFA no benefit
QUASAR 2000	I (0.2%), II and III	A) 5FU/HDFA B) 5FU/LDFA C) 5FU/HDFA or LDFA/Lev D) 5FU/HDFA or LDFA	6 mth 6 mth 6 mth 6 mth	4927	36 36 37 35	70 71 69 72	Both colonic and rectal cases included

LDFA: low dose folinic acid (Leucovorin); HDFA: high dose folinic acid (Leucovorin); Lev: Levamisole
N/R: not reported in publication; p values given where <0.05, not significant if not stated

1998; Wolmark *et al.*, 1999). All these findings have been confirmed by the large QUASAR trial (QUASAR, 2000) (table 1.6).

Newer agents such as irinotecan, oxalpilatin, and the oral fluoropyrimidines have been found to be effective in advanced disease. There are currently several ongoing trials to assess the efficacy of these agents in the adjuvant setting (table 1.7). Oral administration of 5FU as uracil/tegafur has been found to have equivalent efficacy, and as capecitabine a better disease free survival, compared to intravenous 5-FU/FA (Cassidy *et al.*, 2004; Twelves *et al.*, 2005). The MOSAIC trial (6 months 5-FU/FA/Oxaliplatin (FOLFOX4) *versus* 6 months 5-FU/FA) found a significant relative risk reduction of 23% (p=0.002) for 3 year disease free survival in stage III CRC (Andre *et al.*, 2004).

Table 1.7. Some ongoing phase III trials of adjuvant treatment of colon cancer

Trial	Description
EORTC-40963	bolus 5-FU/FA vs infusional 5-FU/FA
FRE-FNCLCC-ACORD-2	infusional 5-FU/FA +/- irinotecan
CALGB-89803	bolus 5-FU/FA +/- irinotecan
PETACC-3	infusional 5-FU/FA +/- irinotecan
NSABP C-06	UFT/FA vs bolus 5-FU/FA
NSABP C-07	bolus 5-FU/FA +/- oxaliplatin
ROCHE –M66001	bolus 5-FU/FA vs oral capecitabine

Stage II Disease

Patients with Stage II/Dukes' B disease have a good outcome from surgery alone, and as the additional benefits from chemotherapy are small, the routine use of adjuvant chemotherapy is not universally accepted. However, a number of poor prognostic factors have been identified which influence the use of chemotherapy in Stage II disease, and many oncologists would encourage the inclusion of these patients in clinical trials.

The IMPACT B2 meta-analysis of five randomised controlled trials of patients with B2 disease did not find any significant benefit of 5-FU/FA (5 year overall survival with treatment 82% versus 80%) (IMPACT, 1999). The pooled results from the four adjuvant NSABP trials suggested that the relative risk reduction with chemotherapy was similar between Dukes' B and C cancers, regardless of the presence or absence of adverse prognostic factors (Mamounas *et al.*, 1999). However, the treatment received in each trial varied widely and not all studies had an observation arm. In order to detect an absolute risk reduction of 4% at 5 years, 4,700 patients would be required (Buyse and Piedbois, 2001).

The meta-analysis performed by the American Society of Clinical Oncology found no evidence of a statistically significant survival benefit of adjuvant chemotherapy for stage II patients (Benson *et al.*, 2004). However, the QUASAR I and MOSAIC trials did find a significant reduction in risk of recurrence, although this did not translate into an overall survival benefit (Andre *et al.*, 2004; Gray *et al.*, 2004).

Prognostic Factors affecting outcome in Stage II colon cancer

A number of factors affecting prognosis in patients with Stage II colon cancer have been identified (table 1.8). Attempts have been made to stratify these risks to identify high and low risk patients for prognosis (Merkel *et al.*, 2001; Petersen *et al.*, 2002). The prognostic index formed by Petersen *et al.* demonstrated a considerable difference in five year survival between patients stratified as high or low risk (49.8% versus 85.7%).

Chemotherapy in Older Patients

The benefit of chemotherapy to older patients is unclear. Many trials do not include separate data on elderly patients, and many exclude these patients altogether. In fact it is difficult to define what age is classified as elderly. Several cut off ages have been used in trials, from 65-75 years. The average life expectancy of a 70-year-old man is now ten years, and 15 for a 70-year-old woman. The elderly population are living longer, and are often fitter than previously. For this reason, and due to improvements in peri- and post-operative care, there has been a large increase in the number of elderly patients receiving surgery for primary CRC. However,

chemotherapy and radiotherapy are still offered to relatively few patients because of the perceived toxicity.

Table 1.8. Clinical, histological and molecular indicators of poor prognosis

Location of tumour
Obstruction / perforation
T4 tumours
Poor differentiation
Vascular / lymphatic / perineural invasion
Detection of micrometastases
Overexpression of thymidylate synthatase
Mutant p53, p27, DCC genes

Brower *et al.* (1993) reported increased toxicity in patients over 75 years of age receiving adjuvant chemotherapy, resulting in reduced dose intensity and poorer outcome. In advanced disease the 5-FU/FA regimen was found to be significantly more toxic than raltitrexed in the higher age group (Zalcberg *et al.*, 1998), particularly amongst women. This is thought to be due to gender differences in DPD activity. More recently, no difference in toxicity, hospital stay, response rate or 1 year overall survival was found in patients over 70 years old receiving adjuvant or palliative chemotherapy compared to the younger age group (Popescu *et al.*, 1999; Fata *et al.*, 2002). However, overall median survival was less with a significantly higher incidence of stomatitis in the elderly patients receiving bolus 5-FU/FA.

The biological age of a patient may be very different from their actual chronological age, and therefore, dictating a cut-off age for treatment is probably inappropriate. The performance status (table 1.9) and co-morbidity of the patient are known to have more influence on toxicity and outcome. These patients have been recruited for the QUASAR I trial, comparing 6 months bolus 5-FU/LFA with observation only, the results of which are awaited (Tebbutt *et al.*, 2002).

Table 1.9. The ECOG/Zubrod performance scale

Point	Description
0	Normal activity: asymptomatic
1	Symptomatic: fully ambulatory
2	Symptomatic: in bed <50% of time
3	Symptomatic: in bed >50% of time - not bedridden
4	100% bedridden.
5	Dead

(iii) Palliative Chemotherapy in Advanced disease

5-Fluorouracil

5-Fluorouracil (5-FU), as single agent, or in combination, has been the mainstay of medical treatment for colorectal cancer for over 40 years (Li and Ross, 1976). This is due to its relatively low toxicity, and until recently, the inability of newer drugs to achieve significantly better response rates. Attempts have been made to ameliorate the effect of 5-FU by biochemical modulation (see section 1.2) and route of administration. Modulation with folinic acid (FA), also called leucovorin, is now standard treatment even though a meta-analysis found it increased response rate but not overall survival (Advanced Colorectal Cancer Meta-analysis Project, 1992).

Protracted venous infusion significantly increases response rates (22% versus 15%) but produces only a modest, but just statistically significant, increase in overall survival compared to bolus administration (12.1 months versus 11.3 months) (Meta-analysis Group in Cancer, 1998). Infusional 5-FU regimens are associated with a more manageable toxicity profile, the dose-limiting toxicities being diarrhoea, stomatitis and hand-foot syndrome. However, the modest benefits have to be weighed against increased cost and complexity of treatment compared to bolus regimens. As a result, a variety of 5-FU regimens are available to the clinician (table 1.10) with a noticeable geographical variation of infusional administration being favoured in the US. Until the recent 2005 review, the NICE guidance on the

Table 1.10. 5FU-based treatment regimens

Regimen	Schedule
Bolus 5FU	
Machover	5FU 400mg/m²/d + FA 200mg/m²/d for 5 consecutive days every 4 weeks
Mayo	5FU 425mg/m²/d + 20mg/m²/d for 5 days every 4 weeks
Roswell Park	5FU (300mg/m² escalating to 750mg/m²) given in the middle of a 2-hour infusion of FA (500mg/m²) once a week for a minimum of 6 and, in case of response, until progression (maximum 1 year)
Infusional 5FU	
AIO	2-hour infusion of FA (500mg/m²) followed by 24-hour infusion of 5FU (2,600mg/m²), weekly for 6 weeks
de Gramont	2-hour infusion of FA (200mg/m²) + bolus 5FU (400mg/m²) followed by a 22-hour infusion of 5FU (600mg/m²) on days 1 and 2 of each fortnight
Modified de Gramont	FA (200mg/m²) + bolus 5FU (400mg/m²) followed by a 46 hour infusion of 5FU (2400-3000mg/m²) fortnightly
Lokich	5FU 250-300mg/m² as prolonged continuous iv infusion until progression/toxicity

treatment of advanced CRC in the UK recommended that 5-FU with folinic acid should remain as first line treatment (NICE, 2002; NICE, 2005).

Oral Fluoropyrimidines

Daily oral 5-FU simulates protracted venous infusion, but bioavailability of oral 5-FU is very variable as a consequence of the enzyme dihydropyrimidine dehydrogenase (DPD). Therefore the administration of DPD inhibitors or pro-drugs of 5-FU that are converted to cytotoxic agents after absorption has been investigated. Capecitabine is a fluoropyrimidine carbamate that is preferentially converted to 5-FU at the tumour site. Pooled results from two large phase III randomised controlled trials show capecitabine to be at least as effective as the Mayo regimen of 5-FU/FA in metastatic CRC, with less grade 3/4 neutropenia but more hand-foot syndrome (Twelves, 2002). Similarly, UFT has been found to have comparable activity to bolus 5-FU/FA with less toxicity (Carmichael *et al.*, 2002; Douillard *et al.*, 2002). Both these drugs are undergoing further investigation in combination with irinotecan and oxaliplatin. The use of oral fluoropyrimidines is cost effective and favoured by patients. However, 30% of patients with metastatic CRC have primary resistance to 5-FU and most of the others will develop it. Therefore other agents are undergoing investigation, especially in combination with 5-FU/FA (table 1.11).

Mitomycin C

Single agent Mitomycin C has produced response rates of up to 23% in colorectal cancer (Moertel *et al.*, 1968; Moore *et al.*, 1968). A randomised phase III trial found first-line treatment with combination MMC and protracted venous infusion 5-FU increased response rates to 54%, but with no benefit to overall and one-year survival (Ross *et al.*, 1997). A further phase III study confirmed an improved response rate and a survival benefit at 2 years (Price *et al.*, 1999).

Irinotecan

The topoisomerase I inhibitor irinotecan was initially investigated as second-line monotherapy in 5-FU resistant disease and demonstrated survival benefits compared to best supportive care (Cunningham *et al.*, 1998). In combination with 5-FU, irinotecan has been shown to be effective as first-line treatment, with an

Table 1.11. Pivotal studies in the treatment of advanced colorectal cancer

Drug	Study	Treatment arms	Patients (n)	Response rates (%)	Median overall survival (mths)	Median disease free survival (mths)
Oral fluoropyrimidines						
Capecitabine	Hoff *et al.*, 2001	Capecitabine	302	25.8*	12.5	4.3
		Bolus 5FU/FA	303	11.6	13.3	4.7
	Van Cutem *et al.*, 2001	Capecitabine	301	18.9	13.2	5.2
		Bolus 5FU/FA	301	15.0	12.1	4.7
UFT/FA	Douillard *et al.*, 2002	UFT/FA	409	12	12.4	3.5
		Bolus 5FU/FA	407	15	13.4	3.8
	Carmichael *et al.*, 2002	UFT/FA	190	11	12.2	3.4
		Bolus 5FU/FA	190	9	10.3	3.3
Eniluracil/5FU	Schilsky *et al.*, 2002	Eniluracil/5FU	485	12.2	13.3	4.6
		Bolus 5FU/FA	479	12.7	14.5	5.2
	Van Cutsem *et al.*, 2001	Eniluracil/5FU	268	11.6	10.9	4.4
		Bolus 5FU/FA	263	14.4	14.7*	5.3
Irinotecan	Douillard *et al.*, 2000	Irinotecan/Infused 5FU/FA	199	49*	17.4*	6.7*
First line		Infused 5FU/FA	188	31	14.2	4.4
	Saltz *et al.*, 2000	Irinotecan/bolus 5FU/FA	231	50*	14.8*	7.0*
		Bolus 5FU/FA	226	28	12.6	4.3
		Irinotecan	226	29	12.0	4.2

Table 1.11 continued

Drug	Study	Treatment arms	Patients (n)	Response rates (%)	Median overall survival (mths)	Median disease free survival (mths)
Second line	Cunningham *et al.*, 1998	Irinotecan	189	N/R	9.2*	N/R
		BSC	90	N/R	6.5	N/R
	Rougier *et al.*, 1998	Irinotecan	127	N/R	10.8*	4.2
		Irinotecan + 5FU +/- FA	129	N/R	8.5	2.9
Oxaliplatin	Giacchetti *et al.*, 2000	Oxaliplatin/chron 5FU/FA	100	53*	19.4	8.7*
First line		Chron 5FU/FA	100	16	19.9	6.1
	De Gramont *et al.*, 2000	Oxaliplatin/flat rate 5FU/FA	210	50*	16.2	9.0*
		Flat rate 5FU/FA	210	21.9	14.7	6.2
	Grothey *et al.*, 2002	Oxaliplatin/5FU/FA		49.1*	19.7	7.8*
		5FU/FA		22.6	16.1	5.3
Second line	Rothenberg *et al.*, 2003	Oxaliplatin/5FU/FA		9.9*		4.6*
		5FU/FA		0		2.7
		Oxaliplatin		1		
Irinotecan/oxaliplatin/5FU	Goldberg *et al.*, 2002	Irinotecan/bolus 5FU/FA	264	31	15	6.9
		Oxaliplatin/infused 5FU/FA	267	45*	19.5*	8.7*
		Irinotecan/oxaliplatin	264	35	17.4	6.5

Chron: chronomodulated infused; flat rate: flat rate infused; N/R: not reported; *: $p<0.05$

improvement in overall survival, despite the use of different methods of administration of 5-FU (Douillard *et al.*, 2000; Saltz *et al.*, 2000). It has recently been recommended as first-line treatment in combination with 5-FU/FA, or as single agent second-line treatment in the UK (NICE, 2005).

Oxaliplatin

Oxaliplatin has mainly been investigated in combination with 5-FU for first-line therapy, as it has only a 10% response rate as a single agent (Machover *et al.*, 1996). Three randomised trials of this combination using different deliveries of 5-FU have shown significant improvements in response rates and progression-free survival, but no overall survival benefit (de Gramont *et al.*, 2000; Giacchetti *et al.*, 2000; Grothey *et al.*, 2002). In combination with 5-FU/FA, oxaliplatin is now recommended for use as first-line or subsequent therapy in the UK (NICE, 2005).

Interpretation of the various combination trials is complicated as all use different control regimens and patients receive cross-over second-line therapies. In order to eliminate these differences and to compare combination irinotecan or oxaliplatin with 5-FU/FA versus 5-FU/FA monotherapy, the Medical Research Council have designed the FOCUS/CR08 trial with second line therapy incorporated into the protocol. Furthermore, there have been doubts about the usefulness of tumour response as a clinical endpoint. However, a meta-analysis using the landmark method to eliminate bias found that tumour response does have a major prognostic effect on the survival of patients with advanced CRC (Buyse *et al.*, 2000). Sargent *et al.* (2004) found 3 year disease free survival as an endpoint did correlate to 5-year overall survival.

Irinotecan/Oxaliplatin

Combinations of 5-FU/FA with irinotecan or oxaliplatin have been shown to improve response rate, although no difference has been found between 5-FU/FA plus irinotecan and 5-FU/FA plus oxaliplatin (Tournigand *et al.*, 2004). Studies have investigated sequence of use and efficacy of combination irinotecan and oxaliplatin. A French study randomised patients to receive Irinotecan/5-FU/FA (FOLFIRI) and then Oxaliplatin/5-FU/FA (FOLFOX) on progression (group A) or the reverse (group

B). The response rates in first line therapy were similar, but better with FOLFOX in second line treatment. However, the overall time to progression after both treatment cycles was similar (Achille *et al.*, 2001). Oxaliplatin/Irinotecan has been found to be superior to 5-FU/FA with alternating Irinotecan or Oxaliplatin (RR 23% vs 6%) (Becouran *et al.*, 2001), but less effective than oxaliplatin with infused 5-FU/FA (Goldberg *et al.*, 2004). Side effects are increased with combination treatment but have generally been found to be manageable.

(iv) Other treatments

Supplementary to the development of new cytotoxic agents and combinations, there is a new era of anticancer therapies, aimed at being tumour specific rather than systemic. These include novel agents targeting signal transduction, e.g. epidermal growth factor receptor inhibitors, angiogenesis inhibitors, and COX-2 inhibitors. In addition, gene and immune therapies are also under investigation. These agents are discussed in section 1.2.1.

(v) Multimodality Treatment of Rectal Cancer

There is no question that radical surgery with negative resection margins is the most important treatment for patients with rectal cancer. However, locoregional failure is still seen after locally curative surgery, and quality of life is often very poor in those with irresectable disease. Multimodality treatments with radiation, chemotherapy or chemoradiation have been investigated in an attempt to reduce local recurrence rates and improve overall survival. The major indications for radiotherapy are: a) the reduction of local recurrences in mobile resectable rectal cancers by eradicating remaining tumour cells, and b) downstaging and downsizing of surgically irresectable tumours to render them operable and to allow sphincter-saving procedures. Radiation therapy may be given pre- or post-operatively, alone or in combination with chemotherapy.

The timing of radiation treatment is important to avoid repopulation of tumours by surviving cells. Treatment schemes of more than four weeks are associated with

tumour cell repopulation that equates to a reduction in radiation dose and are thus less effective. A dose of 50 Gy with conventional fractionation (10-30 x 1.8-2.0 Gy) is considered necessary in order to achieve >90% eradication of subclinical disease (Marijnen and Glimelius, 2002).

Postoperative Radiotherapy

Original studies in rectal cancer showed improved local recurrence rate and survival with postoperative chemotherapy. The results of two randomised controlled trials, the Gastrointestinal Study Group (GITSG) Protocol 7175 (Douglass *et al.*, 1986) and North Central Cancer Treatment Group (NCCTG) Protocol 79-47-51 (Krook *et al.*, 1991), led to postoperative radiotherapy with chemotherapy (5-FU based) becoming standard treatment in the USA (NIH Consensus, 1990). However, these original trials received criticism because the local recurrence rates in the surgery alone groups was very high. This led to future studies being standardised with TME. The NSABP-R-02 trial (Wolmark *et al.*, 2000) demonstrated a local recurrence reduction from 13% to 8% with chemoradiotherapy, but no overall survival benefit (table 1.12). Remaining clonigenic cells may repopulate post operatively and thus commencement of radiotherapy should start as soon as is possible following surgery (within 4-6 weeks). However, the ideal treatment time is often not achieved due to medical complications following surgery.

Preoperative Radiotherapy

Over the last two decades preoperative radiotherapy has been investigated, both as conventional fraction and short course treatments. There are several features of preoperative radiotherapy that may be advantageous over postoperative radiotherapy, including; sterilization of tumour cells and reduction in tumour spillage at surgery; downstaging and downsizing; sphincter preservation; no irradiation of anastomotic region; small bowel is more mobile in the virgin abdomen and less likely to be irradiated; less acute and late toxicity; more patients receive full dose; more effective due to oxygen tension in tumour higher prior to surgical compromise of blood flow. However, some patients with early stage or disseminated disease may receive unnecessary treatment as selection is dependent on imaging rather than pathological

Table 1.12. Randomised postoperative radiation and chemotherapy trials in rectal cancer

Trial	Local Recurrence (%)	Distant Recurrence (%)	5 year Overall Survival (%)
GITSG			
(Douglass *et al.*, 1986)			
Surgery alone	24	34	44
Surgery + RT	20	30	52
Surgery + CT	27	27	50
Surgery + CRT	11	26	59
NSABP R-01			
(Fisher *et al.*, 1988)			
Surgery alone	25	26	48
Surgery + CT	21	24	58
Surgery + RT	16	31	50
Mayo/NCCTG			
(Krook *et al.*, 1991)			
Surgery + RT	25	46	48
Surgery + CRT	14	29	57
Tveit *et al.*, (1997)			
Surgery alone	30	39	50
Surgery + CRT	12	33	64
NSABP R-02			
(Wolmark *et al.*, 2000)			
Surgery + CT	13	29	65
Surgery + CRT	8	31	66

CT: chemotherapy; CRT: chemoradiotherapy; RT: radiotherapy

stage. Conventional fractionation preoperative radiotherapy may be combined with chemotherapy, and surgery should be performed 5-6 weeks after the last dose.

The large German Rectal Study compared pre-operative and post-operative chemoradiotherapy and found there was better compliance, improved local control and less toxicity with pre-operative treatment, but no improvement in overall survival (Sauer *et al.*, 2004).

The Swedish Rectal Cancer Trial was the first to demonstrate a survival advantage for short course radiotherapy (5 x 5Gy) with an improved 5 year survival rate from 48% to 58% in patients with resectable disease of all stages (Swedish Rectal Cancer Trial, 1997). The Dutch Colorectal Cancer Group, performing TME in all cases, has shown a local failure rate of 2.4% when combined with short course preoperative radiotherapy versus 8.2% without (Peeters *et al.*, 2003). Treatment should be followed by surgery within 1-2 weeks, and therefore tumour shrinkage and sphincter preservation is unlikely. Short course radiotherapy has been associated with increased perineal wound complications, infection rates and blood loss. Short course preoperative radiotherapy cannot be combined with chemotherapy.

1.1.7 Liver Metastases

(i) Introduction

Half of all patients with colorectal cancer develop liver metastases, which in 30-40% is the only site of spread (Cromheecke M *et al.*, 1999; South and West Cancer Intelligence Unit, 2000). Surgery is the only treatment that offers potential cure for patients with liver metastases, however, only 10-20% are suitable for resection. Resection in carefully selected patients results in a median survival of 23-25 months and a 20-45% five year survival rate, compared to 1% without treatment (Rees *et al.*, 1997). The role of pre- and post-resection chemotherapy is under investigation, as are several methods of liver ablation.

ii) Surgical Resection

The massive improvements in surgical techniques using argon diathermy and ultrasound parenchymal dissection have reduced the high morbidity and mortality historically associated with liver surgery (Heriot and Karanjia, 2000; Primrose, 2002). In addition, anaesthetic techniques have also changed dramatically assisting in reduced blood loss. The perioperative mortality now associated with resection of colorectal liver metastases is about 2% (Scheele *et al.*, 1995; Rees *et al.*, 1997). The overall survival rate for liver resection is strongly affected by the patient selection criteria. There is widespread variation amongst experts in the indications for liver resection and prognostic factors. The number of metastases is becoming less important, with more than the traditional limit of four being resected. More importantly, at least two segments of liver, preferably in continuity should be preserved, and all disease resected with at least a 1cm clear margin. In addition, the position of the metastases may also be important. Surgical resection should only be attempted with curative intent.

Stangl *et al.* found percentage of liver replaced by tumour (>25%) to be the most significant prognostic factor (Stangl *et al.*, 1994). However, a number of prognostic factors have been identified that may lead to better selection of patients who will benefit from liver resection. Adverse criteria include; positive nodal status of the primary CRC, a disease free interval of <12 months, number of metastases >1,

preoperative CEA >200ng/ml and the size of the largest tumour >5cm. These criteria have been combined to form a scoring system where a 5 year survival of 60% is possible with a score of 0, but survival is only 14% when scoring 5 (Nordlinger *et al.*, 1996; Fong *et al.*, 1999).

(iii) Ablative therapies

There are three main techniques of destroying tissue: thermal ablation, direct injection chemotherapy and irradiation. Each method causes cell death by coagulative necrosis. Local, minimally invasive tissue ablation preserves uninvolved liver and avoids the morbidity and mortality of major hepatic surgery.

Radiofrequency ablation (RFA) is currently the best choice for local tissue ablation and can be performed at laparotomy, laparoscopy, or percutaneously under local anaesthesia with sedation. A needle electrode with an insulated shaft and non-insulated tip is inserted into the lesion under image guidance. Energy at the tip causes ionic agitation and frictional heat which leads to cell death and coagulative necrosis of targeted tissue. A single application can produce an area of necrosis up to 45mm in diameter. With multiple applications areas up to 7-8 cm can be achieved. The aim is to completely ablate the tumour with a 1 cm margin of ablated normal tissue (Curley *et al.*, 1999).

Percutaneous RFA is associated with a significantly higher recurrence rate, 16.4%, when compared to open or laparoscopic RFA, 5% (Mulier *et al.*, 2005). In addition, overall survival in unresectable disease has not been shown to be any different between RFA and chemotherapy only groups. Currently RFA is not recommended except in trial settings for unresectable disease.

(iv) Chemotherapy

Studies have shown that neoadjuvant chemotherapy (5-FU/FA and oxaliplatin, often chronomodulated) in patients initially thought to have irresectable liver disease has increased resectability by 13.5-38%, with 5 year survival rates comparable to those after resection of resectable lesions (Giacchetti *et al.*, 1999; Adam *et al.*, 2001). Despite the reported 25-40% long term survival rates after liver resection, tumour

relapse occurs in 60% of patients. In a non-randomised trial by Figueras *et al.* (2001), adjuvant chemotherapy with 5F/FA increased 5 year survival from 36% to 53%. A current EORTC/Cancer Research Campaign study is examining the role of pre- and post- operative chemotherapy (5-FU/FA/Oxaliplatin) in patients with operable colorectal liver metastases, in order to determine a survival benefit.

1.2 Chemotherapeutic Drugs and Mechanisms of Resistance

1.2.1 Principles of Chemotherapeutic Drug Action

Cancer cells exhibit uncontrolled proliferation, invasiveness and the ability to metastasise. The growth rate of solid tumours is not exponential. Growth slows as the tumour increases in size and outgrows its blood supply, and not all cells proliferate continuously. Cytotoxic drugs kill a constant fraction of malignant cells (logarithmic cell kill), thus killing 99.99% of 10^{11} cells will leave 10^{7} viable cells to proliferate (Goldie and Coldman, 1979). For this reason at least six cycles of chemotherapy are usually required for potential cure. Therefore, it is important to produce as near total kill as possible at each cycle. Many chemotherapeutic agents act by interacting with cell nucleic acids or specific proteins involved in the cell cycle and only affect dividing tumour cells (figure 1.6).

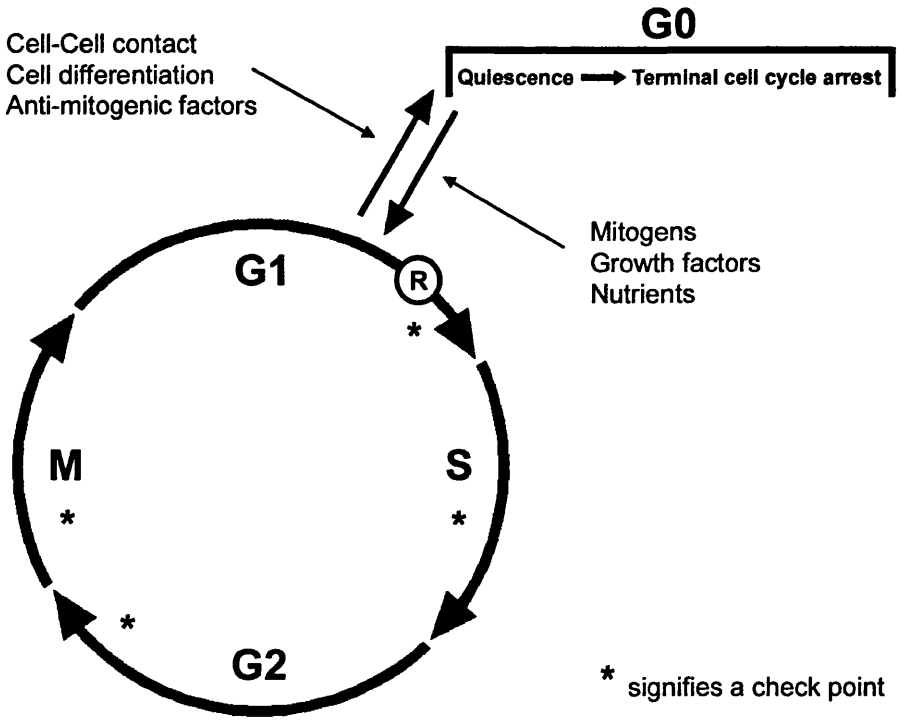


Figure 1.6. Schematic diagram of the cell cycle

Chemotherapy is most effective on cancer cells that are undergoing rapid proliferation, more rapid than host cell division. However, some normal host cells also proliferate rapidly, leading to side effects such as bone marrow depression, sterility, hair loss, nausea and vomiting, and teratogenicity. The term 'cytotoxic' refers to drugs that inhibit cell division and kill cancer cells; 'cytostatic' drugs suppress growth and proliferation of cells. Hormone therapy, also a cytostatic, is useful to suppress growth in some tumour types.

The main anticancer drugs may be grouped into the following categories:

- DNA damaging agents
- antimetabolites
- topoisomerase inhibitors
- antimicrotubule inhibitors

More recently, new agents, complementary to cytotoxic agents, or cytotoxic in their own right, have been investigated and some have already been introduced into clinical practice. These include:

- multidrug resistance inhibitors
- non-steroidal anti-inflammatory drugs
- drugs targeting signal transduction
- immune therapy
- gene therapy

(i) DNA damaging drugs

Alkylating agents

Alkylating agents act by forming covalent bonds with DNA and thus impede DNA replication. They are bifunctional (with two alkylating groups) and can cause intra- or inter- chain cross-linking, which interferes with transcription and replication. They have most effect on cells during replication when some parts of DNA are unpaired resulting in a block at G2 and cell death. Resistance is due to a number of mechanisms including inactivation by binding to electron rich molecules such as glutathione (GSH), catalysed by the enzyme GSH S-transferase.

Nitrogen mustards were the first cytotoxic agents used in clinical trials (Goodman *et al.*, 1946) having been developed from 'mustard gas' used in the First World War. Cyclophosphamide is a commonly used alkylating agent. After metabolism in the liver by P-450, the metabolites are transported to the tissues where they are converted to the cytotoxic molecule phosphoramide mustard.

Mitomycin C (MMC) is an antibiotic extracted from *Streptomyces caespitosus* (Wakaki *et al.*, 1958) containing an aziridine ring. It shows activity in a number of solid cancers and is not cell cycle specific. Reduction is required to activate the drug, which attaches to the extracyclic N2 amino group of a guanyl acid and then to a guanyl acid of the complementary DNA strand. MMC and its metabolites also form intrastrand G-G cross-links that produce bending of DNA. Its main toxicity is myelosuppression but it can also rarely cause the haemolytic uraemic syndrome and interstitial pneumonitis.

Platinum Analogues

Cisplatin (*cis*-diamminedichloro-platinum (II)) (CDDP) is a water-soluble square planar co-ordination complex containing a central platinum atom surrounded by two chlorine atoms and two ammonia groups. When it enters the cell the chloride ions dissociate and are replaced by water forming an aquated species able to form DNA-DNA cross links and DNA-protein crosslinks (intrastrand dinucleotide adducts between adenine and guanine nucleotides and two guanines which affects normal DNA function) resulting in cell cycle arrest and apoptosis. Resistance mechanisms include the limitation of formation of cytotoxic platinum DNA adducts (increased drug inactivation) or prevention of cell death after adduct formation (increased adduct repair or increased damage tolerance). It has low myelotoxicity but causes severe nausea and vomiting and can be nephrotoxic.

Carboplatin (*cis*-diammine(1,1-cyclobutanedicarboxylato)platinum (II)) is a derivative of cisplatin. It is more myelotoxic but causes less nephrotoxicity, neurotoxicity, ototoxicity and less severe nausea and vomiting. Oxaliplatin (*cis*-(1*R*,2*R*)-1,2-cyclohexanediamine-N,N^1)(oxalato(2-)-O,O^1) platinum) is a third generation platinum complex possessing a non-cleaving group (DACH) which forms

DNA adducts in a similar manner to cisplatin. The DACH-Pt adducts are not recognised by, nor dependent for cytotoxicity upon, the MMR protein complex (Fink *et al.*, 1996). It is not associated with renal toxicity and there are minimal haematological side effects but it commonly causes peripheral sensory neuropathy which is usually reversible. It is non-cross resistant with 5-FU and in combination they act synergistically (Levi *et al.*, 1997).

(ii) Antimetabolites

Antimetabolites mimic molecules involved in cellular processes for correct functioning of the cell, thus they block or subvert pathways in DNA synthesis. They may be classified as folate antagonists (e.g. methotrexate, raltitrexed), pyrimidine analogues (e.g. fluorouracil, cytarabine, gemcitabine) and purine analogues (azathioprine).

Gemcitabine is a deoxycytidine analogue which is cell cycle specific, killing cells undergoing DNA synthesis (S-phase) and blocking the progression of cells through the G1-S phase boundary. It enters the cell by facilitated diffusion and is phosphorylated intracellularly to its active metabolite "gemcitabine triphosphate" which competes with dCTP for incorporation into DNA and causes chain termination. DNA polymerase is inhibited by gemcitabine and is unable to remove gemcitabine from and repair the DNA strand. Gemcitabine has a number of other self-regulating interactions within the cell. It is incorporated into RNA and inhibits ribonucleotide reductase, resulting in a depletion of intracellular deoxyribonucletoides, and favouring incorporation of phosphorylated gemcitabine into DNA repair mechanisms. It is a potent radiation sensitiser with a mild toxicity profile (van Moorsel *et al.*, 1997).

The major biological effects of 5-Fluorouracil (5-FU) are caused by its inhibition of thymidylate synthase (TS), a key enzyme in DNA synthesis. 5-FU is phosphorylated intracellularly to 5-fluoro-2'-deoxyuridine monophosphate (FdUMP) which forms a stable complex with thymidylate synthase resulting in inhibition of thymidine nucleotide synthesis and thus DNA synthesis. Other 5-FU metabolites may undergo

further phosphorylation to the triphosphate form (FUTP) which is incorporated into RNA. The metabolism of 5-FU is complex involving many enyzmes and pathways, therefore it is unsurprising that there are multiple mechanisms of resistance. Most commonly described is the overexpression of TS, dihydropyrimidine dehydrogenase (DPD) or thymidine phosphorylase (TP). Figure 1.7 summarises the biochemical pathway of 5-FU. Folinic acid (FA), also called leucovorin, enhances cytotoxicity by expanding intracellular pools of 5,10-methylenetetrahydrofolate, the folate cofactor that forms a stable covalent complex with TS, thereby increasing the extent and duration of TS inhibition.

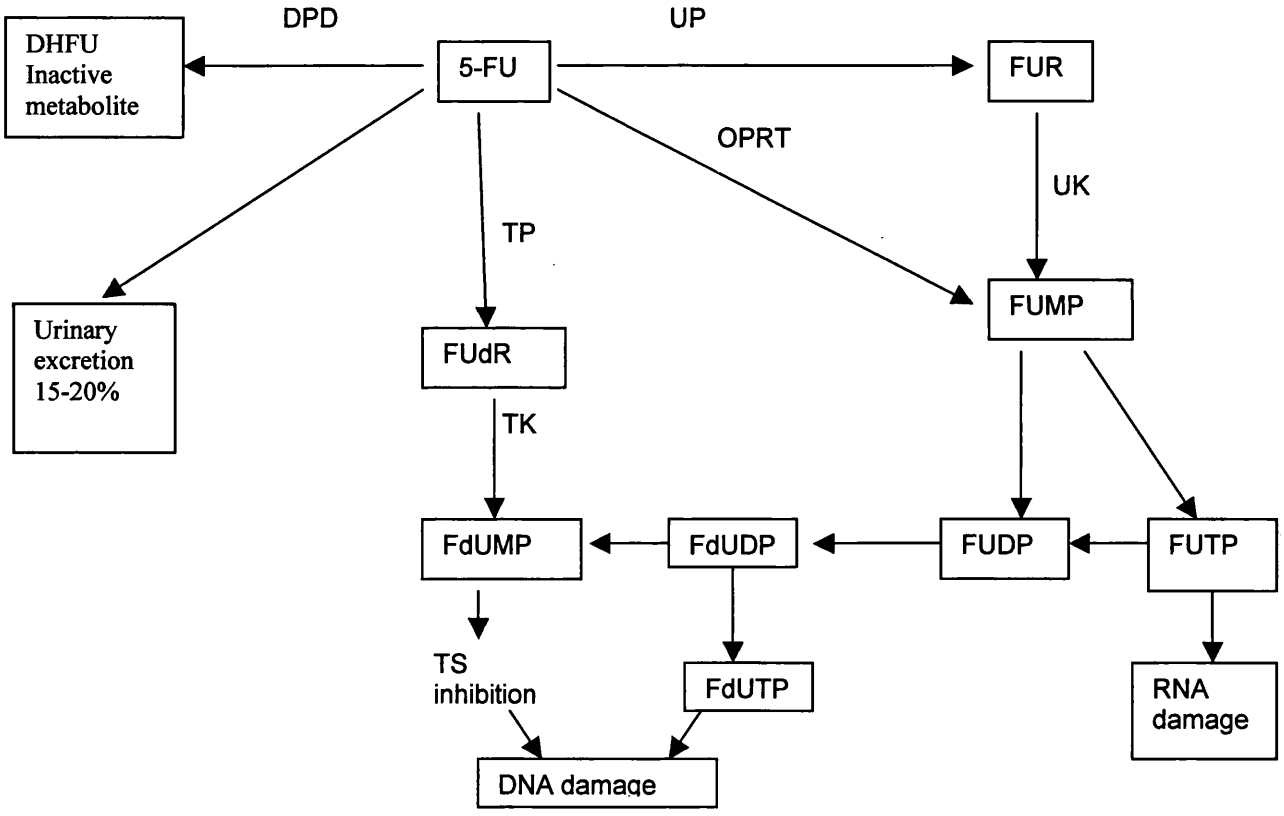


Figure 1.7. Schematic representation of 5-FU metabolism. Dihydropyrimidine dehydrogenase (DPD)-mediated conversion of 5-FU to dihydrofluorouracil (DHFU) is the rate-limiting step in 5-FU catabolism. FdUDP, fluorodeoxyuridine diphosphate; FdUMP, fluorodeoxyuridine monophosphate; FdUTP, fluorodeoxyuridine triphosphate; FUDP, fluorouridine diphosphate; FUDR, fluorodeoxyuridine; FUMP, fluorouridine monophosphate; FUR, fluorouridine; FUTP, fluorouridine triphosphate; OPRT, orotate phosphoribosyltransferase; RR, ribonucleotide reductase; TK, thymidine kinase; TP, thymidine phosphorylase; TS, thymidylate synthase; UK, uridine kinase; UP, uridine phosphorylase

DPD is the first and rate-limiting enzyme in 5-FU catabolism, and accounts for much of the interpatient variability in metabolism. DPD inhibitors enable the use of an oral 5-FU prodrug as 5-FU degradation in the gastrointestinal tract is prevented. Uracil is a potent inactivator of DPD, and oral administration leads to undetectable levels of DPD. When oral 5-FU is given with a DPD inhibitor its absorption becomes predictable and its half-life is dramatically increased. UFT is a combination of uracil and florafur (FT) in a 4:1 molar ratio. FT is a prodrug of 5-FU that is converted to 5-FU by cytochrome P450 in the liver, thus avoiding breakdown by DPD in the small bowel. The delivery of 5-FU is enhanced by the uracil competing for DPD. UFT in combination with folinic acid has achieved response rates similar to other 5-FU/FA regimens, often with a lower toxicity profile, but time to progression and overall survival remains similar.

Raltitrexed is a quinzalone antifolate that inhibits thymidylate synthetase, via a different mechanism from 5-FU. Prolonged TS inhibition is achieved by rapid polyglutamylation of folate and its subsequent retention within cells. Similar response rates and overall survival to 5-FU/FA regimens were found in several large trials, but raltitrexed was associated with greater toxicity and inferior quality of life (Cunningham *et al.*, 2002). Although convenient for patients as it is given on an outpatient basis, renal function must be closely monitored. Dose limiting toxicities include fatigue, myelosuppression and GI symptoms, and treatment related deaths have been reported in 2-6% of patients. Similar agents include pemetrexed and Methotrexate.

(iii) Topoisomerase Inhibitors

DNA strands must unravel for repair and synthesis. When unwinding, torsional strain is increased leading to one side containing more turns (positive supercoiling) or less turns (negative supercoiling). If the correct structure is not maintained, DNA cannot function correctly. Topoisomerases are enzymes that allow unwinding without increasing the torsional strain by breaking one or two of the DNA strands and allowing them to rotate around each other and relax.

Topisomerase I is expressed in nearly all mammalian cells and is involved in DNA replication and RNA transcription. It creates single strand breaks, rotation of the unbroken strand through the break, and then rejoining of the break. Topoisomerase I inhibitors bind to the topoisomerase I –DNA cleavable complex, stabilising it and preventing re-ligation. As the DNA replication fork moves along the DNA it comes into contact with the complex causing breakage of the unbroken DNA strand. This results in a persistent cytotoxic double strand break and thus inhibition of DNA synthesis, cell cycle arrest in G2 and cell death by apoptosis.

Topoisomerase II creates double strand breaks and rotates both strands to relax supercoiling before rejoining the broken ends. Topoisomerase IIβ remains relatively constant over the cell cycle but topoisomerase IIα increases rapidly in proliferating cells. Inhibitors of Topoisomerase II such as doxorubicin and mitoxantrone intercalate into DNA between bases to elicit effects on the enzyme and induce double strand breaks, whereas etoposide acts directly on the enzyme to produce double strand breakages.

Irinotecan (CPT-11) is a semi-synthetic derivative of camptothecin, whose active metabolite (SN38) causes cytotoxicity through interaction with endonuclease topoisomerase I. The pharmacology of irinotecan is important as several factors lead to interpatient variability (figure 1.8).

(iv) Anti-microtubule inhibitors

The microtubule apparatus controls chromosome movement during mitosis and meiosis. Microtubules also have an integral part in maintaining cell shape and intracellular structure as well as contractility, intracellular transport and transferring signals from the cell surface to the nucleus (Gundersen and Cook, 1999). Two classes of microtubule inhibitors are used clinically as cytotoxics: vinca alkaloids and the taxanes, neither of which are used in CRC.

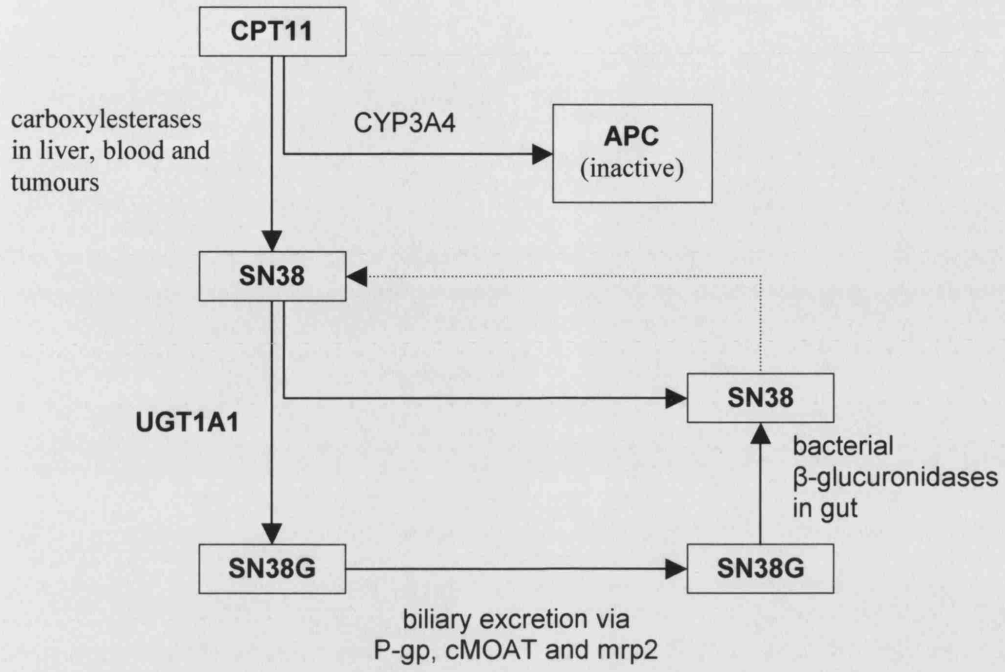


Figure 1.8. Pathway of activation and clearance of Irinotecan

(v) Multidrug Resistance Inhibitors

Only one such agent is relevant to this thesis. XR9576 (Tariquidar) is a novel anthranilic acid derivative which is a specific inhibitor of P-gp and reverses P-gp dependent multidrug resistance (MDR). *In vitro* and *in vivo* it restores the sensitivity of pumped drugs such as doxorubicin, paclitaxel and etoposide (Mistry *et al.*, 2001; Di Nicolantonio *et al.*, 2004b). First and second generation modulators have been investigated previously (i.e. verapamil, cyclosporin D analogues), but severe toxicities have excluded them from clinical use for this purpose.

(vi) Non-Steroidal Anti-inflammatory Drugs / Cyclo-oxygenase-2 Inhibitors

The traditional Non-Steroidal Anti-inflammatory Drugs (NSAIDs) are thought to be chemopreventative by a number of mechanisms which include: inhibition of procarcinogen activation and carcinogen formation, tumour cell invasion and metastasis, and tumour angiogenesis, as well as the induction of tumour cell apoptosis and stimulation of immune surveillance (Shiff and Rigas, 1997).

NSAIDs inhibit cyclo-oxygenase (COX) the rate-limiting step in the production of prostaglandins from arachidonic acid. Prostaglandin E2 (PGE2), found at increased levels in CRC, induces cellular proliferation and suppresses immune surveillance and killing of malignant cells. There are two isoenzymes, COX-1 and -2, also termed prostaglandin H synthetase (PGHS) 1 and 2, which are encoded by two different genes (table 1.13). COX-2 is upregulated in 40% of human colorectal adenomas and 85% of human colorectal carcinomas (Eberhart *et al.*, 1994).

Table 1.13. Cyclo-oxygenase isoenzymes

Isoenzyme	**Gene location**	**Function**
COX-1	chromosome 9	Expressed throughout many normal human tissues. Maintains homeostasis and the physiologic functioning of cells (housekeeping).
COX-2	chromosome 1	Over-expressed by many cancers. Induced by growth factors and tumour promoters.

The use of traditional NSAIDs, such as aspirin and sulindac, which inhibit both COX-1 and –2, has been restricted because of their gastrointestinal toxicity. NSAID related peptic ulcer bleeding or perforation is the cause of 2000 deaths per year in the UK (Tramer *et al.*, 2000). The inhibition of COX-1 is thought to cause their adverse effects, whilst the inhibition of COX-2 is responsible for their anti-neoplastic activity. However, more recently it has been thought that NSAIDs may also have some COX-2 independent effects, including inhibition of angiogenesis factors and endothelial tube formation (Tsujii *et al.*, 1998; Gasparini et al., 2003) (figure 1.9).

The new class of drugs, COX-2 inhibitors, were claimed to have far less adverse effects than conventional NSAIDs whilst retaining their antineoplastic properties. These claims were made after two large long-term trials of the use of celecoxib and rofecoxib in patients with arthritis (Bombardier *et al.*, 2000; Silverstein *et al.*, 2000). However, the CLASS trial of celecoxib (MSD, UK) was heavily criticised for study

design, data analysis and selective result presentation. The twelve month data did not confirm its gastrointestinal protection. Rofecoxib (Searle, UK), although fairing better on GI side effects produced an increased number of cardiovascular events (VIGOR trial). A RCT of rofecoxib in colorectal cancer patients following potentially curative surgery (VICTOR) was commenced in 2002, but was closed early in 2004 when the drug was withdrawn from the market due to an increase in cardiovascular events. In addition, a phase II trial found that rofecoxib in combination with 5-FU/FA increased toxicity without increasing efficacy (Becerra *et al.*, 2003).

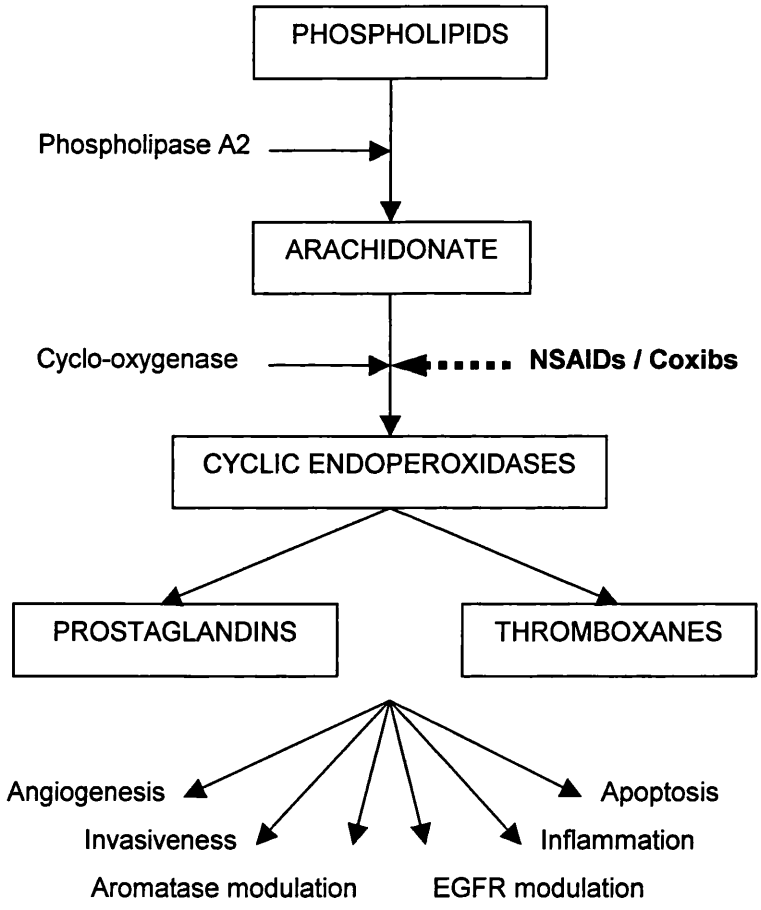


Figure 1.9. Pathways of tumour growth stimulation by COX-2 and mechanisms of action of coxibs

(vii) Drugs targeting signal transduction / novel molecular targets

There are over 200 new agents under evaluation in anti-cancer clinical trials, and even more preclinically. The primary effect of these therapies is tumour growth delay, and therefore major tumour regression is unlikely to be seen. Maximum benefit is most likely when combined with other therapeutic modalities. Careful patient selection based on tumour expression of drug target may be important. The major signalling proteins under investigation include: (i) the epidermal growth factor receptor (EGFR); (ii) Ras via inhibition of farnesyltransferase; (iii) Raf kinase; (iv) the mitogen-activated protein kinase pathway; (v) Akt; and (vi) the apoptosis signalling pathways.

Epidermal growth factor receptor (EGFR)

EGFRs are integral components of the principal signalling cascade involved in regulating solid tumour growth. EGFR blockade inhibits cancer cell proliferation and tumour progression. In addition to anti-EGFR antibodies and receptor tyrosine kinase inhibitors, antisense therapies are available for targeting the EGFR.

Monoclonal antibodies, e.g. Mab 225 (IgG2a), block ligand binding to EGFR and inhibit EGF-stimulated EGFR tyrosine kinase activity, thus causing receptor dimerization and internalisation, inhibiting tyrosine kinase dependent phosphorylation, down-regualating EGFR expression and blocking the EGFR signalling cascade. Cetuximab (C225) is a chimeric monoclonal antibody that arrests cells in G1 phase of the cell cycle. As a single agent, and in combination with irinotecan, it has shown activity in 5-FU and irinotecan refractory tumours (Cunningham *et al.*, 2004).

There are several receptor tyrosine kinase inhibitors under investigation. They prevent receptor autophosphorylation and phosphorylation of downstream signalling proteins. The two which are most advanced in their development are ZD1839 (Iressa) and OSI-774 (Tarceva), which are summarised in table 1.14. Iressa is the most advanced tyrosine kinase inhibitor in clinical development and several phase II trials

in combination with chemotherapy regimens have been performed (Kuo *et al.*, 2005; Veronese *et al.*, 2005).

Table. 1.14. Common small molecule inhibitors of erbB receptor tyrosine kinase activity in clinical development

Agent	Development	Phase of development	Target
ZD 1839 (Iressa)	AstraZeneca	Phase II-III	Competitively inhibits ATP binding to ATP binding site of internal tyrosine kinase domain of erbB1
OSI-774 (Tarceva)	OSI/Pfizer	Phase II-III	Competitively inhibits ATP binding to ATP binding site of internal tyrosine kinase domain of erbB1
PKI 116	Novartis	Phase I	Competitively inhibits ATP binding to ATP binding site of internal tyrosine kinase domain of erbB1
GW2016	GlaxoSmithKline	Phase I	Competitively inhibits ATP binding to ATP binding site of internal tyrosine kinase domain of erbB1 and erbB2
EKB-569	Genetics Insitiute Wyeth-Ayerst	Phase I	Irreversibly binds to erbB1 at the ATP binding of its internal tyrosine kinase domain. Inhibits growth of tumour cells that overexpress erbB1 or erbB2
CI-1033	Pfizer	Phase I	Competitively inhibits ATP binding to ATP binding site of internal tyrosine kinase domain of all erbB receptors, particularly erbB1 and erbB2

Targeting ras signal tranduction

Ras signal transduction proteins are a family of guanosine triphosphatases (GTPases) that function as chemical switches. They transduce signals from receptor tyrosine kinases to a downstream cascade of protein kinases that regulate growth and regulatory processes that are aberrant in malignant cells. Ras is synthesized as an inactive cytosolic peptide and undergoes post-translational modifications dependent on the enzyme farnesyltransferase (FTase). FTase inhibition can block the function of ras, turning off signal transduction. Several FTase inhibitors are under clinical evaluation and have been found to have cytotoxic and radiation-sensitizing properties

in vitro. However, *in vivo*, no increase in overall survival has been demonstrated (Rao *et al.*, 2004).

Targeting Raf-1 signalling

The proto-oncogene Raf-1, a serine-threonine protein kinase, is a downstream effector of Ras in the growth factor signalling cascade. It is involved in the regulation of proliferation, differentiation and apoptosis. Antisense and small pharmacological molecules have been developed and are under investigation.

Targeting the mitogen activated protein kinase pathways

Mitogen activated protein kinases (MAPKs) are critical components of growth factor signalling pathways, and pharmacological inhibitors to block several members of the pathway are available.

Apoptosis signalling pathway

Bcl-2 is commonly over-expressed in several cancers. A high bcl-2:bax ratio inhibits the activation of apoptotic pathways (figure 1.10) and is critical in determining tumour responsiveness to chemotherapy-induced cell damage. A bcl-2 antisense oligonucleotide has been shown to enhance chemosensitivity both *in vitro* and *in vivo*, and studies of its use in combination with 5-FU, irinotecan and oxaliplatin are on-going.

The proto-oncogene AKT (protein kinase B) is negatively regulated by PTEN, which is frequently over-expressed or mutated in CRC. Small molecule inhibitors of the Akt pathway (e.g. rapamycin) are undergoing preclinical testing.

The nuclear factor (NK)-κB family of transcription factors plays an important role in the regulation of a variety of biological responses, including apoptosis, cell-cycle progression and differentiation. NK-κB is activated by a number of stimuli including growth factors and cytokines, resulting in translocation to the nucleus and modulation of genes including apoptosis regulators, growth factors, cell adhesion molecules, and other transcription factors. Blockade of NK-κB, by a number of mechanisms, can potentiate the activity of cytotoxics.

The death receptor ligands, tumour necrosis factor (TNF), Fas ligand (FasL) and TNF-related-apoptosis inducing ligand (TRAIL) are all able to induce apoptosis by binding to their cell membrane receptors. Recombinant forms of these ligands potentiate antitumour effects of cytotoxics *in vitro* and *in vivo*.

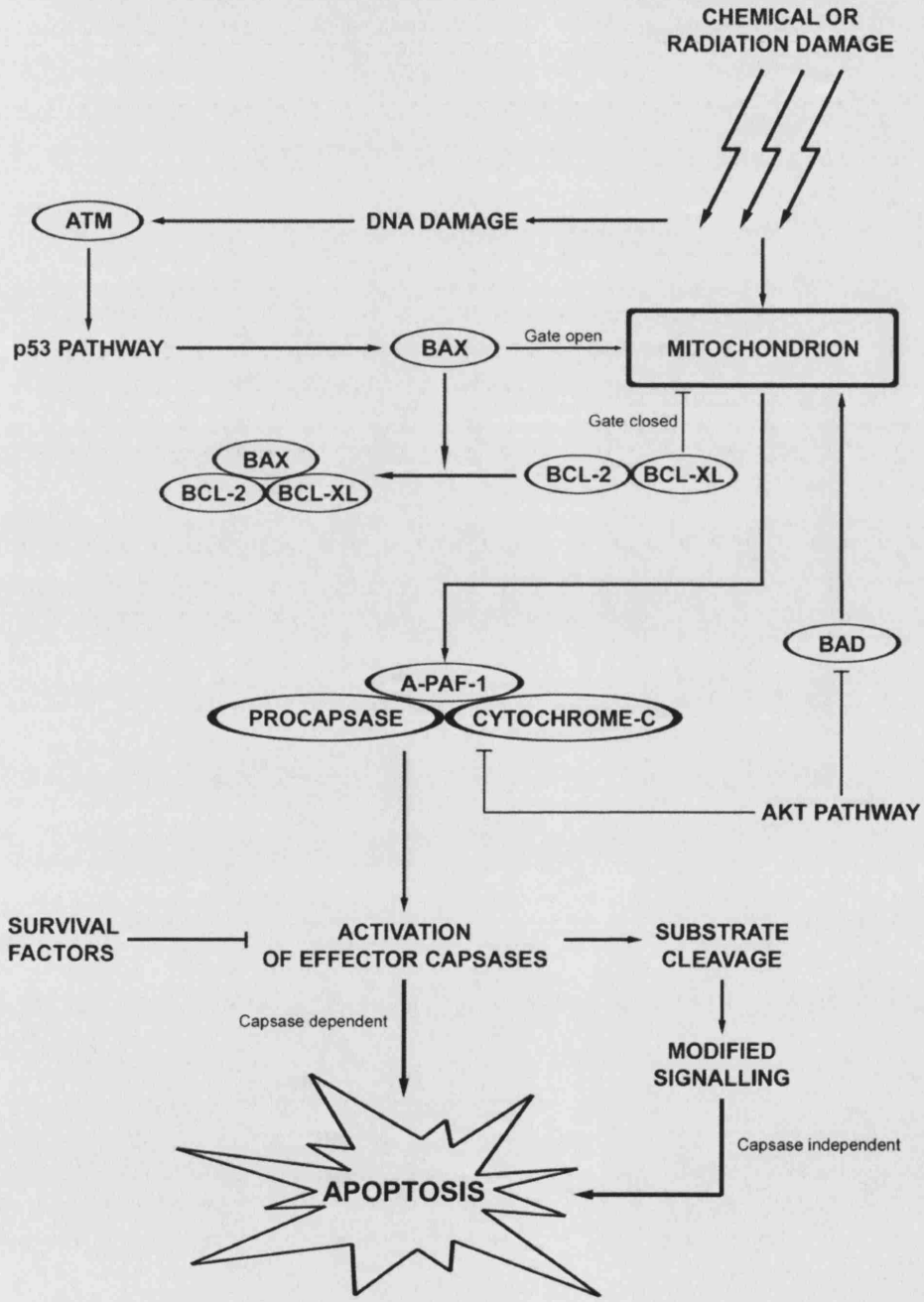


Figure 1.10. The Apoptotic signalling pathway in response to cell damage. ATM, ataxia telangiectasia mutated; BAX, BCL-2 associated X protein; BCL-2, B-cell CLL/lymphoma 2; BCL-XL; BCL-2 antagonist of cell death; A-PAF-1, apoptotic protease activating factor

(viii) Immune therapy

The evidence that both the cellular and humoral parts of the immune system are capable of interacting with tumour cells has led to the development of immunotherapies to produce tumour antigen-specific T-cell responses, in addition to antibody and whole-cell vaccines. There are three main groups of antigens relevant to CRC, a) cancer-testis antigens, which are not present in normal tissues except spermatogonia which do not express HLA class I molecules, b) over-expressed antigens, which are present to a variable degree in normal tissues, e.g. carcino-embryonic antigen (CEA), HER-2/neu, MUC-1, and p53, and c) tumour-specific antigens resulting from mutation of proteins, e.g. TGF-βR-II, APC, ras.

The different tumour immunotherapeutic strategies available are directed explicitly at antigen-specific or antigen non-specific modalities. Antigen non-specific immunotherapies include cytokines (e.g. IL-2, interferons, GM-CSF) and whole-cell vaccines (e.g. combined with bacillus Calmette-Guérin). Antigen-specific immunotherapy may involve peptides, soluble proteins, recombinant plasmid DNA and viral vectors, dendritic cells, and antibodies.

(ix) Gene therapy

There are about 400 cancer gene therapy trials, using viral or non-viral vectors, underway in the UK and US. These are mainly phase I dose finding/safety studies, with little clinical efficacy demonstrated so far. The obvious advantage of gene therapy is the specific targeting of the tumour site, reducing unwanted systemic toxicity. The mechanisms controlling accuracy of gene delivery and expression at the target site are prerequisites that also limit its success. There are a number of mechanisms of gene therapy including: immune stimulation, mutant gene correction, prodrug activation, genetically modified oncolytic viryus therapy, myeloproliferative gene transfer in conjunction with chemotherapy, and antisense oligonucleotide therapy.

1.2.2 Molecular mechanisms of resistance to chemotherapy

Tumours may have inherent resistance to chemotherapy at presentation, or they may acquire resistance after drug administration. The mechanisms of resistance are potential targets for modulation in order to increase the efficacy of chemotherapeutic agents (Harrison, 1995; Cree *et al.*, 2002), and are summarised in figure 1.11.

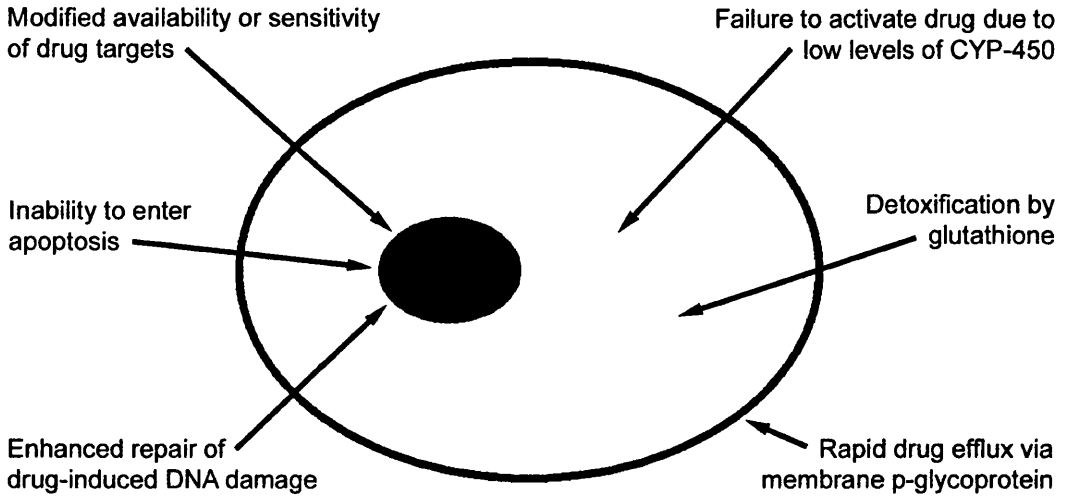


Figure 1.11. Mechanisms of drug resistance. Adapted from Loeb, 1991.

The main molecular mechanisms of drug resistance are:

(i) multidrug resistance
(ii) increased drug detoxification
(iii) up regulation of DNA repair mechanisms
(iv) increased/decreased drug target
(v) up regulation of anti-apoptotic mechanisms
(vi) failure of prodrug activation
(vii) reduced transport

(i) Multidrug resistance

Cells contain defence mechanisms to eliminate xenobiotics and prevent harm to the cell. However, this means a number of cytotoxic agents can be affected by this mechanism. Cells often show cross-resistance to drugs of a structure they have not previously been exposed to (eg. anthracyclines, vinca alkaloids, taxanes). This is termed multidrug resistance (mdr) and is associated with an increased expression of one or more of a family of transporter proteins, including P-glycoprotein (P-gp), the product of the *mdr*1 gene; the multidrug resistance-related protein (MRP); the lung resistance protein (LRP, also known as major vault protein); and the breast cancer resistance protein (BCRP). These proteins act as a transmembrane ATP-dependent efflux pump (Hrycyna *et al.*, 1996) for a range of compounds and result in a decreased accumulation of drug within the cell. P-gp is the most investigated protein and is overexpressed in a large number of intrinsically resistant tumours. Overexpression may be transcriptional or as a result of gene amplification. XR9576 (tariquidar; Xenova Group Plc, Slough, UK), an anthranilic acid derivative, is a potent and specific inhibitor of P-glycoprotein (P-gp), which reverses P-gp dependent mdr.

(ii) Increased detoxification

Some cytotoxic agents are electrophiles and require conjugation with molecules for transport, metabolism and excretion. Such molecules include glutathione and glucuronic acid. Glutathione S-transferases (GSTs) are a family of enzymes expressed in most normal tissues. Pi is the most commonly elevated (Barone and Tew, 1996) of the four isoenzymes, alpha, gamma, mu and pi. The level and activity of available glutathione and glutathione S-transferases is critical in drug resistance, for example to cisplatin, mephalan and treosulfan. Depleting glutathione synthesis or inhibiting GSTs (eg. with sulphasalazine) may lead to an increase in the efficacy of drugs. The MRP efflux pump functions in association with the glutathione system as depletion of intracellular glutathione reverses mdr.

(iii) Up regulation of DNA repair mechanisms

A damaged cell may enter cell cycle arrest and undergo DNA repair or it may undergo apoptosis (programmed cell death). Maintaining normal DNA is very

important to the cell and it has several mechanisms of repair, either directly on the damaged bases (direct), or by removing the section of DNA containing the damaged bases (indirect). DNA damaging drugs are susceptible to resistance by these methods.

Mechanisms of DNA Repair

Mismatch repair (MMR): Errors in DNA, such as base substitutions, are corrected by the mismatch repair genes, mainly hMLH1 and hMSH2 in humans. Mutations in these genes leads to an accumulation of errors in microsatellites, resulting in a mutator phenotype, termed microsatellite instability (MSI). The effect of MMR on chemosensitivity is uncertain, with some agents appearing more sensitive (e.g. topoisomerase I inhibitors and mitomycin C) and others less sensitive (e.g. 5-FU and topoisomerase II inhibitors).

Nucleotide Excision Repair (NER): Damage recognition is based on both structural and chemical abnormalities of DNA. A protein complex binds to the abnormality and an oligonucleotide containing the area of damage is excised. The defect is repaired by DNA polymerase and ligase. NER may be important in the repair of platinated bases.

Base Excision Repair (BER): BER targets damage due to cellular metabolism. BER proteins remove damaged/incorrect bases without sections of DNA on either side. After removal of the base, the adjacent DNA strand is nicked by apurinic-apyrimidine (AP) endonuclease and the new base is incorporated by DNA polymerase and ligase. BER appears to be important in MMR-deficient cells where it repairs N7- and N3-methylguanine lesions caused by alkylating drugs.

Recombination: Homologous recombination (HR) and Non-homologous end joining (NHEJ) repair double strand breaks resulting from the direct action of ionising radiation or chemicals. Inhibition of these processes may increase sensitivity to anthracyclines and mitomycin C, but could also lead to increased toxicity and carcinogenesis as these processes are important in the stability of the normal cell.

Dealkylation / Direct Repair: O^6-methylguanylmethyltransferase (MGMT) removes methyl groups from O^6 on guanine residues. Upregulation of MGMT is a common form of resistance to alkylating agents that methylate DNA at the O^6-position on guanyl bases.

(iv) Changes in drug target

Resistance of some drugs is due in part to increased or decreased expression of the target protein. Topoisomerase inhibitors act by inhibiting the topisomerase enzymes which cause single or double strand DNA breaks allowing strand separation during DNA replication. The amount of topoisomerase present is crucial as the drugs directly interact with the target to form the cleavable complex. Therefore, an increase in topoisomerase I leads to increased sensitivity to irinotecan, but a reduction will result in relative resistance. If the specific topoisomerase is down-regulated, DNA strand breakage cannot occur in the presence of the inhibitor. However, the cell still requires topoisomerase activity. Some cancer cells have ability to down-regulate the enzyme being inhibited (e.g. topoisomerase I by irinotecan) and up-regulate the alternative enzyme (topoisomerase II).

(v) Up regulation of anti-apoptotic mechanisms

Apoptotic cell death is the aim of many chemotherapuetic drugs and radiation therapy. This involves a large number of genes, many of which permit a cell to cycle, for example, p53, c-myc and Bcl-2. p53 is a tumour suppressor gene that prevents a cell from proliferating whilst containing damaged or mutated DNA. When damaged, a cell normally up-regulates p53 to initiate growth arrest. However, many cells have lost p53 function by mutation or deletion and thus do not enter apoptosis. The expression of the oncogene Bcl-2 prevents the cell from entering the apoptotic pathway and thus confers drug resistance.

(vi) Failure of prodrug activation

Some chemotherapeutic agents require metabolic activation before becoming effective. Many of these activation processes are dependent on the action of members of the cytochrome P450 superfamily. P450 is present at its highest levels in the liver with most bioactivation occurring at that site. Metabolites may reach the tumour via the circulation, but a maximal biological effect is not achieved and systemic side effects are enhanced.

(vii) Reduced drug influx

Some drugs enter the cell without a specific transport mechanism, others require a carrier. If a drug cannot effectively enter a cell its observed effects will be less than a drug that can enter freely.

1.3 Predictive Oncology

1.3.1 Introduction

Cancer is the result of multiple genetic and epigenetic defects leading to a loss of growth control, and thus there are many differences between tumours of the same tissue type (Hanahan and Weinberg, 2000). In addition, many cancers have existing, or develop, resistance to some cytotoxic agents. It is the heterogeneity of tumours that forms the basis for individualising chemotherapy. Several methods of individualised therapy are being developed or are currently in use in clinical practice. For example, Oestrogen Receptor (ER) status is routinely measured in breast tumours in order to guide treatment with hormonal drugs. ER positivity has a 70% correlation with response to tamoxifen which is considered an acceptable level to direct treatment (Hamm and Allegra, 1991). The value of HER-2 as a predictor of response to herceptin remains debatable, although now in clinical practice (Miles, 2001). Levels of tumour markers, e.g. carcinoembryonic antigen, may predict recurrence of disease (Northover, 1986), but they do not indicate which drugs or drug combinations are likely to be effective or ineffective. Currently there is much work being performed on the expression of gene products (eg. p53 and bcl, etc.) as a predictor of individual tumour response, but whereas this may be useful as an indicator of prognosis, it is not yet useful in directing treatment.

Prognostic and Predictive Markers

Prognostic factors give a guide to the likely overall survival for an individual with a certain disease without any interventional therapy. A very good example is Dukes' staging, where patients with Dukes' stage A have a much better 5 year survival than those with stage C. Predictive markers identify tumours likely to be sensitive or resistant to certain treatments. For example, patients with low expression of thymidylate synthase are more likely to respond to 5-FU therapy (Aschele *et al.*, 1999). Figure 1.12 illustrates the differences between prognostic and predictive factors.

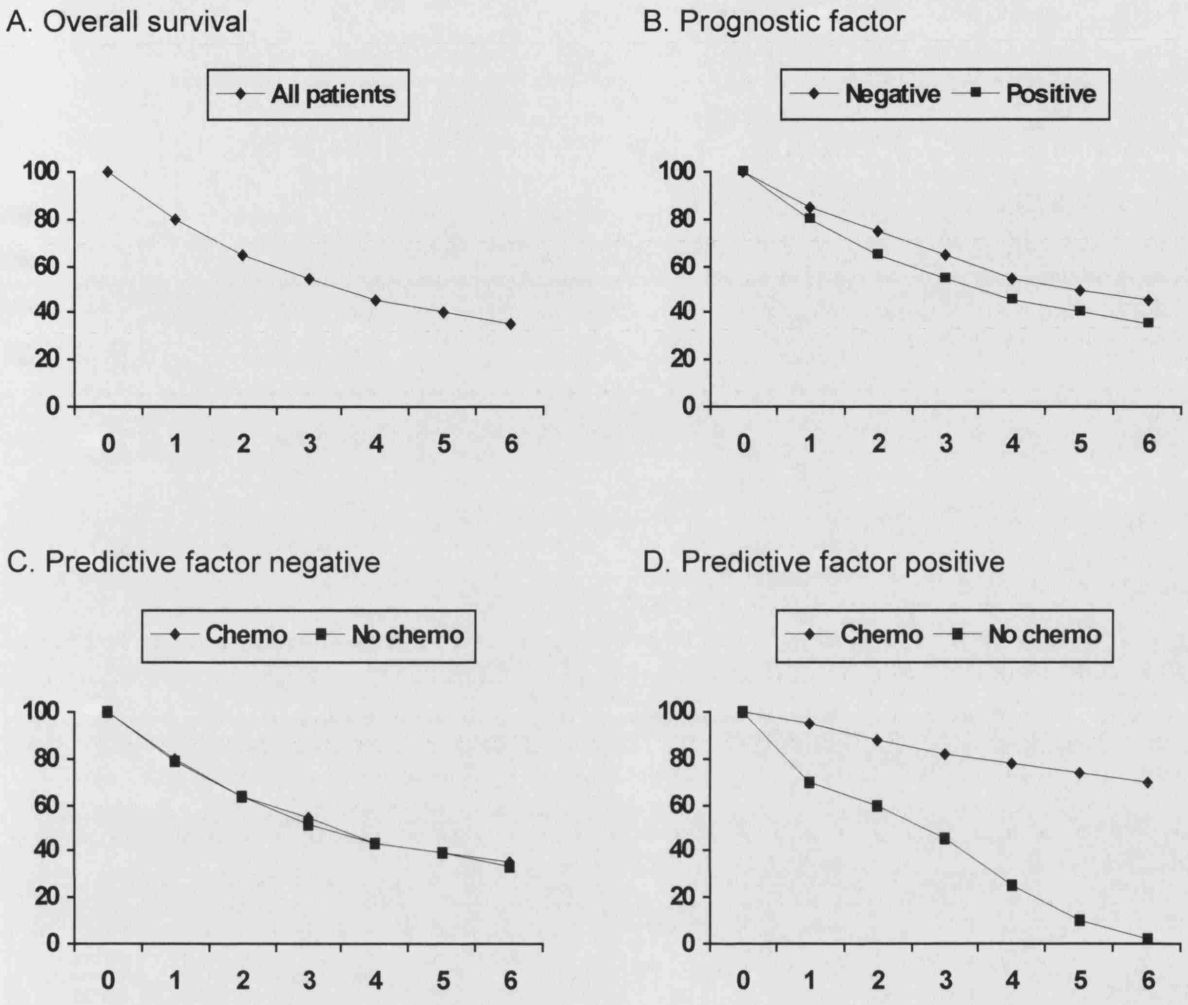


Figure 1.12. Graphs illustrating the effects of prognostic and predictive factors (adapted from Adlard *et al.*, 2002). (A) typical survival curve for Dukes' C CRC; (B) a prognostic factor separates the patients into two groups with differing survival; The effect of a predictive factor is shown in (C) and (D). Without a predictive factor (C), survival is similar with or without chemotherapy. The presence of a predictive factor (D) identifies a group of patients likely to benefit from chemotherapy.

Tumours can be analysed at cell, protein, RNA or DNA levels for predictive markers. RNA expression can be assessed by real-time reverse transciptase PCR and compared to a consistently expressed 'housekeeping ' gene. However, formalin fixation leads to structural changes in RNA and so the use of fresh cells is best. DNA microarrays are a new technology of assessing thousands of genes on a single slide. Protein expression can be measured easily by immunohistochemistry using fixed

tissue. Table 1.15 summarises some possible molecular predictive factors for response to treatment in colorectal cancer.

Table 1.15. Factors predictive for response investigated in the treatment of colorectal cancer

Treatment and Potential marker	**Association with response to treatment**	**References**
Fluorouracil		
TS	low RNA expression/negative IHC staining	Leichman *et al.*, 1997 Aschele *et al.*, 1999
DPD	low RNA expression/negative IHC staining	Danenberg *et al.*, 1998 Salonga *et al.*, 2000
TP	low RNA expression/negative IHC staining	Metzger *et al.*, 1998
p53	wild-type p53/negative IHC staining	Ahnen *et al.*, 1998
BCL-2	high RNA expression/positive IHC staining	McKay *et al.*, 2000
MSI	MSI positive/negative IHC staining (hMLH1/hMSH2)	Elsaleh *et al.*, 2001
Capecitabine		
TS	low RNA expression/negative IHC staining/ homozygosity for gene promotor double tandem repeat	Park *et al.*, 2001
DPD	low RNA expression/negative IHC staining	
TP	high RNA expression/positive IHC staining	Nishimura *et al.*, 2001
Irinotecan		
Topoisomerase 1	high RNA expression/positive IHC staining	Paradiso *et al.*, 2001
Oxaliplatin		
ERCC-1	low RNA expression/negative IHC staining	Shirota *et al.*, 2001
Growth-factor antibodies/tyrosine kinase inhibitors		
VEGF		
EGFR	high RNA expression/positive IHC staining	
COX-2 inhibitors		
COX-2	high RNA expression/positive IHC staining	

1.3.2 Tumour Chemosensitivity Assays

Chemosensitivity assays have been a topic of interest for several years (Von Hoff, 1990; Bellamy, 1992; Bosanquet, 1993). However, they are often met with scepticism, mainly due to poor results from technical problems and low evaluability rates. There is a profusion of interchangeable terms describing tests aimed at predicting the cytotoxic agents most effective for individual tumours: 'chemosensitivity assay', 'drug response assay' and tumour response assay'. In addition, 'chemoresistance assays' and 'drug resistance assays' are tests to identify drugs which are inactive, and thus provide more restrictive information. Although *in vitro* and *ex vivo* are also terms used interchangeably, *ex vivo* should be reserved for cultures of fresh cells isolated from tumour samples rather than those exposed to long-term artificial culture conditions.

Determining the chemosensitivity of individual tumours is potentially of benefit to patients in terms of survival, but also in reducing toxic side effects from ineffective drugs. A drug to which the tumour shows resistance can be excluded from the regimen and the doses of the other drugs increased, or it may be substituted with another more effective drug. *Ex vivo* chemosensitivity assays are not a direct indicator of the processes occurring *in vivo*, but are models that mimic them. There are several features that make an ideal tumour chemosensitivity assay: use of small amount of tumour material; ability to measure multiple drugs and combinations; measurement of dose response over multiple concentrations; high evaluability rate (>90%); clearly defined criteria for analysis of results or interpretation; and good relevance to the clinical situation.

Important points for consideration regarding results of chemosensitivity tests include:

- Are the results obtained from *in vitro* assays comparable to *in vivo* responses?
- Are the responses observed *in vitro* for a particular histological type of tumour similar to the responses seen clinically in the same type of tumour?
- Can individualising the choice of chemotherapy by the use of chemosensitivity assays increase patient survival?

There are four stages peculiar to all chemosensitivity assays (Fruehauf and Bosanquet, 1993). First, the cancer cells are isolated from the tumour. Viable cancer cells should be present in sufficient numbers and without bacterial contamination. Secondly, cancer cells are exposed to cytotoxic agents. Both incubation times and the concentration of drugs varies between assays. All assays include a control of tumour cells cultured in identical conditions but without any additional cytotoxics. Third, the effect of the drugs on the cancer cells, the assay endpoint, is determined; either by measurement of cell proliferation or cell death. Finally, a report of the results must be issued. Investigators often develop their own indices of sensitivity and resistance, and therefore it may be difficult to compare results from different types of tests.

There are two main types of chemosensitivity assay. Clonogenic assays show the effect of anticancer agents on cell division, using cell proliferation as an endpoint, while non-clonogenic assays show the effect on cell viability, using cell death as an endpoint. There tends to be good correlation between the various assays. The major chemosensitivity assays are briefly described below.

Clonogenic / Human Tumour Stem Cell Assay

Clonogenic assays, based on cell proliferation, and developed from antibiotic testing in microbiology, were the first chemosensitivity tests to be performed on tumour material (Hamburger and Salmon, 1977; Selby *et al.*, 1983). A cell suspension of the tumour is incubated with and without test drugs for one hour at 37°C. The cells are then washed and plated on two-layer soft agar for 14 days at 37°C. The colonies formed are counted by an automated image analysis system or by eye, and compared to the control sample.

The clonogenic assay is regarded as the "gold standard" chemosensitivity test, but there are several disadvantages of the assay. Large numbers of cells are needed, which may take a long time for sufficient numbers to grow. Not all cancers grow in agar, in fact only 40-70% do. The culture conditions may affect the results. The total cell population is examined and it may be difficult to determine whether a cell is actually neoplastic or not. It is labour intensive and takes 14 days. However, there are

many clinical correlation studies, and a review of 54 trials found it to have high true positive (69%) and true negative (91%) rates (Von Hoff, 1990).

Recently a newer version has been developed, using a collagen droplet embedded culture system (Inaba *et al.*, 1996), although this was originally developed several years ago (Jason, 1979). Following enzymatic dissociation, cells are incubated in collagen gel coated flask for 12-24 hours. Living cells are than collected and incubated in collagen gel droplets for a further 24 hours, before the test drug is added at a single clinically relevant concentration. The drugs are removed after 24 hours and the droplets incubated for a further 7 days, after which they are stained, fixed and quantified by image analysis. Cell growth is assessed calculating the ratio of image density measured on day 7 and day 1 after the addition of drugs. The cloning efficiency of the assay is considerably higher than that for soft agar culture, with an overall evaluability rate of 83%, and predictive accuracy of 84.1% (Kobayashi, 2003).

Thymidine incorporation / Kern Assay

This modification of the clonogenic assay is used widely in the USA (Oncotech and Impath). Tumour cells are suspended in soft agarose and cultured for 3-4 days in the presence of single supra-clinical concentrations of cytoxic drugs. Radiolabelled thymidine ([^{3}H]thymidine) is added and incorporated into the DNA of proliferating cells, the radioactivity of which is measured after a further 2 days incubation. Cancer cells that can proliferate in such conditions are considered resistant to the treatment agent, thus this assay only provides chemoresistance and not chemosensitivity information.

The correlation of assay results and clinical data led to the definition of 'extreme drug resistance', or EDR, as a result one standard deviation more resistant than the median result calculated from a database (Kern and Weisenthal, 1990). This assay, using single agents, has a very high specificity (>98%), but a low sensitivity (<40%). This means that a drug with EDR will almost definitely be inactive, but that some drugs without EDR will also be inactive. More recent clinical correlation studies have been more variable. Eltabbakh *et al.*, (1998) reported EDR to paclitaxel did not

appear to be a prognostic factor in ovarian cancer patients. However, other studies have shown EDR to have prognostic implications in ovarian and breast cancer (Fruehauf *et al.*, 2001; Mehta *et al.*, 2001).

Disadvantages of the Kern assay include the fact that it only applies to solid and not haematological malignancies. Also, there is the use of radioactivity. It is not specific for tumour cells, and the cells need to be dividing to incorporate the radiolabelled thymidine.

Differential staining cytotoxicity (DiSC) Assay

The Differential staining cytotoxicity (DiSC) Assay is a modification of an earlier dye exclusion method (Weisenthal *et al.*, 1983; Weisenthal and Kern, 1991). Dissociated cells are cultured for 4 days in liquid media with and without test drugs. Usually three drug concentrations are tested, with the upper and lower concentrations ten-fold higher and lower concentrations than the intermediate concentration that represents clinically achievable levels. After incubation the cells are stained with fast green/nigrosin dye which stains dead cells green and leaves viable cells unstained. The cells are then counter-stained with H&E to differentiate tumour and non-tumour cells, and the proportion of dead and live cells estimated with direct microscopy, which may result in assessor bias as well as being labour intensive.

MTT

In the MTT assay, small fragments of tumour, grown on collagen gel sponges or disaggregated cells cultured as monolayers, are incubated with and without test drugs for 4-5 days. The MTT assay detects mitochondrial succinate dehydrogenase (SDH) activity as a determinant of mitochondrial function and cell viability. The reduction of yellow 3-[4,5-dimethyl (thiazol-2-yl)-3,5-diphenyl] tetrazolium bromide (MTT), by SDH, produces a blue crystallized formazan product. The product is dissolved in DMSO at the end of the assay, and the colour measured using a spectrometer (Carmichael *et al.*, 1987).

An advantage of the assay is that it is quick, with results being available after only 4-5 days, with potential for automation. However, there are several disadvantages to

the MTT. It requires a relatively large number of cells, about 25,000 per well. The dye is not metabolised well in all cell types, and some drugs may interfere with the dye. The reduction of MTT is affected by the metabolic conditions of the cell, for example, a lack of glucose results in the cells being unable to produce reducing agents.

It has frequently been used in cell line studies, and has also been used to predict response to chemotherapy in solid and haematological malignancies (Xu *et al.*, 1999; Taylor *et al.*, 2001).

Flurometric Microculture Cytotoxicity Assay

The Flurometric Microculture Cytotoxicity Assay (FMCA) detects metabolic activity of live cells remaining after incubation with cytotoxics. After 72 hours, the media is removed from wells and lipid soluble flurescein diacetate added. Hydrolysis of flurescein diacetate to non-lipid soluble flurescein by esterases within living cells produces flurescence proportional to the number of live cells present (Larsson and Nygren, 1993).

As the FMCA only takes 72 hours, it is a very quick technique, and also is suitable for automation. However, late effects of drugs, e.g. apoptosis, may not be observed. It has been evaluated in a number of different tumours (Nygren *et al.*, 1999) and used to evaluate new agents and combinations (Fridborg *et al.*, 1996; Jonsson *et al.*, 1998).

Sulphorodamine B Assay

The Sulphorodamine B assay determines the protein content of a sample as an indicator of cell growth and viability. Cells are cultured with and without drugs in 96-well plates, usually for 3 days, after which they are fixed, stained, and quantified with a spectrometer in situ. A problem with this assay is that it may measure all protein, including that from dead cells (Keepers *et al.*, 1991). However, it may be automated, and currently forms the basis for the NCI drug screening program (Monks *et al.*, 1991).

1.3.3 ATP Bioluminescence Assay

Adenosine Triphosphate (ATP) is present in all cells as the major intracellular source of energy required for metabolism, and thus is considered a valid indicator of living cells (biomass) (Lundin *et al.*, 1986). When cells die their ATP is rapidly degraded by ATPases. ATP is hydrolysed to AMP by firefly luciferin-luciferase in the presence of the substrate D-luciferin, producing light according to the reaction:

$$\text{ATP} + \text{D-Luciferin} + O_2 \xrightarrow{\text{Luciferase (E.C. 1.13.12.7)}} \text{AMP} + 2\text{Pi} + CO_2 + \text{Light}$$

The amount of light produced is proportional to the amount of ATP hydrolysed, and thus the concentration of ATP can be determined for comparison between samples. This mechanism has been used in various forms to determine *in vitro* and *ex vivo* chemosensitivity of different tumours (Kangas *et al.*, 1984; Crouch *et al.*, 1993; Andreotti *et al.*, 1995; Möllgård *et al.*, 2000).

All ATP chemosensitivity assays use the measurement of ATP as their endpoint, but there are marked differences in culture media, assay formats, drug concentrations and incubation times. The method used and described here is that of Andreotti *et al.* (1995), currently sold as a kit (TCA-100) by DCS Innovative Diagnostik Systeme, Hamburg, Germany (see Chapter 2.2).

The tumour sample is minced and dissociated to produce a single cell suspension. Cells are cultured in 96 well polypropylene plates with cytotoxics at 37°C with 5% CO2 for six days. Each plate contains two internal controls, a maximum inhibitor (MI) kills all the cells giving a zero ATP count, and a medium only (MO) that equates to 100% viable cells. Cells are lysed using a commercial extraction reagent that inhibits intracellular ATPases and prevents ATP degradation. Luciferin-luciferase is then added and the amount of light produced measured with a luminometer. ATP levels lower than the 100% control indicate a growth inhibitory effect by the drug, and dose response curves and indices of efficacy may be calculated from the data.

The ATP assay is very sensitive, requiring less than 1500 tumour cells per well for an evaluable reading. In fact it is able to detect < 5 viable cells remaining in one well after drug exposure (Cree and Kurbacher, 1997). This is much more sensitive than the MTT assay which requires about 25,000 cells per well and is less reproducible (Petty *et al.*, 1995). It has also been found to be more reliable and have a higher evaluability rate than clonogenic assays when tested against solid tumours (Cree *et al.*, 1995). The sensitivity of the ATP assay translates into high evaluability rates, which are usually greater than 90% (table 1.16).

Table 1.16. Evaluability rates of the ATP-TCA

Tumour Type	**Assay evaluability (%)**	**Reference**
Breast	97%	Cree *et al.*, 1996
	94.5%	Kurbacher et al., 1996
Colorectal	87%	Whitehouse *et al.*, 2003
Choroidal melanoma	84%	Neale *et al.*, 1999
Cutaneous melanoma	96%	Cree *et al.*, 1999
Oesophageal	73%	Mercer *et al.*, 2003
Ovarian	93%	Kurbacher *et al.*, 1998
	89%	Konecny *et al.*, 2000
Retinoblastoma	70%	Di Nicolantonio *et al.*, 2003

A further advantage of the ATP assay is that interference from non-tumour cells is minimised by the use of round-bottomed polypropylene plates, which inhibits cell adherence. Fibroblasts, mesothelial and other stromal cells are able to proliferate in adherence-based culture systems, which may confuse measurements of cell growth or death in most assay types. Another mechanism to suppress non-transformed cell proliferation is the use of serum-free medium. Andreotti *et al.* assessed the ratio of malignant and non-malignant cells before and after culture in specialised media (Complete Assay Medium; DCS, Hamburg, Germany), and found the proportion of malignant cells increased from 54% to 83% by the end of the six day incubation period (Andreotti *et al.*, 1995). The consequence of these techniques means the ATP-TCA may be performed with <20% tumour cells in the cell suspension.

One disadvantage of the ATP assay is that it is unable to distinguish between cytocidal or cytostatic effects of drugs, i.e. a reduction in ATP may be due to a decrease in the ATP content per cell as well as a decrease in the number of cells. Metabolically active cells produce more ATP, as do cells metabolising glucose through the citric acid cycle linked to aerobic mitochondrial function. *In vitro* drug effects that inhibit glucose metabolism or mitochondrial function result in decreased intracellular ATP concentrations.

The ATP-TCA has shown good correlation between *ex vivo* sensitivity and clinical response in breast and ovarian carcinomas (table 1.17). Accrual for a phase III randomised trial comparing assay-directed therapy to physician's choice treatment in patients with recurrent platinum resistant ovarian carcinoma was completed in 2003, the results of which are awaited (Kurbacher *et al.*, protocol 97PRT/1, Lancet website).

Table 1.17. A summary of recent studies with clinical correlation using the ATP tumour chemosensitivity assay

Tumour	**Sample No.**	**Summary of results**	**Reference**
Breast	29	76% positive predictive value	Cree *et al.*, 1996
Ovarian	114	> 90% accuracy for predicting cisplatin resistance	Andreotti *et al.*, 1995
Ovarian	55	64% overall response rate vs 37% in control group, increased PFS and OS	Kurbacher *et al.*, 1996
Ovarian	38	76% classed sensitive to drug regimen *ex vivo* had significantly longer PFS (28.5 vs 12.6 mths, $p<0.033$) and OS (46.1 vs 17.6 mths, $p<0.03$) than those classed as resistant	Konecny *et al.*, 2000
Ovarian	54	61% overall response rate in patients treated with assay-selected therapy	Sharma *et al.*, 2003

The ATP-TCA has been used to predict the *ex vivo* activity of novel drug combinations (see Chapter 5), some of which are now in clinical use (Neale *et al.*, 1999; Di Nicolantonio *et al.*, 2002). In addition, it is currently being used to evaluate new cytotoxic agents and chemosensitizers (Neale *et al.*, 2000; Di Nicolantonio *et al.*, 2004a; Di Nicolantonio *et al.*, 2004b; Di Nicolantonio *et al.*, 2004c).

1.3.4 Chemosensitivity Assays in Colorectal Cancer

A major reason why the use of chemosensitivity assays has not become standard is that although many preliminary studies have been performed, these are usually with a small sample size and have not gone on to randomised controlled trials. Chemosensitivity assays in colorectal cancer have been performed less frequently than in other tumour types due to the technical difficulties associated with the tumour material available. The majority of studies performed demonstrate the heterogeneity of chemosensitivity between individual tumours, and suggest that the assay in question may be appropriate for use to analyse the anti-tumour effects of drugs, but most do not contain any clinical correlation. Table 1.18 summarises some of the recent studies of chemosensitivity assays (not including molecular techniques) in colorectal cancer.

Table 1.18. Some recent studies of chemosensitivity assays in colorectal cancer

Reference	Method	n	Drug	Comments
Yoshinare *et al.*, 2003	MTT	88	5-FU	
Isshi *et al.*, 2002	Fluorescein diacetate assay, HDRA	62	5-FU	Evaluability rate 97.5%
Kabeshima *et al.*, 2002	MTT	200		Survival of sensitive group vs resistant p=0.0158; vs surgery only p=0.0004
Araki *et al.*, 1999	Collagen-gel droplet embedded culture drug sensitivity test	24	adriamycin, etoposide, 5-FU, MMC cisplatin,	Evaluability rate 87.5% Adriamycin most sensitive
Kawabata *et al.*, 1998	3H-thymidine incorporation assay	184	5-FU, etoposide, MMC, cisplatin	5-FU most sensitive
Yamaue *et al.*, 1996	MTT	93	cisplatin, MMC, 5-FU, adriamycin	Cisplatin most sensitive 5/15 response with assay directed therapy
Furukawa *et al.*, 1995	HDRA	29	5-FU	96.3% evaluability PFS in sensitive group better than insensitive group ($p<0.05$)

1.4 Hypothesis and Aims

1.4.1 Hypothesis

This thesis examines the hypothesis that the chemosensitivity of colorectal cancer can be measured by the ATP-TCA, and that the observed heterogeneity of chemosensitivity will match clinical efficacy. If this is the case, then the assay should be capable of aiding drug and regimen development for colorectal cancer. Molecular profiling of the tumours may relate to chemosensitivity and add further information to the design of new regimens.

1.4.2 Aims

1. To modify and validate the ATP-TCA for use with colorectal tumour-derived cells.
2. To define the spectrum of sensitivity of colorectal cancer to existing cytotoxic agents using the ATP-TCA.
3. To determine whether the chemosensitivity profile relates to the molecular profile of individual tumours.
4. To identify novel agents or drug combinations for the treatment of colorectal cancer.

CHAPTER 2

Materials and Methods

2.1 Introduction

This chapter describes the methods used in this thesis. The main technique used was the ATP tumour chemosensitivity assay (ATP-TCA), which has been introduced in chapter 1. The ATP-TCA was used to assess the *ex vivo* chemosensitivity of colorectal cancer (CRC). In the process of determining the heterogeneity of chemosensitivity of this cancer we have evaluated new drug combinations.

Immunohistochemistry (IHC) has been used to investigate the expression levels of several proteins by the tumour samples for comparison with ATP-TCA data. IHC was performed by Mrs Penny Johnson of the Histopathology Department, Queen Alexandra Hospital, Portsmouth. The method used is included here in brief.

qRT-PCR has been used to measure expression of genes in some samples where enough RNA was extracted from spare cells. Changes in expression of some genes after short-term exposure to drugs in the ATP-TCA have also been investigated.

2.2 ATP-Tumour Chemosensitivity Assay

2.2.1 Cell culture media

All chemosensitivity assays were performed in Complete Assay Medium (CAM) (DCS Innovative Diagnostik Systeme, Hamburg, Germany), a serum free medium without growth factors. To this medium 300 IU/ml penicillin and 300 mg/ml streptomycin (P0781; Sigma, Poole, UK), 75 mg/ml gentamicin (G1272; Sigma) and 10 mM HEPES (H6147; Sigma) were added as previously validated (Andreotti *et al.*, 1995).

2.2.2 Tumour Material

Fresh colorectal tumour material was obtained from Queen Alexandra Hospital and malignant effusions from St. Mary's Hospital, Portsmouth. All patients underwent informed consent and received a detailed information sheet approved by LREC.

2.2.3 Transportation of Tumour Material and Preparation of Specimens

Colorectal cancer specimens from Queen Alexandra Hospital were received whole and fresh. A pathologist or surgeon assessed the specimen to ensure a sample could be taken for assay testing without compromising the histopathological diagnosis or resection margins. Solid tumour material was transported in sterile universals containing 10 ml of CAM. To overcome infection of sample material, 2.5μg/ml amphotericin B and 1 μg/ml metronidazole was also added. The effect of additional antibiotics on chemosensitivity has been validated in this thesis (see chapter 3 - Technical Development).

Malignant effusions were transported in 250 ml bottles containing 25 ml CAM to which an additional 250 mM HEPES was added. 5,000 IU heparin sodium (Monoparin®, CP Pharmaceuticals Ltd, Wales) was also added to prevent the formation of blood clots whilst in transit.

Tumours transported from St Mary's were packed in a polystyrene box containing an ice pack. The specimen was separated from the ice pack by paper towels as a precaution against freezing of the sample, which results in cell death.

2.2.4 ATP-Tumour Chemosensitivity Assay (ATP-TCA)

The ATP-TCA was performed as previously described by Andreotti *et al.* (1995) and modified for colorectal tumours as described here.

2.2.5 Enzymatic tumour dissociation of Solid Samples

On receipt, solid samples were diced into 0.5-2.0 mm^3 pieces in a class II safety cabinet using a sterile Petri dish and scalpel. With colorectal samples, care was taken to remove any loose material and the surface was scraped to remove surface faecal matter. The samples were washed in CAM with antibiotics to remove as many contaminating bacteria as possible. Each sample was then added to sterile universal tubes containing CAM and 1.5 mg/ml collagenase (C-8051; Sigma). The tumour and collagenase solution was incubated overnight at 37°C. The following morning the mixture was shaken and left for half an hour to encourage dissociation.

2.2.6 Preparation of Samples

The dissociated cell suspension or ascites sample was centrifuged at 1100 rpm for 7 minutes and the supernatant discarded. The cells were washed twice by re-suspension in CAM and centrifuging at 1100 rpm for 7 minutes. After the second wash the cells were resuspended in 10 ml CAM prior to the removal of red blood cells by Ficoll-Hypaque density gradient separation.

2.2.7 Ficoll-Hypaque Density Gradient Separation

Ficoll-Hypaque density gradient separation (Boyum, 1968) was performed following the manufacturers instructions. 10 ml Ficoll-Hypaque (1077-1; Sigma) was placed in a sterile universal. 10 ml cell suspension was carefully layered on top by pipetting drop by drop into the 25 ml universal held at an angle. The sample was then centrifuged at 1500 rpm for 30 minutes. Most of the red blood cells form a pellet at the bottom of the universal whilst the tumour cells form an interface between the Ficoll-Hypaque and Medium. Using a sterile Pasteur pipette, the tumour cells (including 1cm depth of medium either side of the interface) were transferred into a new sterile universal and washed twice with CAM. After the final wash the cells were resuspended in CAM and the cell number and viability was assessed using the trypan blue exclusion method.

2.2.8 Trypan Blue Exclusion Method

Cell number and viability was assessed using the trypan blue exclusion method (Kaltenbach *et al.*, 1958). Equal volumes (12-28 µl) of 0.4% trypan blue solution (T8154; Sigma) and cell suspension were mixed in an eppendorf vial and pipetted onto a haemocytometer. Viable cells appear clear and dead or dying cells appear blue as they are unable to pump the dye out of their cytoplasm.

This method also allowed the 'degree of clumping' of the cells to be assessed. If this was excessive, as was sometimes the case with the colorectal samples, the cells were trypsinated. This consisted of mixing equal volumes of cell suspension and trypsin (T4174; Sigma) in a flask and placing in the incubator for 3 minutes. The trypsin was neutralised by centrifuging in medium containing heat inactivated fetal serum (S026195; Labtech, Basingstoke, UK).

2.2.9 Preparation of Chemotherapeutic Test Drugs

Each chemotherapeutic drug or combination was tested in triplicate at six dilutions corresponding to 200%, 100%, 50%, 25%, 12.5% and 6.25% of the estimated Test Drug Concentration (TDC). The TDC for each individual drug is based on the peak plasma concentration or area under the curve (AUC) obtained from phase I clinical trials (Alberts and Chen, 1980). A similar AUC is calculated for the ATP-TCA, taking into account differences in protein binding between plasma and CAM (Andreotti *et al.*, 1995).

The cytotoxic drugs used were obtained as vials for injection from the Pharmacy Department at Queen Alexandra Hospital, Portsmouth. All chemotherapeutic drugs were prepared following manufacturers instructions, aliquoted into eppendorf vials, and stored at room temperature, 4°C, -20°C or –80°C according to previously published stability data (Hunter *et al.*, 1994). 800% TDC solutions were prepared by adding stock drug solution to 5 ml CAM. The volume of stock drug solution added to CAM to obtain 800% TDC for individual drugs is shown in table 2.1.

Table 2.1. Test Drug Concentrations, stock drug concentrations of the drugs used and storage conditions

Cytotoxic drug	Test Drug Concentration in µM	Test Drug Concentration (TDC) µg/ml	Stock drug solution mg/ml	µl stock drug solution added to 5 ml CAM (= 800% TDC)	Storage conditions (°C)
Celecoxib	2.6	1.0	10	4	-20
5 Fluorouracil	346	45	25	72	RT
Gemcitabine	40	12	40	12	-20
Irinotecan	148	100	20	200	RT
Mitomycin C	2	0.7	1	28	-20
Oxaliplatin	12.6	5	5	40	-80

RT: room temperature

2.2.10 Choice of Drugs for ATP-TCA

The drugs used in the ATP-TCA were chosen by Professor I A Cree in consultation with Oncology colleagues. The drugs tested reflect the NICE guidelines for the treatment of advanced CRC (NICE, 2002). The order of drugs was such that the most commonly clinically used drugs appear in plate 1 and would be tested if insufficient cells were present to test all drugs on the list (table 2.2).

Table 2.2. Colorectal Cancer Drug List (based on NICE Guidance No. 33, 2002)

Plate	Drug
1a	5 Fluorouracil
1b	Irinotecan
1c	Oxaliplatin
1d	Oxaliplatin + 5 Fluorouracil
2a	Mitomycin C + 5 Fluorouracil
2b	Mitomycin C
2c	Irinotecan + 5 Fluorouracil
2d	Mitomycin C + Gemcitabine
3a	Oxaliplatin + Gemcitabine
3b	Gemcitabine
3c	Celecoxib
3d	Celecoxib + 5 Fluorouracil

2.2.11 Preparation of 96-well Microculture plates

Round bottomed polypropylene culture plates (3790; Corning-Costar, High Wycombe, UK) were used for the ATP-TCA. 100 µl Maximum Inhibitor (MI) (0.02% Triton X-100 in DMEM) was added to row A. 100 µl CAM was added to the remaining wells. See figure 2.1 for plate layout. 100 µl of the 800% TDC solution of each drug was added in triplicate to row B, and serially diluted down the plate to row G with a multi-channel pipette. The remaining 100 µl was discarded.

MI	MI	MI	MI	MI	MI	MI	MI	MI	MI	MI	MI
DRUG 1 200% TDC	DRUG 1 200% TDC	DRUG 1 200% TDC	DRUG 2 200% TDC	DRUG 2 200% TDC	DRUG 2 200% TDC	DRUG 3 200% TDC	DRUG 3 200% TDC	DRUG 3 200% TDC	DRUG 4 200% TDC	DRUG 4 200% TDC	DRUG 4 200% TDC
DRUG 1 100% TDC	DRUG 1 100% TDC	DRUG 1 100% TDC	DRUG 2 100% TDC	DRUG 2 100% TDC	DRUG 2 100% TDC	DRUG 3 100% TDC	DRUG 3 100% TDC	DRUG 3 100% TDC	DRUG 4 100% TDC	DRUG 4 100% TDC	DRUG 4 100% TDC
DRUG 1 50% TDC	DRUG 1 50% TDC	DRUG 1 50% TDC	DRUG 2 50% TDC	DRUG 2 50% TDC	DRUG 2 50% TDC	DRUG 3 50% TDC	DRUG 3 50% TDC	DRUG 3 50% TDC	DRUG 4 50% TDC	DRUG 4 50% TDC	DRUG 4 50% TDC
DRUG 1 25% TDC	DRUG 1 25% TDC	DRUG 1 25% TDC	DRUG 2 25% TDC	DRUG 2 25% TDC	DRUG 2 25% TDC	DRUG 3 25% TDC	DRUG 3 25% TDC	DRUG 3 25% TDC	DRUG 4 25% TDC	DRUG 4 25% TDC	DRUG 4 25% TDC
DRUG 1 12.5% TDC	DRUG 1 12.5% TDC	DRUG 1 12.5% TDC	DRUG 2 12.5% TDC	DRUG 2 12.5% TDC	DRUG 2 12.5% TDC	DRUG 3 12.5% TDC	DRUG 3 12.5% TDC	DRUG 3 12.5% TDC	DRUG 4 12.5% TDC	DRUG 4 12.5% TDC	DRUG 4 12.5% TDC
DRUG 1 6.25% TDC	DRUG 1 6.25% TDC	DRUG 1 6.25% TDC	DRUG 2 6.25% TDC	DRUG 2 6.25% TDC	DRUG 2 6.25% TDC	DRUG 3 6.25% TDC	DRUG 3 6.25% TDC	DRUG 3 6.25% TDC	DRUG 4 6.25% TDC	DRUG 4 6.25% TDC	DRUG 4 6.25% TDC
MO	MO	MO	MO	MO	MO	MO	MO	MO	MO	MO	MO

Figure 2.1. 96 well plate layout for the ATP-TCA. Four drugs are tested at six concentrations (6.25-200%) of the TDC in triplicate. The plate has 12 no drug controls (MO) and 12 maximum inhibitor wells (MI).

Tumour cells were diluted in CAM to give a final concentration of 200,000 cells per ml for solid tumours and 100,000 cells per ml for ascites. Ascites and effusion samples usually contain >90% tumour cells and proliferate rapidly, so fewer cells are plated to prevent overgrowth in the wells. The final cell concentration per well was 20,000 for solid tumour specimens and 10,000 for ascites. The plates were incubated for six days at 37°C in the presence of 5% CO_2, with periodic checks for overgrowth and infection.

Excess cells not required for ATP-TCA were frozen at –80°C in Phosphate Buffered Solution (D8537; Sigma) for use in molecular biology tests.

2.2.12 ATP Extraction

ATP was extracted from the cells by adding 50 µl of ATP extraction reagent to each well. Well contents were immediately mixed gently 6-8 times using a multichannel pipette and left for at least 5 minutes before reading. Pipette tips were discarded after each drug triplicate.

2.2.13 Preparation of Luciferin-Luciferase Counting Reagent

Total well ATP was measured using a luciferin-luciferase counting reagent (D-luciferin (800-LN) and recombinant luciferase (700-LF); DCS Innovative Diagnostik Systeme, Hamburg, Germany) diluted with 10x HEPES buffer. 10x HEPES buffer was prepared by dissolving 23.8 g HEPES (H6147; Sigma) and 6.1 g magnesium sulphate in 400 ml of sterile water. The pH was adjusted to pH 7.7 using sodium hydroxide and the volume made up to 500 ml. The solution was then filtered through a 0.2 µM filter unit (Nalgene, UK). Luminescence measurements were carried out following manufacturer's instructions in a Berthold LB953 luminometer (Berthold Diagnostic Systems, Pforzheim, Germany).

A 2 ml aliquot of luciferin-luciferase reconstituted with 18 ml of dilution buffer (100 ml 10x HEPES buffer to 400 ml sterile water) is sufficient for four 96 well plates to be read in addition to performing an ATP standard curve. The mixture was mixed well, left at room temperature for 30 minutes and protected from light at all times.

2.2.14 ATP standard curve

An ATP standard curve was performed before reading the ATP-TCA plates to ensure the reagents and equipment were working correctly. 50 µl of dilution buffer was added to columns 1-9 of a single row of a white plate (7905; Dynatech,USA). Adenosine 5'-triphosphate standard disodium salt hydrate (Sigma) was reconstituted using sterile water to give a final concentration of 250 ng/ml. The solution was aliquoted into 40 µl aliquots, stored at –20°C and protected from light. 10 µl of the ATP standard was added to 4 ml dilution buffer and protected from light. 25 µl of this ATP solution was added to the first well and serially diluted along the row. 50 µl of the luciferin-luciferase solution was added to each well resulting in 1:3 dilutions with ATP concentrations of 83.33, 27.76, 9.253, 3.084, 1.028, 0.342, 0.114, 0.038 and 0.012 ng/ml. The log of ATP concentration against the log of the luminescence count produces a straight standard curve (figure 2.2).

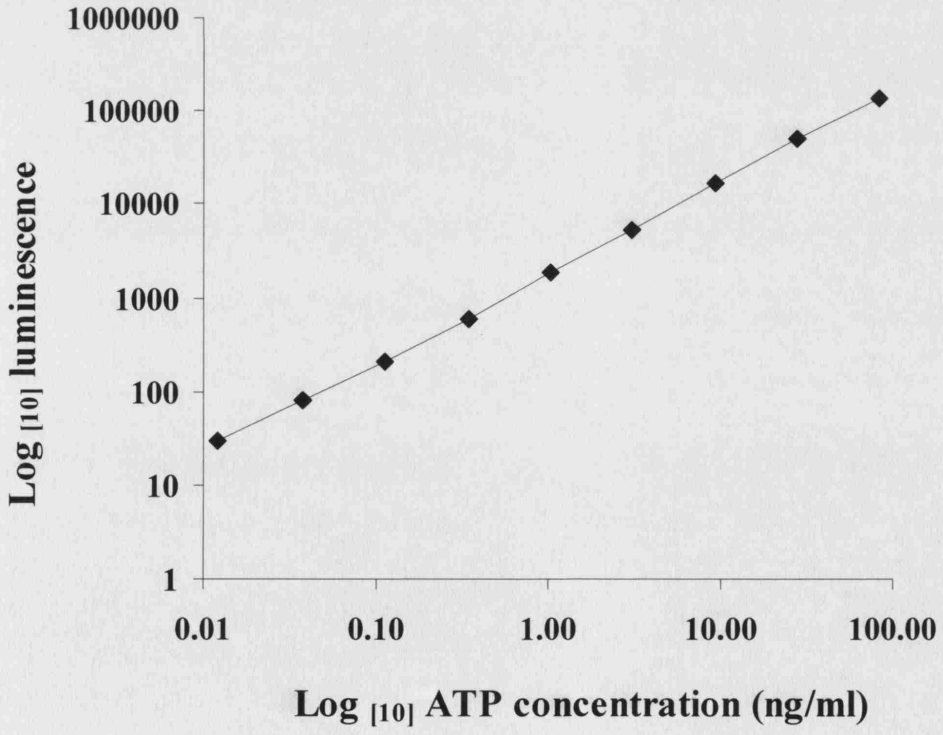


Figure 2.2. ATP standard curve produced on a Berthold MPLX plate luminometer.

2.2.15 Reading ATP levels and evaluability

Following cell lysis (ATP extraction), 50 µl from each well was transferred into a white 96 well plate, using fresh pipette tips for each drug. 50 µl of luciferin-luciferase was added to each well and the plate read in the luminometer within 10 minutes. For an assay to be evaluable the average positive control luminescence has to be at least as high as the luminescence produced by 0.342 ng/ml ATP in the standard curve.

2.2.16 Interpretation of Luminometry Results

All data from the luminometer was transferred directly to an Excel (Microsoft®) spreadsheet that calculated the percentage tumour growth inhibition at each concentration, the IC50, IC90 and area under concentration vs inhibition graph (IndexAUC). From this information % inhibition curves for each drug and combination were plotted. The typical coefficient of variance is less than 15%. The data was also entered into an Access (Microsoft®) database.

The percentage tumour growth inhibition was calculated as follows:

$$1.0 - \frac{(TEST) - (MI)}{(MO) - (MI)} \times 100 = \text{Percent tumour growth inhibition}$$

(TEST) = mean counts for test drug wells
(MI) = mean counts for maximum inhibitor wells
(MO) = mean counts for no drugs

A sensitivity index ($Index_{SUM}$) was calculated for each drug for every tumour. This involves summing the percentage tumour growth inhibition and subtracting this figure from 600.

$$(Index_{SUM}) = 600 - Sum[Inhibition_{6.25.....200}]$$

Assessment of Drug Combinations

The effect of drug combinations compared to the constituent single agents was analysed in the Excel spreadsheet using the method determined by Poch *et al.* (1995). This method has been shown to be better suited to the data produced by the ATP-TCA compared to methods such as Chou and Talalay (1984).

The median effect method of Chou and Talalay is not useful when dealing with drugs that produce a shallow dose response curve, such as gemcitabine. However, we performed Chou and Talalay analysis when the Poch method suggested synergism between two agents. The results were compared. The combination index (CI) was determined at 90% cell death, and was defined as follows:

$$CI_{A+B} = [(D_{A/A+B})/D_A] + [(D_{B/A+B})/D_B] + [\text{alpha}(D_{A/A+B} \times D_{B/A+B})/D_A D_B]$$

where CI_{A+B} = CI for a fixed effect (F=90%) for the combination of cytotoxic A and cytotoxic B; $D_{A/A+B}$ = concentration of cytotoxic A in the combination A + B giving an effect F; $D_{B/A+B}$ = concentration of cytotoxic B in the combination A + B giving an effect F; D_A = concentration of cytotoxic A alone giving an effect F; D_B = concentration of cytotoxic B alone giving an effect F. alpha = parameter with value 0 when A and B are mutually exclusive and 1 when A and B are mutually non-exclusive.

The combination index indicated: synergism <0.8; additivity >0.8 and <1.2; antagonism >1.2 (Greco *et al.*, 1995).

2.3 Immunohistochemistry

2.3.1 Introduction

The immunohistochemistry (IHC) technique used is based on the dual antibody (Ab) system using the Avidin-Biotin Complex method. The primary Ab binds to specific antigens present in the specimen, and excess is washed away. A secondary Ab, conjugated to biotin, is added and reacts with the primary Ab. Any unbound Ab is washed away. The specimen is incubated with an enzymatic label conjugated to avidin, which binds to the biotin-labelled secondary Ab present on the tissue. Chromogenic substrates are added, which in the presence of the enzymatic label, deposit coloured insoluble precipitate at antigenic sites recognised by the primary Ab. The antibodies investigated are shown in table 2.3.

Table 2.3. Primary antibodies used for staining colorectal cancer sample slides, including dilution, positive control and supplier.

Antibody	Dilution	Staining	Positive Control	Supplier
BCRP	1:40	Cytoplasmic	Kidney	MAB4146; Chemicon
COX-2	1:200	Cytoplasmic	Colon carcinoma	160112; Cayman Chemical
hMLH1	1:50	Nuclear	Colon	554072; BD Pharmingen
hMSH2	1:100	Nuclear	Colon	NA27; Oncogene
hMSH6	1:200	Nuclear	Colon	610919; BD Biosciences
Thymidine Phosphorylase	1:200	Nuclear	Breast	MS-499-P1; Labvision
Thymidylate Synthase	1:20	Nuclear	Colon carcinoma	MS-471-P; Labvision
Topoisomerase I	1:100	Nuclear	Tonsil	NCL-TOPOI; Novocastra

2.3.2 Preparation of Cytoclots

For malignant effusions where no tissue blocks were available and where number of cells permitted, cytoclots were made. Cell pellets were resuspended adding an equal

amount of human plasma using a Pasteur pipette. Three drops of human thrombin were added to the mixture and mixed rapidly. The subsequent clot was eased from the tube using an orange stick and transferred to a labelled pot containing 10% buffered formalin. After 24-48 hours the clot was then processed in the same way as formalin-fixed tissue.

2.3.3 Immunohistochemistry Method

Sections were floated onto Surgipath® positively charged slides (Surgipath® Europe Ltd, Peterborough, UK) from formalin-fixed tissue embedded in paraffin blocks, and dried at 60°C for 40 minutes. The sections were de-waxed and endogenous peroxidases blocked with methanol and hydrogen peroxide. All antibodies, except Thymidine Phosphorylase, were pretreated by pressure-cooking (Tefal Clipso pressure cooker, 70 Psi power) in citrate buffer at pH 6 for 2 minutes to 'unmask' the antigen. The sections were rapidly cooled and washed in running tap water. The specimens were stained using the Lab Vision Autostainer (Newmarket, UK) and Chemicon secondary detection system (Det-HP1000; Chemicon International, Chandlers Ford, Southampton, UK). In brief, the slides were exposed to i) normal goat serum, ii) the primary antibodies at the above dilutions, iii) anti-mouse biotin, iv) biotin-horseradish peroxidase and finally v) the chromagen, diaminobenzidine (DAB; 4170; Kementec, Aylesbury, UK). The slides were then dipped in haemotoxolyn, dehydrated and mounted with DPX.

The concentration of the antibody was determined by titration on positive control material and was made up to its optimal dilution in tris-buffered saline (TBS) pH 7.6. A positive control was run with each batch of staining and a duplicate of each test section was included as a negative control by omitting the antibody and replacing with TBS.

2.3.4 Data Analysis

The slides were examined and graded by Dr Silvana Di Palma (Consultant Histopathologist, Department of Histopathology, Queen Alexandra Hospital, Portsmouth). For hMLH1, hMSH2 and hMSH6, slides were graded as either positive or negative. For the remaining antibodies, slides were graded by the percentage of

cells stained and the intensity of staining (0 if negative, 1+ for weak staining up to 3+ for very strong staining). For slides graded by intensity and percentage, the Histoscore (H-score) was used to achieve a score between 0 and 300, where H = % cells staining positive x 0-3 on intensity. An H-score of 100 or more was regarded as positive.

Statistics

The appropriate statistical analysis was performed using InStat (GraphPad ®, San Diego, USA) or Statsdirect (CamCode, Herts, UK).

2.4 Quantitative Real-Time Polymerase Chain Reaction

2.4.1 Introduction

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) was used to measure gene expression for a limited number of genes in some samples where cells pre- and post-treatment *in vitro* were stored and enough RNA extracted. Primer design, optimisation and validation were performed by Federica Di Nicolantonio and Stuart Mercer. The RNA extraction, reverse transcriptase steps and PCR were carried out by a team consisting of myself, Federica Di Nicolantonio, Louise Knight and Stuart Mercer. Some results presented here are also presented in their theses to other Universities. The method is described here briefly.

2.4.2 Primers

Forward and reverse primers (table 2.4) were chosen to span exon boundaries in the target sequences, and were obtained freeze dried from Qiagen (Hilden, Germany) and reconstituted with RNA-free water to stock solutions of 100 μM.

Sighting shots of the new primers were performed with cDNA1 and cDNA1:4 dilutions, with reverse transcriptase (RT) negative and water controls. The primers were then tested at three different concentrations with three different concentrations of MgCl for optimisation. Standard calibrations were performed aiming for an efficiency of 95-110% and correlation coefficient of at least 0.99, as this is necessary to permit comparison of genes (including housekeeping versus test genes).

2.4.3 Housekeeping Genes

Housekeeping genes are used as controls against which the expression level of genes can be normalised. There are a large group of genes that code for proteins whose activities are essential for maintenance of cell function. Many studies use only one housekeeping gene, but the expression of some genes has been shown to vary enormously between cell type and in different conditions. It is therefore necessary to use at least three housekeeping genes to determine the most stable internal control genes (Vandesompele *et al.*, 2002). The housekeeping genes used for qRT-PCR of colorectal samples included: glyceraldehyde-3 phosphate dehydrogenase (GAPDH),

Table 2.4. Primer Sequences

Oligonucleotide Name (Accession Number)	**Forward Primer**	**Reverse Primer**
BCRP (AF098951)	CAC AAC CAT TGC ATC TTG GC	GCT GCA AAG CCG TAA ATC CA
COX-2 (M90100)	CCT TCC TCC TGT GCC TGA TG	ACA ATC TCA TTT GAA TCA GGA AGC T
DPD (NM_000110)	CCA AAG GCA GTA AAG CAG GAA	TCA CGA CTC CCC GTA TCG A
ERCC1 (NM_001983)	GGG AAT TTG GCG ACG TAA TTC	GCG GAG GCT GAG GAA CAG
GAPDH (NM_002046)	GAA GGT GAA GGT CGG AGT C	GAA GAT GGT GAT GGG ATT TC
HPRT1 (NM_000194)	TCA GGC AGT ATA ATC CAA AGA TGG T	AGT CTG GCT TAT ATC CAA CAC TTC G
MDR1 (AF016535)	TGG TTC AGG TGG CTC TGG AT	CTG TAG ACA AAC GAT GAG CTA TCA CA
MLH1 (NM_000249)	GGC ACA GCA TCA AAC CAA GT	GCA AGC ATG GCA AGG TCA A
MSH2 (NM_000251)	ACC AGA AAT TAT TGT TGG CAG TTT	CTG AGA TTA GGA TCA AAT GAA GGT TT
PBGD (NM_000190)	CTG CAC GAT CCC GAG ACT CT	GCT GTA TGC ACG GCT ACT GG
PDGFB (NM_002608)	TCA GGT GGG TTA GAG ATG GA	AGT GGA GTA TGG GGA GGA AG
TBP (X54993)	CACGAACCACGGCACTGATT	TTTTCTTGCTGCCAGTCTGGAC
TOPO I (J03250)	CTC CAC AAC GAT TCC CAG AT	TTA TGT TCA CTG TTG CTA TGC TT
TP (NM_001953)	CCTTGGATAAGCTGGAGTCT	CCTACTCTGACCCACGATAC
TS (NM001071)	CCA GAG ATC GGG AGA CAT GG	TAC GTG AGC AGG GCG TAG CT

Hypoxanthine phoshoribosyl-transferase 1 (HRPT1), human porphobilinigen deaminase (PBGD), and TATA box binding protein (TBP).

2.4.4 Total RNA Extraction

RNA extraction, from at least 10^7 cells, was performed following the protocol for Nucleospin® RNA II mini kit (740955.10; Macherey-Nagel, Dűren, Germany). RNA was extracted from the MO wells and treatment wells (usually 50% TDC) of the TCA 96 well plates. The protocol includes a DNAse digestion step to prevent carry-over of genomic DNA in further analysis. The resulting RNA was frozen at –80°C as 60 µl aliquots in RNA-free water.

2.4.5 Reverse Transcription

A single-strand complementary DNA copy (cDNA) of the RNA is produced by the action of the retroviral enzyme, reverse transcriptase (RT). This was performed following instructions from the manufacturer (Promega; Southampton, UK) using avian myeoblastosis virus RT (AMV-RT). A master mix solution for the required number of reactions was prepared using all the reagents, except AMV-RT enzyme and RNA, listed in table 2.5. An RT negative sample was also made (omitting AMV) to check that DNA was not being produced spontaneously.

The samples were then transferred to a thermal cycler (ThermoHybaid, Ashford, UK) and incubated at 42°C for 60 minutes to improve the efficiency of the reaction. After this, samples were heated at 95°C for 5 minutes and then incubated at 4°C for 5 minutes to inactivate the AMV RT and prevent it from binding to the cDNA. The cDNA samples were then stored at -80°C.

2.4.6 Real Time PCR

The cDNA was amplified by real time quantitative PCR in 96 well plates (2239441; Biorad) in a Biorad iCycler instrument (Biorad Laboratories, Hemel Hampstead, UK). All reagents were obtained from Applied Biosystems (Warrington, UK), and used in accordance with the manufacturers instructions. The amount of amplified PCR product is monitored by SYBR Green® which preferentially binds to newly synthesized double-stranded DNA (dsDNA). The fluorescent signal produced is

Table 2.5. List of reagents, volumes and concentrations for each RT reaction

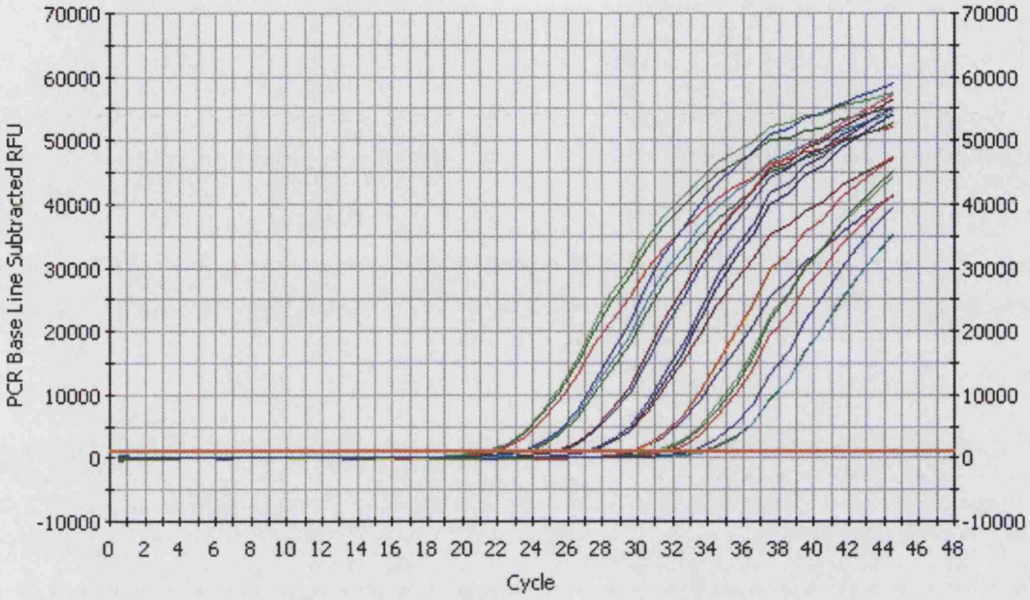
Reagent	**Volume/sample**	**Master Mix Final Concentration**
Sterile Nuclease-Free Water	1.9 µl	
MgCl2 25mM	4.0 µl	5 mM
Reverse Transcription 10X Buffer	2.0 µl	1X
DNTP Mixture – 10mM each	2.0 µl	1 mM
Random Primers 0.5µg/µl	1.0 µl	25 ng/µl
Recombinant Rnasin® Ribonuclease Inhibitor (50U/µl)	0.5 µl	25 U
AMV Reverse Transcriptase (25U/µl)	0.6 µl	15 U
RNA samples	8.0 µl	
TOTAL	20.0 µl	

proportional to the concentration of the newly synthesised dsDNA. The amount of cDNA used in each experiment was adjusted according to the sighting shot results.

The final constituents of each PCR reaction (25µl) were 1µl of cDNA (or H_20), 400 nM of each primer, 200 µM each dATP,dCTP,dGTP, 400 µM dUTP, 4.0 mM MgCL2, 0.125 units AMPErase® UNG, 0.625 units of AmpliTaq Gold DNA polymerase, 1x SYBR Green PCR buffer.

Product amplification was performed up to 45 PCR cycles, after uracil removal (2 minutes at 50°C) and polymerase activation (10 minutes at 95°C). Each two-step PCR cycle comprised denaturing (15 seconds at 95°C), annealing, and extending (1 minute at 60°C). At the end of each run a final melt curve cycle (cooling to 50°C and then increasing stepwise 1°C to 95°C) was performed to exclude the presence of primer-dimer artefacts and to check that a single product was produced (figure 2.3). Each product was checked for size by gel electrophoresis (Di Nicolantonio, *thesis,* 2004) and by Taqman® (ABI Biosystems) probes.

a)



(b)

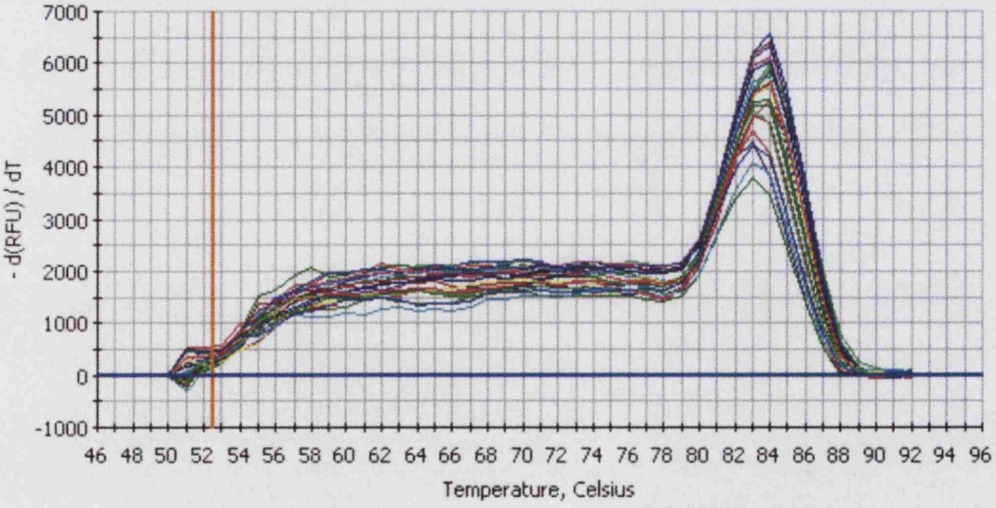


Figure 2.3. Example of (a) PCR MP/Cycle and (b) melt curve graphs for DPD.

Positive and negative controls were added in every experiment. All assays were run in triplicate. Validation experiments were run to show that the efficiencies of the target and reference gene amplifications were approximately equal. The PCR cycle number that generated the first fluorescence signal above a threshold (threshold cycle, Ct; 10 standard deviations above the mean fluorescence generated during the baseline cycles) was determined, and a comparative Ct method was then used to measure relative gene expression (ABI PRISM 7700 User Bulletin #2, 2001 update). The following formula was used to calculate the relative amount of the transcript in the sample:$2^{-\Delta\Delta Ct}$ where ΔCt is the difference in Ct between the gene of interest and the mean of at least two reference genes, and $\Delta\Delta Ct = \Delta Ct$ of drug exposed cells – ΔCt of control cells (exposed to medium only).

2.5.7 Data Analysis

The threshold cycle (Ct) values available from RT-PCR allow accurate quantification of specific gene expression within a sample. These values can be compared between samples, showing the trend or heterogeneity that exists between samples of the same tumour type. The level of change of gene expression in response to treatment *ex vivo* can also be determined and plotted.

CHAPTER 3

Technical Development

3.1 Validation of Antibiotics

3.1.1 Introduction

Infective contamination was a commonly encountered problem when first performing the ATP-TCA on gastrointestinal specimens, despite the presence of penicillin and streptomycin in the culture medium. Due to the nature of the assay, which measures the total amount of ATP in the well, any contamination with bacteria, which contain ATP, severely alters the result and renders it useless. To overcome this, the use of additional antibiotics was investigated. Antifungals were investigated first as many infections were fungal in nature. However, this did not overcome the problem entirely and as a result metronidazole was also added to the media.

(i) Antifungals

Amphotericin B (AMB) was the antifungal chosen because the mechanisms of action of other antifungal agents make them unsuitable for use in the assay. The Azoles (ketoconazole, fluconazole) are broad spectrum synthetic antifungals and act by binding to the enzyme cytochrome P-450, thus preventing the synthesis of ergosterol. Cytochrome P-450 is required for the conversion of some drugs into metabolites (e.g. irinotecan), and therefore these antifungals were not suitable for use in the assay. Flucytosine is another synthetic antifungal agent, which is converted to 5-fluorouracil, an antimetabolite cytotoxic used against cancer cells, and therefore is unsuitable.

Amphotericin B is an amphoteric macrolide polyene antifungal antibiotic (MW 924) which is fungistatic and has a half-life of 24 hours. It is insoluble in water and unstable at 37°C. AMB acts by binding irreversibly to the sterol component of cell membranes, which results in a change in membrane permeability allowing leakage of intracellular electrolytes (potassium) and metabolites, and increases cellular permeability to various drugs. The peak plasma concentration possible by intravenous infusion is 2-4 mg/l.

AMB has been shown to reverse resistance to, and enhance the cytotoxicity of, cisplatin and its analogues *in vitro* (table 3.1). This is due to an increased intracellular accumulation of drug and increase in interstrand cross-link formation (Morikage *et al.*, 1993). AMB does not seem to have a synergistic effect with 5-FU or a number of other drugs (Ganu *et al.*, 1984; Iida *et al.*, 2001). The addition of amphotericin B to doxorubicin, lomustine, hexamethylmelamine and methotrexate (ACHM) has not improved the response rate or survival of patients with non-small cell lung cancer (Presant *et al.*, 1984). Camptothecan analogues have been shown to exhibit synergistic antifungal activity with AMB (Del Poeta *et al.*, 1999), but the cytotoxic effect of AMB on Camptothecans was not tested in this paper.

Table 3.1. Studies demonstrating cytotoxic resistance reversal by Amphotericin B

Reference	**Cytotoxic Agent**	**Effect of AMB**
Ganu *et al.*, 1984	5-FU, Vincristine	no effect
Kikkawa *et al.*, 1993	Cisplatin and analogues	enhanced intracellular accumulation and cytotoxicity
Morikage *et al.*, 1993 Assem *et al.*, 1994	Cisplatin	
Zhengdong *et al.*, 1996 Poulain *et al.*, 1997	Platinum analogues	
Ferguson *et al.*, 1999	Cisplatin, Carboplatin	
Iida *et al.*, 2001	Danorubicin, Doxorubicin, 5-FU, Paclitaxel, Vinblastine	no effect

(ii) Metronidazole

Metronidazole is a synthetic antiprotozoal and antibacterial agent commonly used clinically to cover anaerobic gastrointestinal infections. It is metabolised by the liver and known to interact with several drugs, including warfarin, lithium, antiepileptics and cimetidine, as well as alcohol and disulfiram. There is no evidence that metronidazole has a cytotoxic effect of its own, and little to suggest a synergistic effect with anti-tumour agents. Tannock (1980) demonstrated an increase in the cytotoxic effect of 5-fluorouracil when combined with metronidazole, but Bardakji *et*

al. (1986) showed metronidazole impaired the clearance of 5-fluorouracil without enhancing its therapeutic efficacy. Metronidazole has been shown to weakly enhance the cytotoxicity of the alkylating agent melphalan (Smith *et al.*, 1982). Enhanced inhibition of the cytotoxicity of cisplatin and cytosine arabinoside has been noted in a clonogenic study including metronidazole in the assay media (Trujillo *et al.*, 1989). However, these authors used a concentration of 10 mg/ml metronidazole. Metronidazole does not significantly alter hepatic cytochrome P-450 activity (Haas *et al.*, 2001).

3.1.2 Method

(i) Drug Concentrations

Amphotericin B

Amphotericin B (Fungizone; A2942; Sigma, Poole, UK), MW 924.1 g/ml, was supplied as 250 μg/ml solubilised by the addition of sodium deoxycholate, which produces a colloidal dispersion. Clinically the peak plasma concentration is known to be 2-4 mg/l (ABPI Compendium, 1996) and the minimum inhibitory concentration (MIC) against *Candida Albicans* is 0.13 μg/ml (Burgess *et al.*, 2000). At concentrations of 10 μg/ml Amphotericin B has direct toxic effects and enhances cisplatin toxicity (Morikage *et al.*, 1993; Poulain *et al.*, 1997; Ferguson *et al.*, 1999). At <5 μg/ml no direct effect is seen (Kikkawa *et al.*, 1993; Assem *et al.*, 1994). A final concentration of 2.5 μg/ml was achieved by adding 100 μl stock solution to 10 ml media.

Metronidazole

Metronidazole (Flagyl; Rhône Poulenc Rorer Limited, Eastbourne, UK), MW 171.16 g/l, was supplied as 5 mg/ml for intravenous infusion. The clinically effective concentration is 3-6 μg/ml and the MIC against *Clostridium spp.* is 1 mg/l (Speciale *et al.*, 2002). 2 μl of stock solution was added to 10 ml media for a final concentration of 1 μg/ml.

(ii) Cell Culture Experiments

Experiments were performed using the ATP-TCA as described in section 2.2, first with SK-MEL-28 melanoma cell lines (kindly supplied by David Jackson, ICRF, Leeds, UK), and then using tumour-derived cells. The cell line was incubated at 37°C in humidified 5% CO_2 in standard RPMI-1640 (R7638; Sigma) supplemented with 2 mM L-Glutamine (G7513; Sigma), 100 IU/ml penicillin and 100 μg/ml streptomycin (P0781; Sigma) and 10% heat inactivated Fetal Bovine Serum (S026195; Labtech International, East Sussex, UK) until confluent. Tumours were dissociated overnight in CAM and collagenase. After washing and counting the cells were plated at 20,000 cells per well with six concentrations of cytotoxic agents with or without the addition of antibiotics as in table 3.2.

Table 3.2. Amphotericin B experiments

Control plate	No additional antibiotics
Experimental plate 1	Tumour dissociated overnight with 2.5 μg/ml AMB added to CAM and collagenase, and plated with 2.5 μg/ml AMB added to CAM

Table 3.3. Metronidazole experiments

Control plate	Tumour dissociated overnight with 2.5 μg/ml AMB added to CAM and collagenase, and plated with 2.5 μg/ml AMB added to CAM
Experimental plate 1	Tumour dissociated overnight with 2.5 μg/ml AMB and 1 μg/ml metronidazole added to CAM and collagenase, and plated with 2.5 μg/ml AMB and 1 μg/ml metronidazole added to CAM

The cell lines were plated in flat bottomed 96 well polystyrene microplates (353072; Falcon, BD Biosciences, Oxford, UK) rather than the round bottomed 96 well plates previously described. After six days incubation the amount of ATP was read in a luminometer (proportional to number of living cells).

3.1.3 Results

The addition of antibiotics, either amphotericin B alone, or in combination with metronidazole, did not have any significant effect on the cytotoxicity of a number of drugs when tested on cell lines or tumour-derived cells (Wilcoxon matched pairs test). The original data, including the area under curve (AUC), IC50, IC90 and $Index_{SUM}$ for each individual experiment performed using cell lines and tumour cells with a range of drugs, is presented in Appendix A. The median $Index_{SUM}$ for experiments with and without antibiotics are shown in tables 3.4 and 3.5, and figures 3.1 and 3.2.

Table 3.4. Median $Index_{SUM}$ of SK-MEL-28 cell lines tested with and without Amphotericin B

Drug	n	Median $Index_{SUM}$	
		Control (no antibiotics)	2.5 µg/ml AMB
Cisplatin	4	410	412.5
5-FU	3	228	198
Gemcitabine	4	204	201.5
MMC	3	238	212.5
Oxaliplatin	3	502.5	460.5

Table 3.5. Median $Index_{SUM}$ of tumour-derived cells with and without Metronidazole

Drug	n	Median $Index_{SUM}$	
		Control (no antibiotics)	1 µg/ml Metronidazole
5-FU	5	357	345
Irinotecan	4	306.5	285.5
MMC	3	333	368
Oxaliplatin	4	548	561

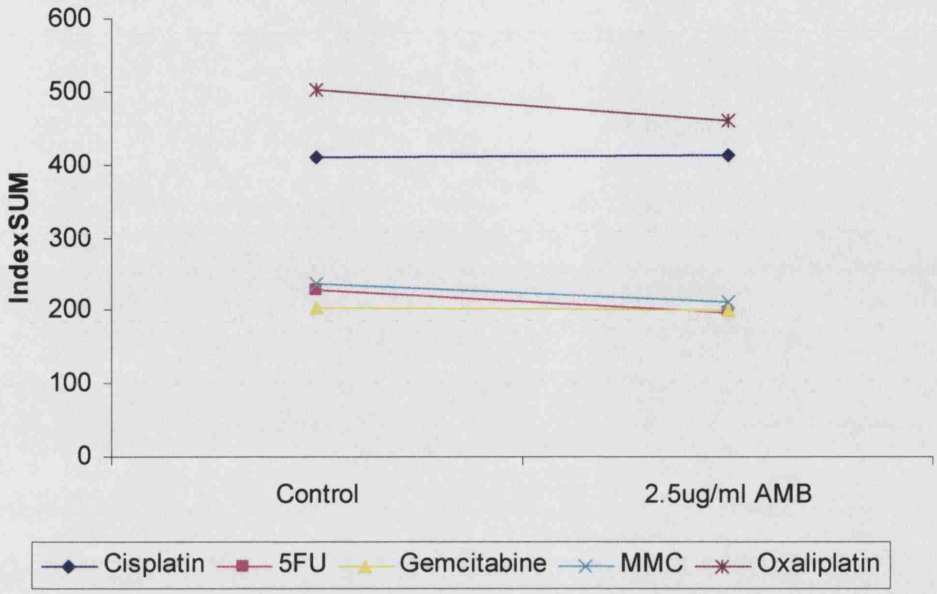


Figure 3.1. Paired results of effect of Amphotericin B on SK-MEL-28 cell lines compared to control (no additional antibiotics)

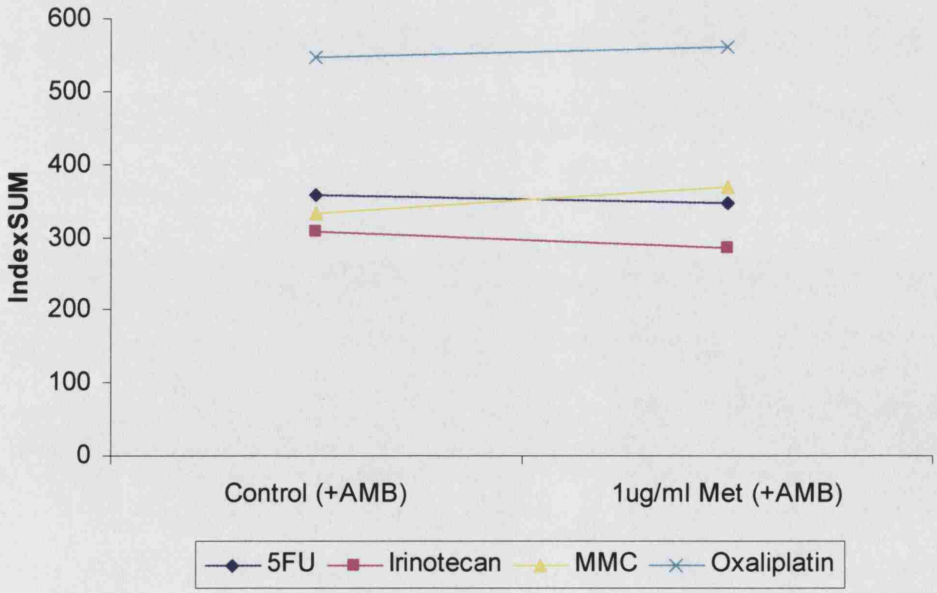
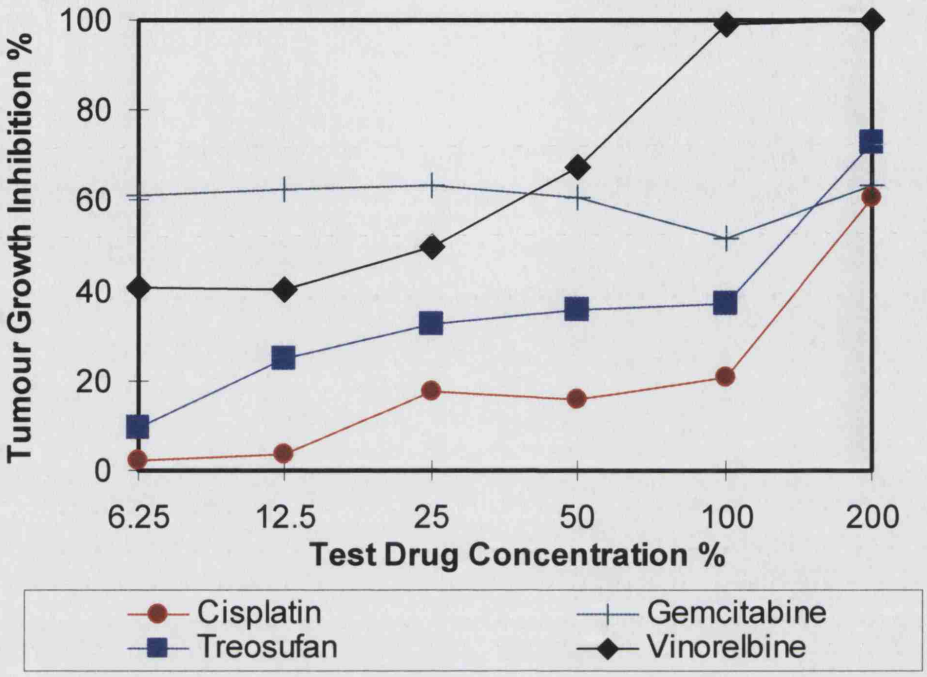


Figure 3.2. Effect of Metronidazole on colorectal cancer tumour-derived cells compared to control (CAM plus 2.5 µg/ml amphotericin B)

(a)



(b)

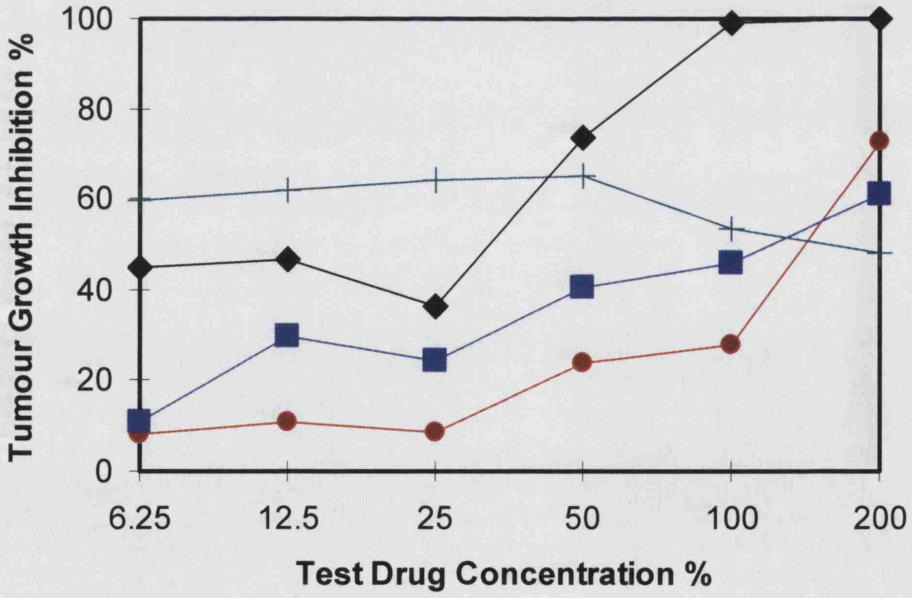
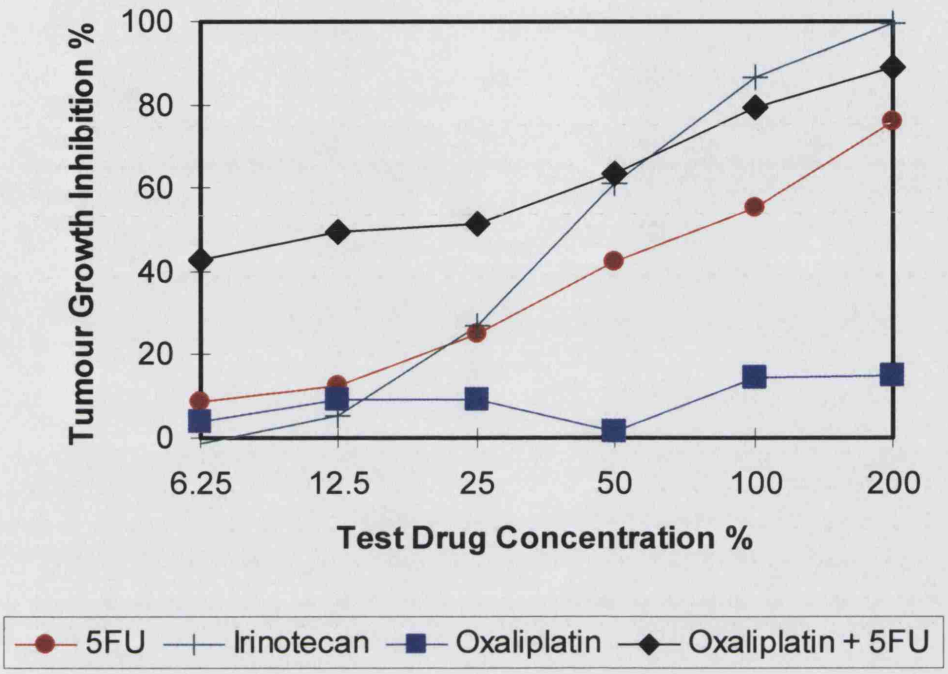


Figure 3.3. Example of growth inhibition curves of SK-MEL-28 cells (a) with and (b) without additional 2.5µg/ml Amphotericin B. The degree of variation is within expected experimental variation (+/- 15%).

(a)



(b)

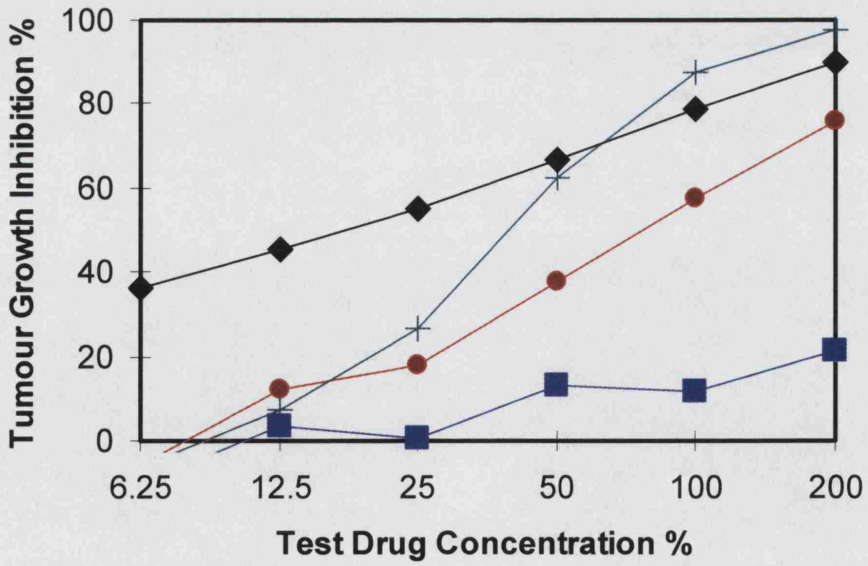


Figure 3.4. Example of tumour-derived cells (TORC02-0098) (a) with and b) without additional Metronidazole (1μg/ml). The degree of variation is within expected experimental variation (+/- 15%).

3.1.4 Discussion

Infection of cell culture plates is a regular problem that has led to cell assays failing to become a favourable method of testing. Despite high evaluability rates of the ATP-TCA with most tumours, the application of cell culture methods to gastrointestinal tumour cells has been limited by microbial contamination from the bowel lumen. There are two main methods for reduction of infection. One is by meticulous washing and manual removal of contaminated material. This is achieved by washing/vortexing the tumour several times in clean media. If necessary, visible contamination can be scraped away with a scalpel. The second method is the use of antibiotics. All culture media contains some antibiotics of one kind or another, most usually streptomycin and penicillin. Colorectal specimens are often colonised with anaerobic or fungal organisms, and therefore antibiotic modification of the culture is necessary.

Some antibiotics may alter the sensitivity of cytotoxic drugs. Indeed, amphotericin B has previously been investigated as a modulator of cytotoxic sensitivity and has been shown to reverse resistance to and enhance the cytotoxicity of cisplatin and its analogues *in vitro* due to an increased intracellular accumulation of drug and an increase in interstrand cross-link formation. The addition of 2.5 μg/ml amphotericin B was not found to have any significant effect on the cytotoxicity of the drugs tested, including cisplatin and oxaliplatin. Metronidazole is a synthetic antiprotozoal and antibacterial agent known to interact with several drugs but not chemotherapeutic agents. Low concentration of metronidazole (1 μg/ml) did not appear to alter the chemosensitivity of the drugs tested in the ATP-tumour chemosensitivity assay.

The addition of 2.5 μg/ml amphotericin B and 1 μg/ml metronidazole to Complete Assay Media has become standard practice when performing the ATP-TCA on colorectal cancer samples, producing an acceptable evaluability and infection rate.

3.2 Comparison of sensitivity of Irinotecan and its active metabolite SN38

3.2.1 Introduction

The topoisomerase I inhibitor, irinotecan (CPT-11), is converted to its inactive metabolites by cytochrome P-450, and to its active metabolite, SN38, by carboxyesterases in the liver, blood and tumours. As these enzymes are not necessarily present in our culture environment, we tested both irinotecan and SN38 in 15 samples.

3.2.2 Method

Irinotecan and its active metabolite SN38 were tested in the ATP-TCA at equivalent doses derived from pharmacokinetic data. The Test Drug Concentrations were 148 μM for irinotecan and 0.141 μM for SN38.

3.2.3 Results

Of all the samples tested, 47% were sensitive to single agent irinotecan and none to SN38 (table 3.6), based on a threshold for sensitivity of $Index_{SUM} < 300$. Fifteen samples were tested with both irinotecan and SN38. Of these 15, none were sensitive to SN38 at the concentration tested (0%), but seven were sensitive to irinotecan (47%). Table 3.7 shows the median, minimum and maximum AUC, IC50, IC90 and $Index_{SUM}$ values for irinotecan and SN38.

Table 3.6. Summary of sensitivity data (using an arbitrary threshold of sensitivity defined as a $Index_{SUM} < 300$ for six concentrations used).

Drug	No. sensitive in ATP-TCA	No. assessed	Sensitivity (%)
Irinotecan	27	58	47
SN38	0	15	0

Table 3.7. Median AUC, IC50, IC90 and $Index_{SUM}$ values (and ranges) for Irinotecan and SN38

Drug	AUC	IC50	IC90	$Index_{SUM}$
Irinotecan	14515 -931 - +17885	40 +5 - +981	118.5 +42 - +1765	310 +106 - +674
SN38	5166.5 -1698 - +19611	299 -44717 - +1132	482 -80491 - +2037	489.5 -7 - +678

Figure 3.5 shows the marked difference in median values of samples tested with equivalent doses of irinotecan and SN38 (p=0.0001, Wilcoxon signed rank test).

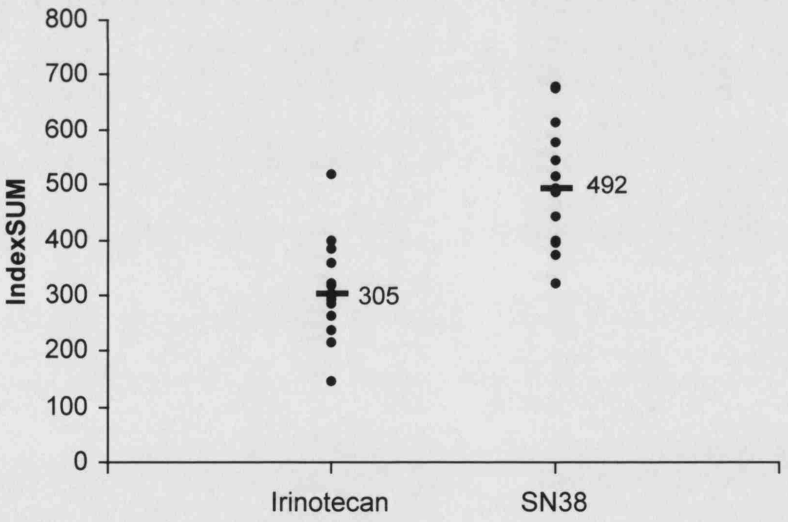


Figure 3.5. Sensitivity ($Index_{SUM}$) of samples tested with both irinotecan and SN38 at equivalent doses (n=15). Each dot represents an individual sample and the bars indicate the median values. An obvious difference in median values can be seen, which is confirmed by the Wilcoxon signed rank test (p=0.0001)

3.2.4 Discussion

From our results, single agent irinotecan showed activity in 47% of samples tested, but SN38 produced very little growth inhibition in any. These results echo those found by Jonsson *et al.* (2000) who demonstrated almost identical activity of irinotecan and SN38 on cell lines but lower SN38 sensitivity when tested *ex vivo* on colorectal cancer cells.

Irinotecan, a water-soluble semisynthetic derivative of the plant alkaloid camptothecin, is converted to its active metabolite SN38 by carboyesterases, which are abundant in plasma, liver and tumour cells. SN38 is eliminated through conjugation by hepatic uridine glucuronosyl-transferase into SN38 glucuronide (SN38G), which is actively secreted into the bile by a canalicular-multispecific organic anion transporter. SN38G is 100-fold less active than SN38. Other metabolites of irinotecan have been identified, the most important of which is the aminopentane carboxylic acid metabolite (APC) produced by CYP3A4-mediated metabolism. However, this metabolite has shown very limited antiproliferative properties and is 100-fold less active than SN38 (Rivory *et al.*, 1996; Slatter *et al.*, 2000).

Some investigators have found a correlation between the activity of irinotecan and the amount of carboxyesterase activity in tumour cells (Kanzawa *et al.*, 1990; Chen *et al.*, 1994), although others found a low level of enzyme activity in tumour tissue suggesting a minor role in the efficacy of irinotecan (Jansen *et al.*, 1997). In addition, it has been found that irinotecan is more selective for solid tumours than SN38, and that irinotecan is more active *in vivo* than SN38, which indicates there may be other mechanisms for the antitumour activity of irinotecan other than being a prodrug for SN38 (Kawato *et al.*, 1991; Bissery *et al.*, 1997). In humans, the AUC (area under the concentration-time curve) ratio of SN38 to irinotecan is approximately 2-4% (Rowinsky *et al.*, 1994), whilst in mice the ratio seems to be much higher, suggesting a higher esterase activity in the mouse (Zamboni *et al.*, 1998).

The hCE-2 isoform of carboxylesterase has been found to be a higher-affinity, higher-velocity enzyme for irinotecan hydrolysis compared to hCE-1 (Wu *et al.*, 2002). There are at least four different enzymes/isoforms found in human liver known to convert irinotecan to SN38. Conversion of irinotecan to its active metabolite may result from a combination of these isoforms or from others not yet identified. To further improve the therapeutic index of irinotecan, enzyme prodrug combinations of irinotecan and hCE-1 or purified rabbit carboxylesterase are being developed (Danks *et al.*, 1998). In addition, prodrug/gene therapy with hCE-2 may be clinically useful. However, these therapies have not been developed for clinical use at present.

The ATP-binding cassette (ABC) transporter proteins, including P-glycoprotein (P-gp) and the multidrug resistance protein (MRP) family, have been implicated in the transport and resistance to SN38. Similarly, overexpression of BCRP produces SN38 resistance by efflux transport of SN38 and/or SN38G out of the cells (Kawabata *et al.*, 2001).

These results, and the findings of Jonsson *et al.* question the choice of model for testing drugs preclinically. As significant differences in sensitivity for the same drug have been found between cell lines, mouse models and *ex vivo* human tumour cells, results from the first two models may not always be relevant to human tumours. This is now a well established theory, thus proving the need for assays which use fresh human tumour cells, such as the ATP-TCA.

In light of these results SN38 was removed from the list of drugs tested against colorectal adenocarcinoma in the ATP-TCA, and irinotecan was used instead.

CHAPTER 4

Colorectal Cancer ATP-Tumour Chemosensitivity Results

4.1 Introduction

As stated in chapter 1, the ATP-TCA has been used to investigate the *ex vivo* chemosensitivity of a number of different tumour types (Hunter *et al.*, 1993; Kurbacher *et al.*, 1996; Myatt *et al.*, 1997; Cree *et al.*, 1999; Mercer *et al.*, 2003), as well as to direct chemotherapy or predict response to treatment (Andreotti *et al.*, 1995; Cree *et al.*, 1996, Kurbacher *et al.*, 1998, Konecny *et al.*, 2000; Sharma *et al.*, 2003). Following the preliminary work on antibiotic validation (see chapter 3), the ATP-TCA has been successfully performed on CRC samples for the first time.

4.2 Materials and Methods

The colorectal cancer samples were tested using the ATP-TCA, following the method outlined in Chapter 2.

4.3 Results

4.3.1 Tumour Specimens

A total of 71 specimens were studied between January 2002 and April 2003: 69 were from patients undergoing resection of their primary colorectal adenocarcinoma (of all pathological stages) and two were malignant aspirates in patients with metastatic disease. Of the solid tumours, 48 were colonic, 20 were rectal and one was a peritoneal biopsy. The mean age of the patients was 69 years (range 39-88). 43 patients were male (61%) and 28 female (39%). There was a significant difference in the distribution of rectal cancer – 85% of samples were from men ($p=0.0137$, Fisher's exact test). Of the rectal cancers, two patients had received neoadjuvant chemoradiotherapy. Table 4.1 summarises the patient characteristics, and the full patient data can be found in Appendix B.

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Table 4.1. Summary of patient characteristics

	Number tested	Mean age (range) years	Sample Site			
			Colon	Rectum	Peritoneal biopsy	Malignant aspirate
Male	43 (61%)	67.8 (52-79)	25 (52%)	17 (85%)	0 (0%)	1 (50%)
Female	28 (39%)	70.8 (39-88)	23 (48%)	3 (15%)	1 (100%)	1 (50%)
Total	71	69 (39-88)	48	20	1	2

Sample site expressed as percentage by sex of total

4.3.2 Specimens and Evaluability

The evaluability rate (i.e. the number of tumours with interpretable results) of colorectal specimens was 82% (58/71). Of the 13 that were non-evaluable, eight were due to infection and five from lack of yield of cells after digestion of the tumour material. Most specimens were large and it was possible to extract enough cells for testing with a large number of cytotoxics. However, nine samples yielded only enough cells for one plate, in which were tested the four drugs/combinations most commonly used clinically in the UK. The two rectal samples that had undergone neoadjuvant chemoradiotherapy were also small and very fibrotic resulting in few cells available for testing. Further patients who had already undergone pre-operative radiotherapy were excluded from entering the study.

4.3.3 Heterogeneity of Chemosensitivity of Colorectal Cancer

For comparison between drugs and tumours, an $Index_{SUM}$ of <300 representing an average 50% inhibition across all concentrations tested, was used to indicate sensitivity, as previously published (Cree *et al.*, 1999; Neale *et al.*, 1999). The samples were tested using the drug list shown in table 2.2. For samples with a small

yield of cells only the first plate was tested, and therefore the number of samples tested with each drug or combination vary.

The results show considerable heterogeneity of chemosensitivity to single agents and drug combinations between the tumours tested. A summary of the data including the number of samples tested with each drug, the number and percentage showing sensitivity, and the number and percentage achieving >90% inhibition is shown in table 4.2. Table 4.3 summarises the median values (and ranges) of area under the curve (AUC), IC50, IC90 and $Index_{SUM}$ for each drug and combination.

Table 4.2. Summary of sensitivity data (using an arbitrary threshold of sensitivity defined as a TCA $Index_{SUM} < 300$ for six concentrations used)

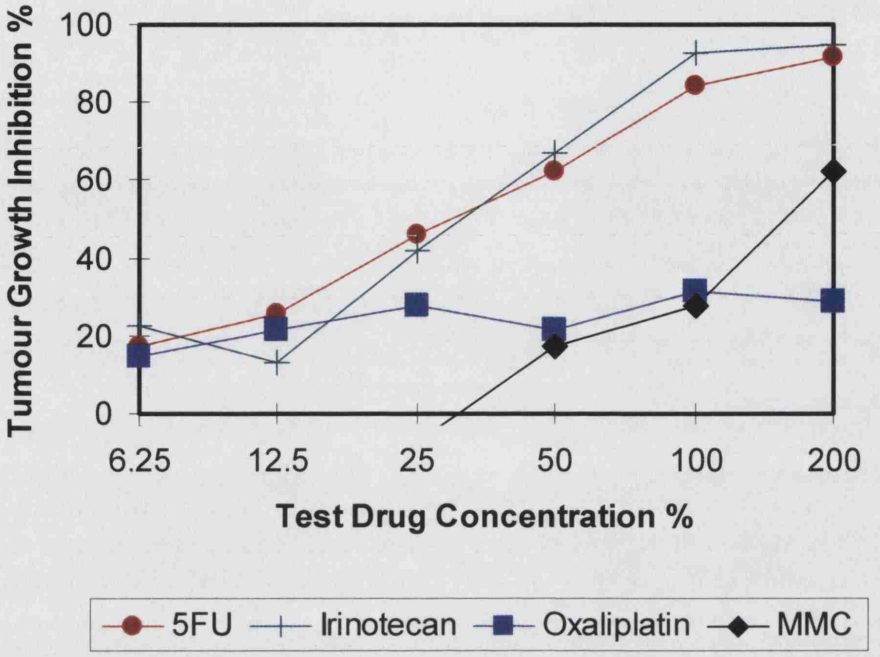
Drug	**No. sensitive in ATP-TCA**	**No. assessed**	**Sensitivity (%)**	**No. reaching 90% inhibition**
5-FU	33	58	57	8 (14%)
Irinotecan	27	58	47	25 (43%)
Oxaliplatin	1	53	2	0 (0%)
Mitomycin C	25	48	52	10 (21%)
Gemcitabine	21	48	44	4 (8%)
SN38	0	11	0	0 (0%)
5-FU + Oxaliplatin	44	54	81	22 (41%)
5-FU + Irinotecan	22	26	85	23 (89%)
5-FU + MMC	36	48	75	30 (63%)
MMC + Gemcitabine	45	46	98	38 (83%)
Oxaliplatin + Gemcitabine	31	41	76	12 (29%)

Table 4.3. Median AUC, IC50, IC90 and $Index_{SUM}$ values (and ranges) for each drug and combination

Drug	AUC	IC50	IC90	$Index_{SUM}$
5-FU	13999 +8751 - +17788	35 +4 - +107	188 +45 - +251	290.5 +94 - +528
Irinotecan	14515 -931 - +17885	40 +5 - +981	118.5 +42 - +1765	310 +106 - +674
Oxaliplatin	2977 -5693 - +12702	304 -8104 - +41192	546 -14588 - +74145	524 +272 - +862
Mitomycin C	13650 +4711 - +18102	43 +5 - +223	45 +187 - +402	298.5 +115 - +543
Gemcitabine	11337 +193 - +18145	33 +4 - +345	280 +31 - +2500	329.5 +78 - +651
Oxaliplatin + 5-FU	15303 +10440 - +17910	23 +5 - +78	130 +48 - +231	236 +92 - +403
Irinotecan + 5-FU	17029.5 +10876 - +17879	14.5 +6 - +82	65.5 +45 - +201	179.5 +127 - +414
MMC + 5-FU	15894.5 -10437 - +18099	23 -1223 - +181	88.5 -2201 - +304	242.5 +104 - +814
MMC + Gemcitabine	17471 +10829 - +19277	9 +3 - +53	49 +6 - +262	139 +8 - +327
Oxaliplatin + Gemcitabine	14863 +7442 - +19092	17.5 +4 - +116	178 +12 - +1313	239 +40 - +508

(i) Single agents

Figure 4.1 (a-d) shows examples of inhibition curves from CRC samples tested with single agents. A wide range of responses to single agents was demonstrated, with all single agents tested, except oxaliplatin and SN38, classified as active in about 50% of samples (44-57%) based on the $Index_{SUM}$ <300 threshold. The most active single agent tested was 5-fluorouracil (5-FU), to which 57% of samples were sensitive. However, only 14% achieved >90% growth inhibition. Of the 27 samples (47%) that showed sensitivity to irinotecan, nearly all (25/27) achieved >90% inhibition.



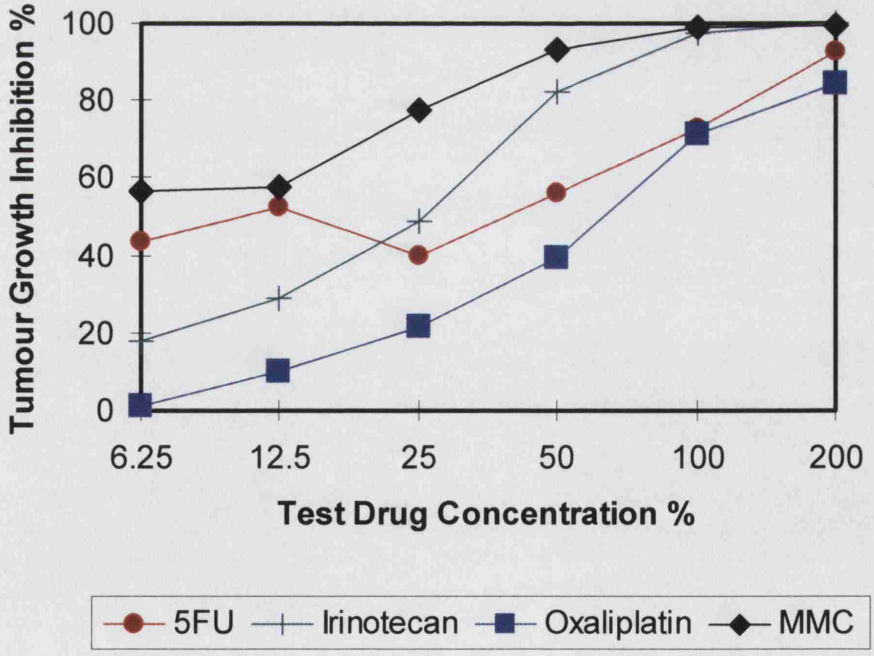
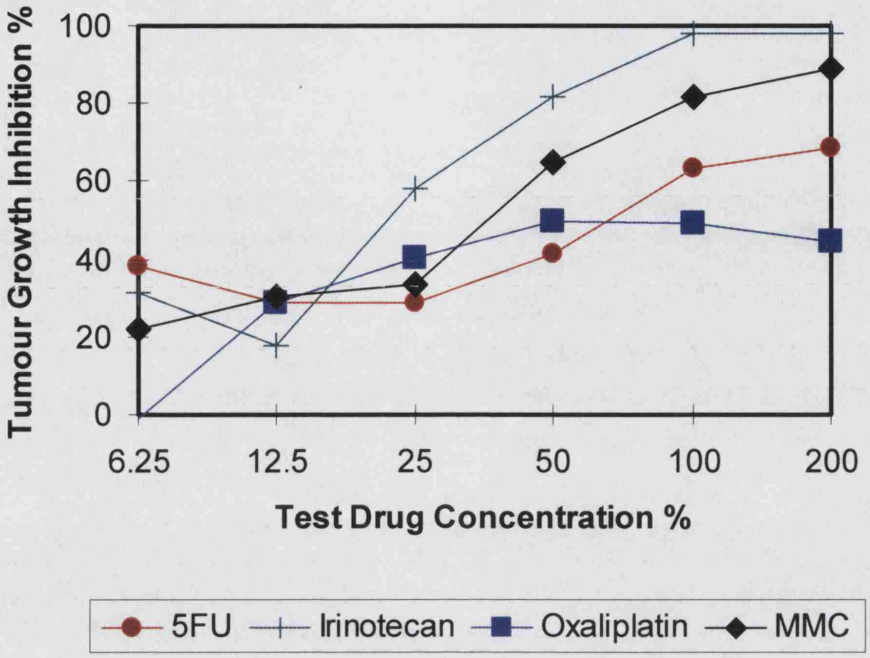


Figure 4.1. (a-b) Examples of ATP-TCA growth inhibition curves showing heterogeneity of chemosensitivity to single agent 5FU, irinotecan, MMC and oxaliplatin. Further variation in sensitivity is seen in the examples shown in figure 4.1 (c-d).



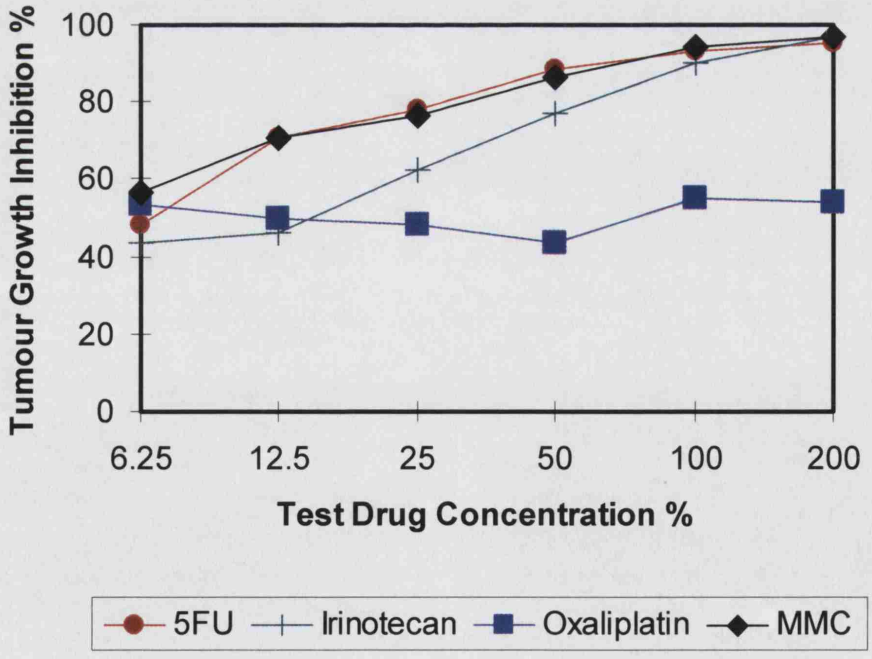


Figure 4.1. (c-d) Examples of ATP-TCA growth inhibition curves showing heterogeneity of chemosensitivity to single agent 5FU, irinotecan, MMC and oxaliplatin. Further variation in sensitivity is seen in the examples shown in figure 4.1 (a-b).

Gemcitabine generally showed a shallow dose response curve with little difference in growth inhibition between 6.25% TDC and 200% TDC (2.5-80μM). This is reflected in only 8% (4/48) achieving >90% growth inhibition. There was however a lot of variation between tumours, from very little effect to almost total inhibition (figure 4.2). Only two samples were sensitive to oxaliplatin as a single agent, and none of the samples were sensitive to SN38, which was dropped from our panel of agents (see technical development, chapter 3).

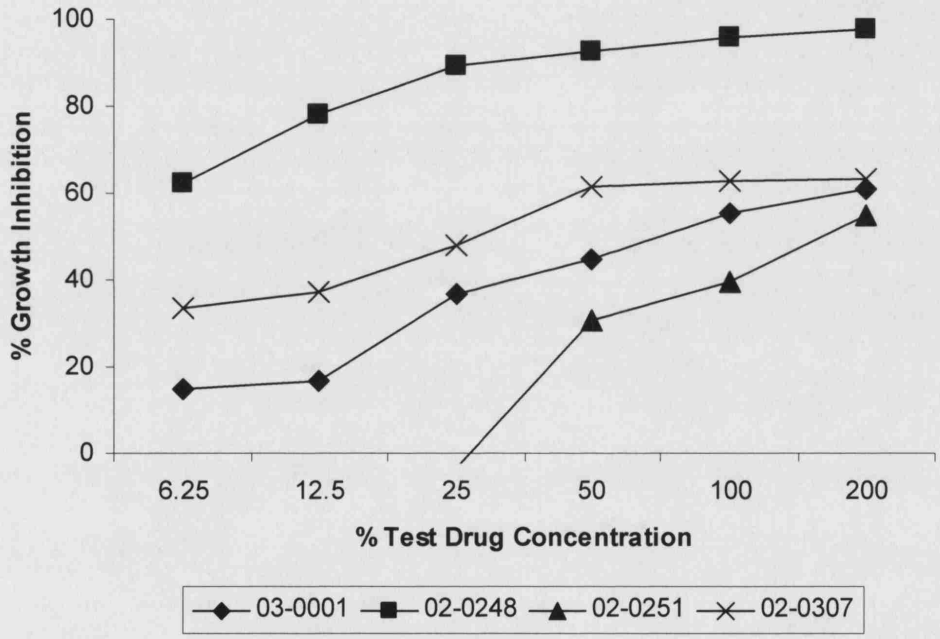


Figure 4.2. Growth inhibition curves for gemcitabine from 4 different tumour samples. There is wide variation in sensitivities achieved, but all curves are shallow.

The $Index_{SUM}$ results have been separated into categories for each of the five single agents, and used to draw histograms showing the distribution of the $Index_{SUM}$ in the CRC samples (figure 4.3). The histograms for 5-FU, gemcitabine and MMC are skewed to the lower values, with irinotecan spread across the range of $Index_{SUM}$ values. The histogram for oxaliplatin is skewed to the higher values suggesting ineffectiveness as a single agent in CRC, as is known from clinical data.

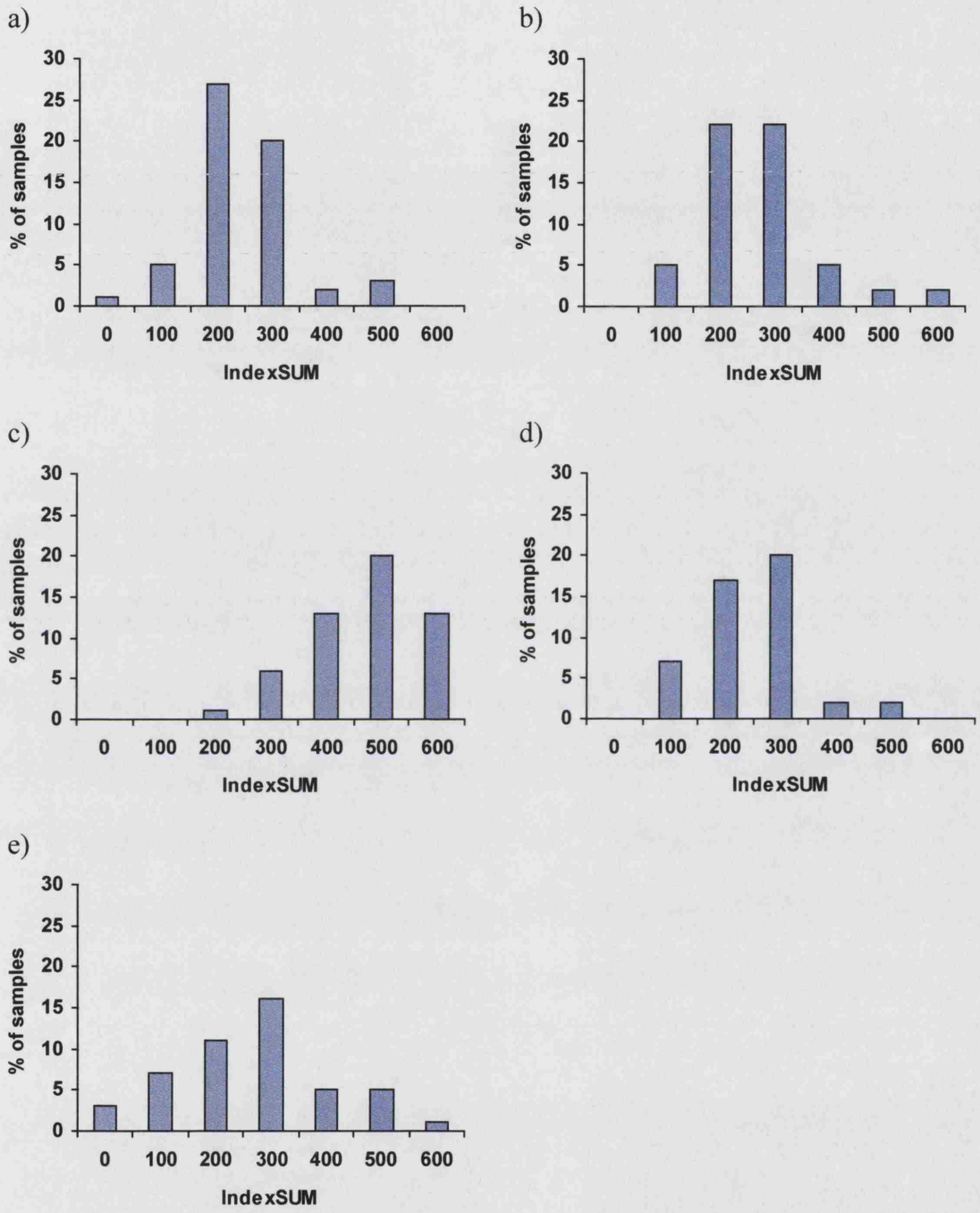


Figure 4.3. Histograms demonstrating the heterogeneity of chemosensitivity of single agent a) 5FU, b) irinotecan, c) oxaliplatin, d) MMC, and e) gemcitabine. The $Index_{SUM}$ is represented on the x axis (<300 indicating sensitivity) and the percentage of samples tested with that particular agent on the y axis.

These histograms further highlight the extent of variation in the *ex vivo* sensitivity shown by the colorectal cancer samples.

(ii) Drug combinations

The drug combinations 5-FU + oxaliplatin, 5-FU + irinotecan and 5-FU + MMC were tested as well as the experimental combinations MMC + gemcitabine and oxaliplatin + gemcitabine. Examples of growth inhibition curves for the combinations tested are shown in figure 4.4.

All drug combinations achieved greater growth inhibition than drugs tested as single agents (75-98% using $Index_{SUM}$ <300 threshold). The combination showing the most sensitivity was mitomycin C + gemcitabine, with all but one sample (45/46; 98%) showing sensitivity. This combination achieved >90% inhibition in 83% of samples (38/46) and was the most effective combination in 78% of the tumours tested (36/46). The combination of oxaliplatin + gemcitabine appeared active in 76% of samples (31/41) but only 29% achieved >90% inhibition. Similarly, 5-FU + oxaliplatin showed activity in 81% (44/54) with only 41% reaching >90% inhibition (22/54).

The $Index_{SUM}$ results obtained from the combinations tested have been drawn as histograms as shown previously for the single agents. The histograms for the combinations, shown in figure 4.5, are all skewed to a lower $Index_{SUM}$ indicating generally greater sensitivity to combination rather than single agent treatment. However, there are some outlying samples with a higher $Index_{SUM}$, consistent with heterogeneity of chemosensitivity to combinations as well as single agents.

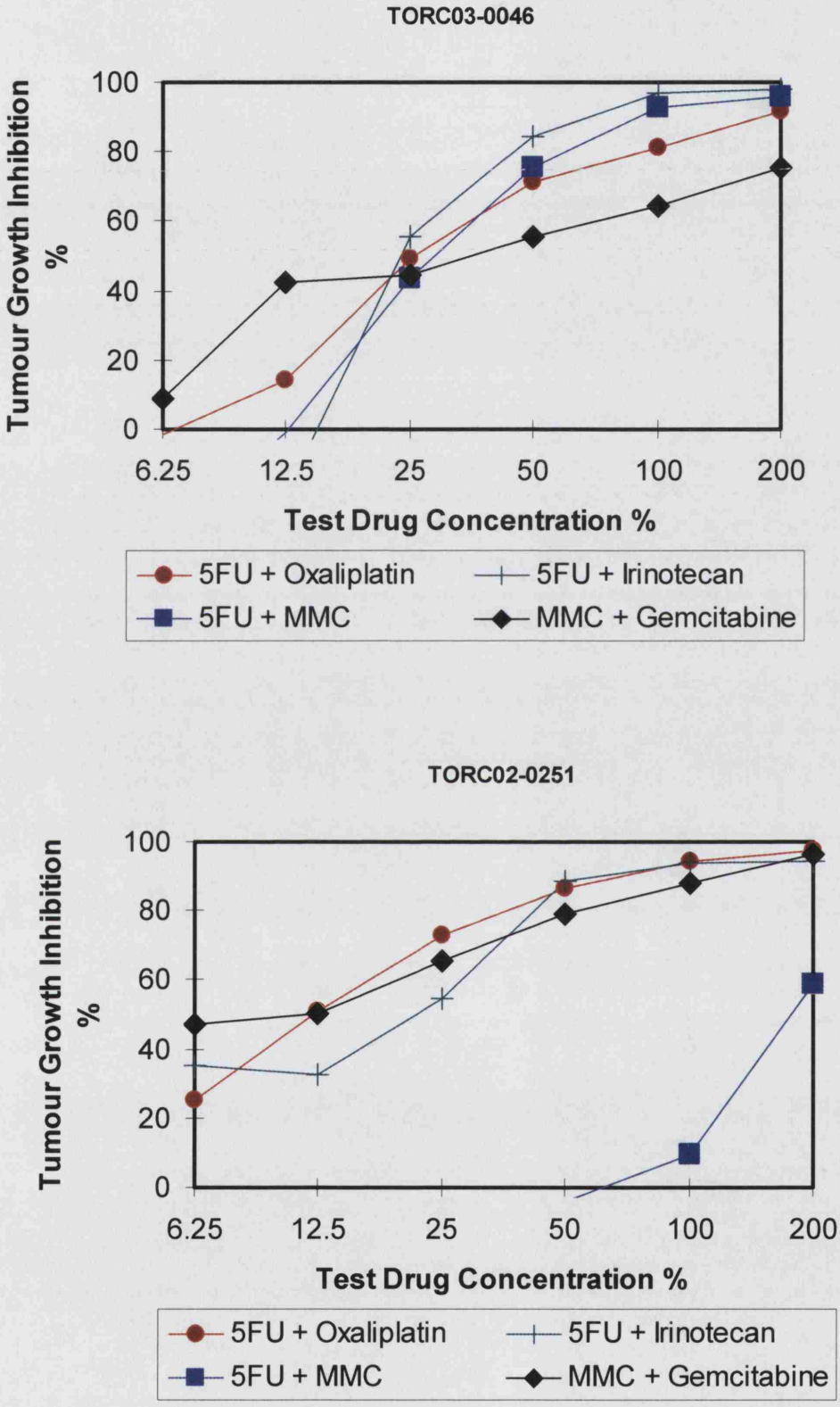


Figure 4.4. Examples of ATP-TCA growth inhibition curves showing heterogeneity of chemosensitivity to combinations of cytotoxic agents.

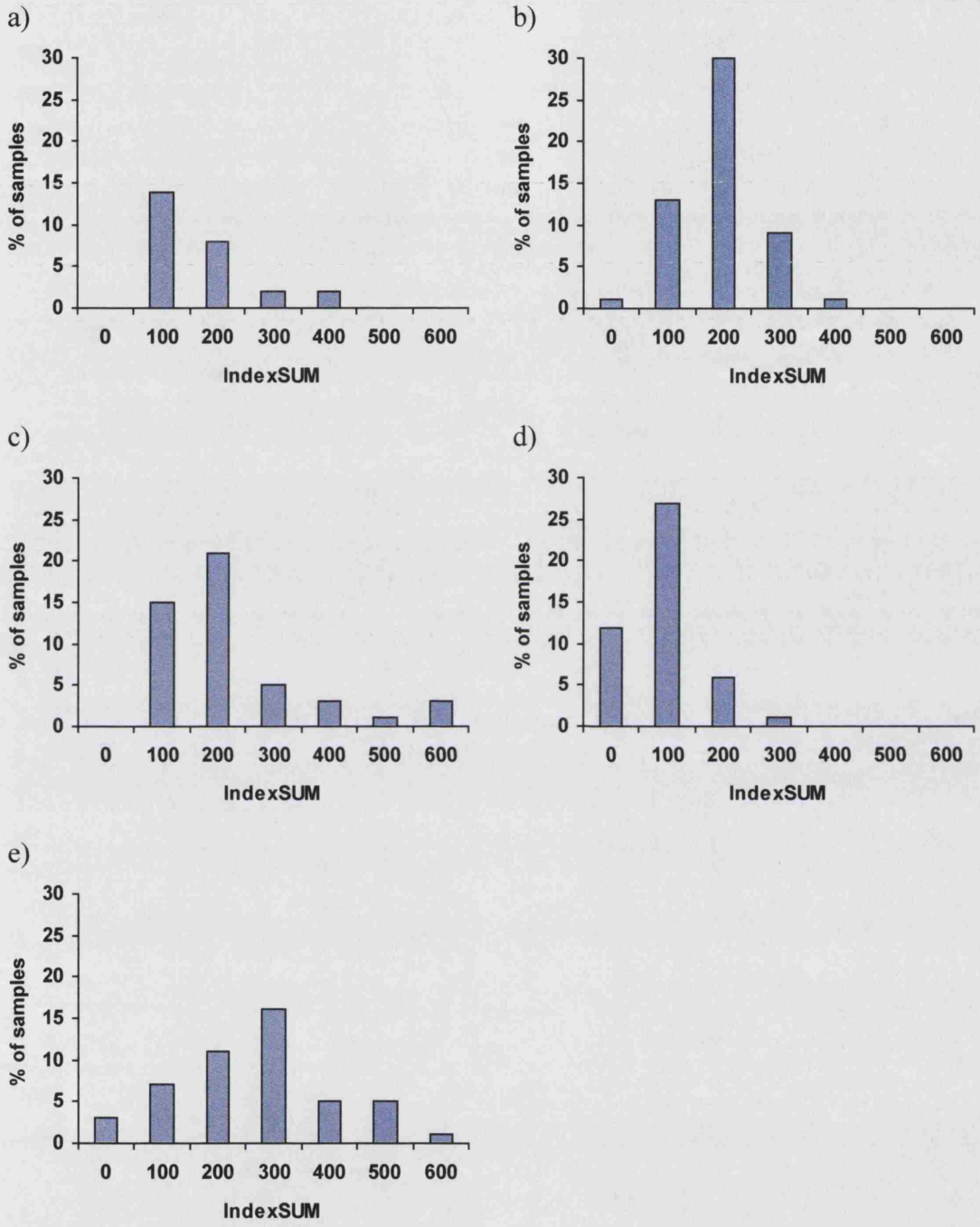


Figure 4.5. Histograms demonstrating the heterogeneity of chemosensitivity to combinations of cytotoxic drugs. a) 5FU + irinotecan, b) 5FU + oxaliplatin, c) 5FU + MMC, d) MMC + gemcitabine, and e) oxaliplatin + gemcitabine. The x axis represents the $Index_{SUM}$ (<300 indicating sensitivity) and y axis the % of samples tested with that combination.

4.4 Multi-drug cross-sensitivity / Multidrug Resistance

Whilst some tumours responded well to particular single agents or combinations, others showed no response to these drugs and instead responded to an alternative regimen. Five cases were sensitive to only one drug/combination and resistant to all the others tested. Of these five, two were sensitive only to 5-FU, one to 5-FU + irinotecan, one to 5-FU + oxaliplatin and one to 5-FU + MMC. Two cases were resistant to all single agents and combinations tested (4%).

Of the 48 samples tested with the three single agents 5-FU, irinotecan and MMC, 11 (23%) were resistant to all three single agents. Another 11 (23%) were only sensitive to one of the agents, and 9 (19%) were sensitive to all three single agents. The $Index_{SUM}$ results have been used to draw a Venn diagram illustrating the cross-sensitivity between single agents 5-FU, irinotecan and MMC, all of which have different mechanisms of action (figure 4.6).

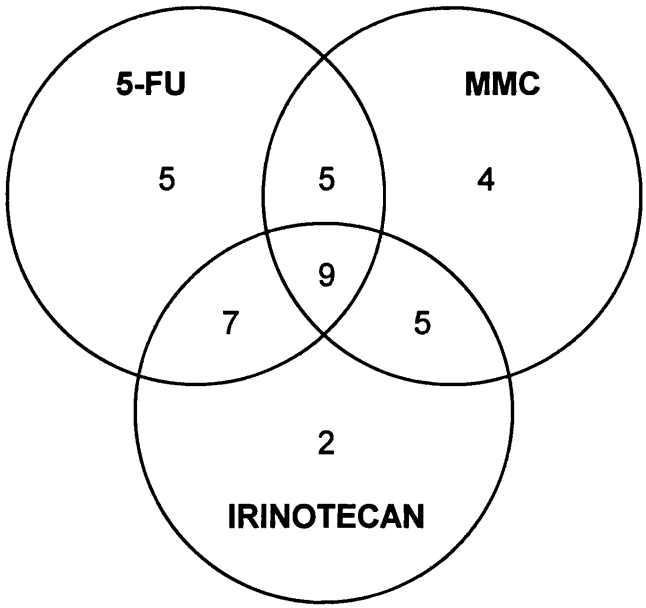


Figure 4.6. Venn diagram showing cross-sensitivity ($Index_{SUM}$ <300) of colorectal cancer samples to the 3 single agents 5-FU, irinotecan and MMC (n=48). 11/48 were not sensitive to any of these single agents, however, 9 of the 11 were sensitive to at least one drug combination.

4.5 Results for Celecoxib (Cyclo-oxygenase-2 Inhibitor)

Cyclo-oxygenase-2 (COX-2) expression is up-regulated in 80% of colorectal cancers and is thus a potential therapeutic target. Clinical trials of COX-2 inhibitors are in progress, both as treatment and prophylaxis.

26 samples were tested with both 5-FU and Celecoxib as single agents, and with the combination of the two drugs (table 4.4). 5-FU showed activity ($Index_{SUM}$ <300) in 50% of samples. Celecoxib alone was ineffective in all of the samples, and the combination demonstrated activity in 62% (table 4.5).

Table 4.4. Median AUC, IC50, IC90 and $Index_{SUM}$ values (and ranges) for the 26 samples tested both with 5-FU, Celecoxib and the combination of the two drugs

	AUC	**IC50 (μM)**	**IC90 (μM)**	**$Index_{SUM}$**
Celecoxib	-1005.5 -16086 - +9237	-271.5 -12647 - +879	-488.5 -22764 - +1582	628 +307 - +1139
5-FU	13949 +5909 - +17510	121.07 +21 - +370	639.92 +190 - +841	303.5 +116 - +490
5-FU + Celecoxib	14307 +1826 - +16695	107.23 +17 - +356	683.15 +301 - +2072	259 +140 - +577

Table 4.5. Sensitivity data for subset of samples tested with celecoxib, 5-FU and the combination of the two drugs

Drug	**No. sensitive in ATP-TCA**	**No. assessed**	**Sensitivity (%)**
Celecoxib	0	26	0
5-FU	13	26	50
Celecoxib + 5-FU	16	26	62

There was no statistical difference in IC50, IC90 and $Index_{SUM}$ between 5-FU tested alone or in combination with celecoxib, and no general trends noted. The IC50 of 5-

FU decreased in 12/23 samples with the addition of celecoxib (p=0.6221, Mann Whitney U test; p=0.5530, Wilcoxon matched pairs test) (figure 4.7).

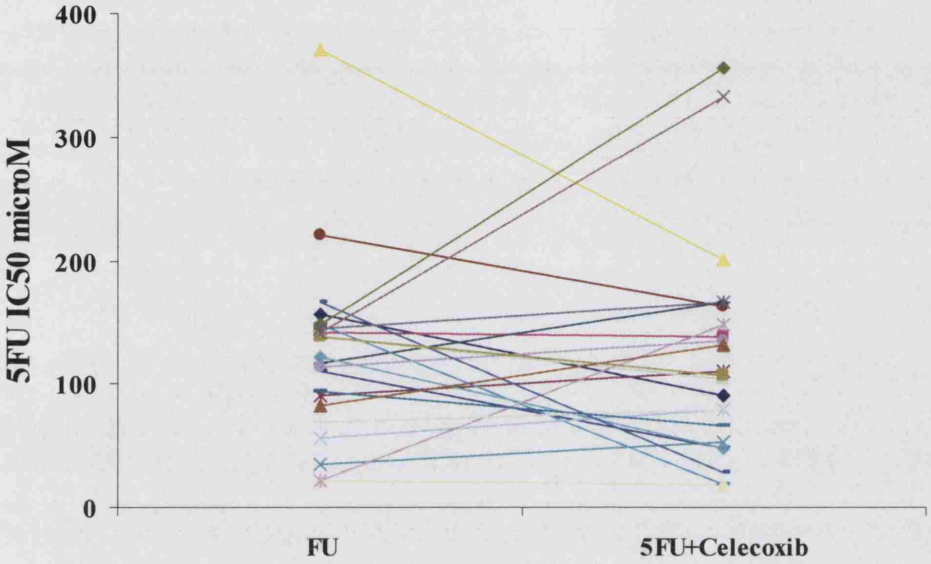


Figure 4.7. Paired data of 5-FU IC50 and the addition of celecoxib (N/S, Mann-Whitney U test and Wilcoxon matched pairs test).

Similar results were found for IC90 (p=0.7488, Mann Whitney; p=0.2134, Wilcoxon) (figure 4.8) and $Index_{SUM}$ (p=0.9205, Mann Whitney; p=0.5218, Wilcoxon) (figure 4.9). The $Index_{SUM}$ was lowered in 13/26 samples after the addition of celecoxib (i.e. showing greater sensitivity).

The combination of drugs for individual samples was assessed using the method described by Poch *et al.* (1995). Considerable heterogeneity between samples was found. Examples of ATP-TCA growth inhibition curves and Poch combination graphs are seen in figure 4.10a-c. These demonstrate the importance of the area under the curve (IndexAUC) in addition to $Index_{SUM}$ and 90% inhibition values.

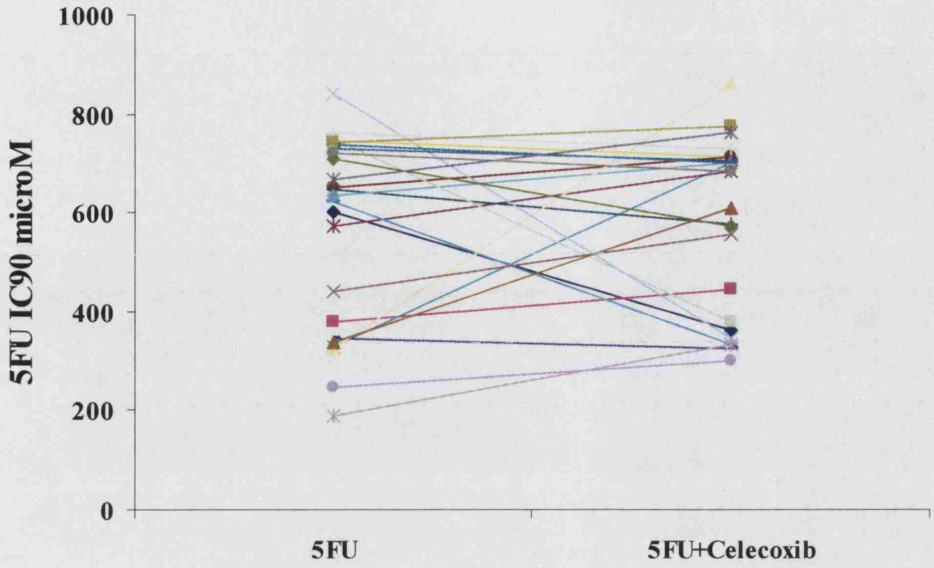


Figure 4.8. Paired data of 5FU IC90 and the addition of celecoxib (N/S, Mann-Whitney U test and Wilcoxon matched pairs test).

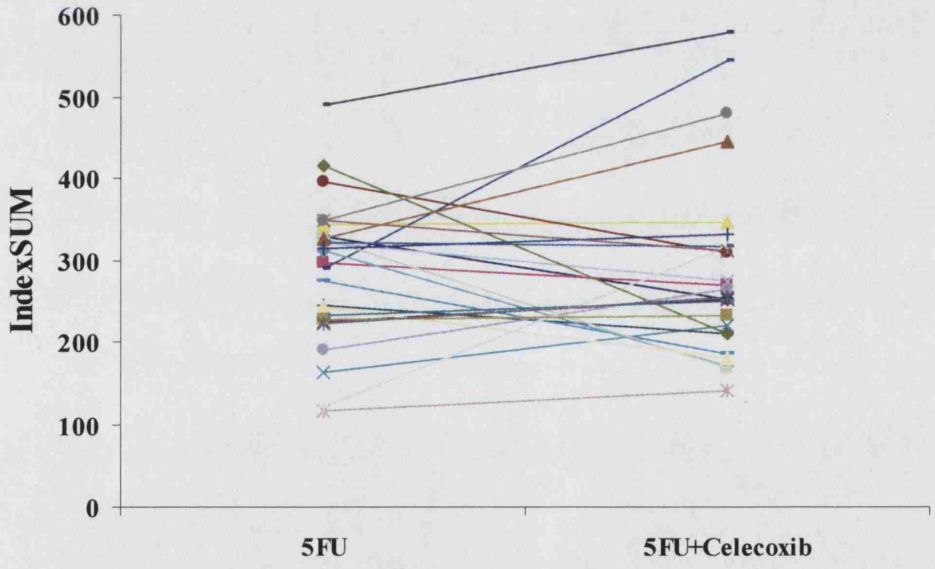
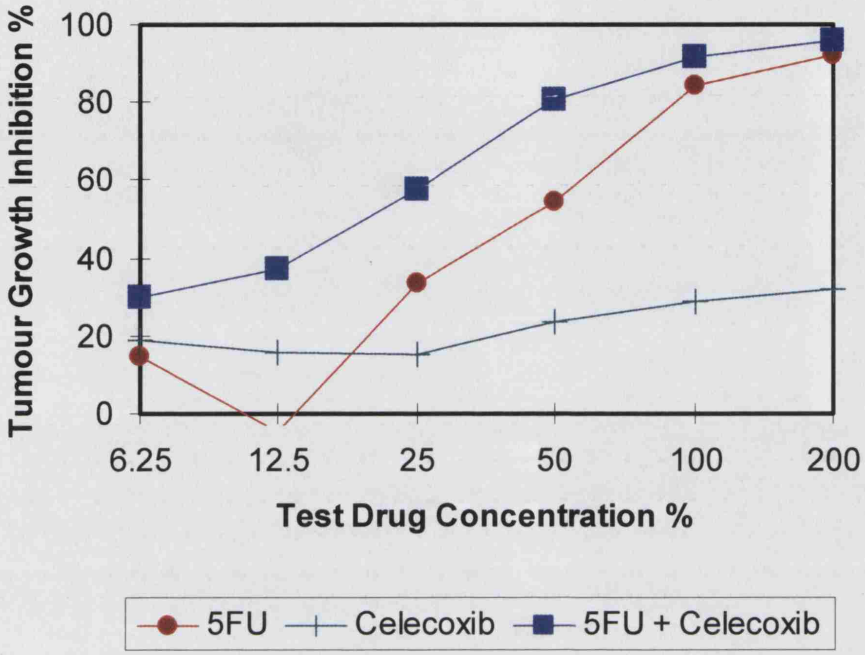


Figure 4.9. Paired data of 5FU $Index_{SUM}$ and the addition of celecoxib (N/S, Mann-Whitney U test and Wilcoxon matched pairs test).



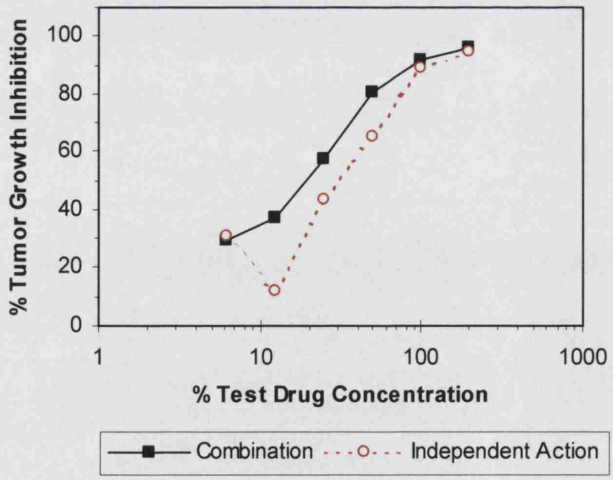
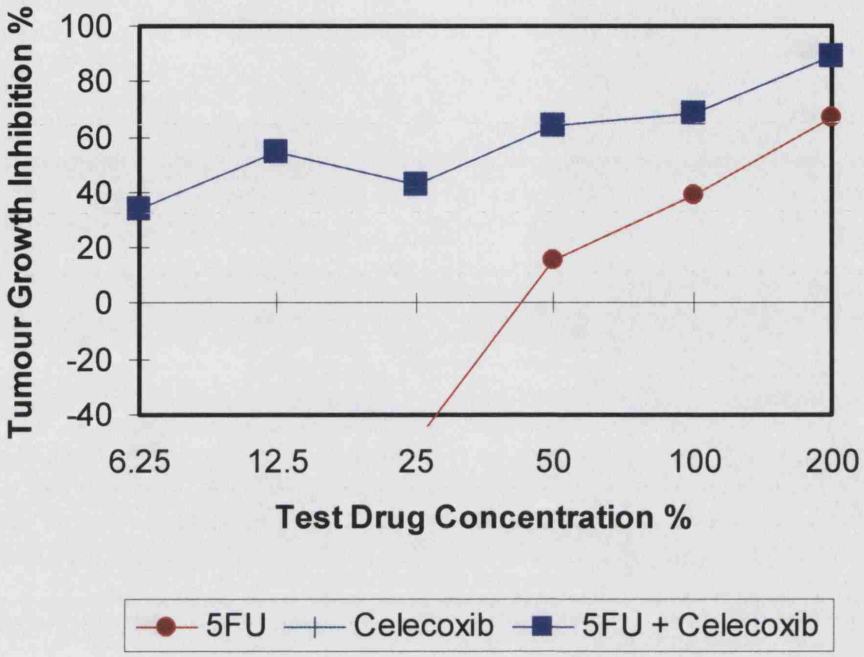


Figure 4.10a. ATP-TCA growth inhibition curve showing greater growth inhibition by combination 5FU + celecoxib compared to single agents. Confirmed by the synergistic effect seen on the Poch graph where the continuous line is above and to the left of the dotted line, which indicates the effect expected from addition of the two agents.



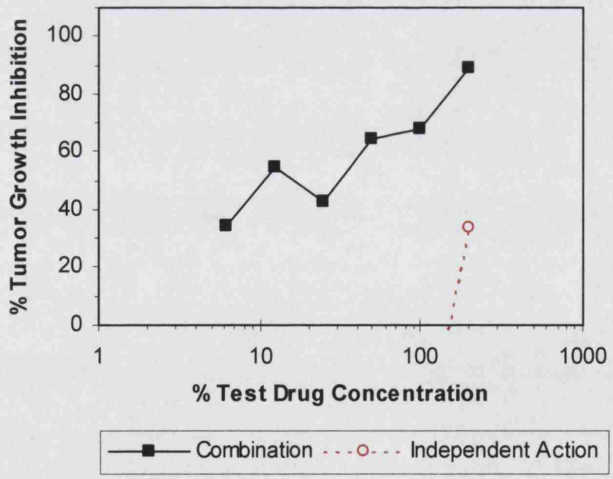
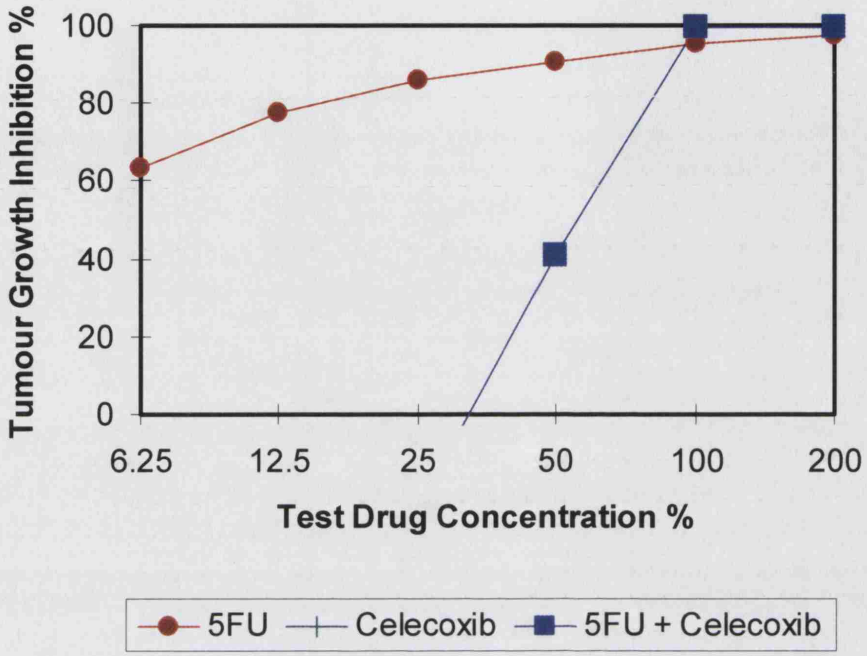


Figure 4.10b In this example, there is no growth inhibition effect by celecoxib: in fact it may even have a positive effect on cell growth as it shows negative growth inhibition values. However, the combination of 5FU + celecoxib is shown to be much more active than 5FU alone. The Poch graph shows the combination to be much more active than that expected by the additive effect of the two drugs.



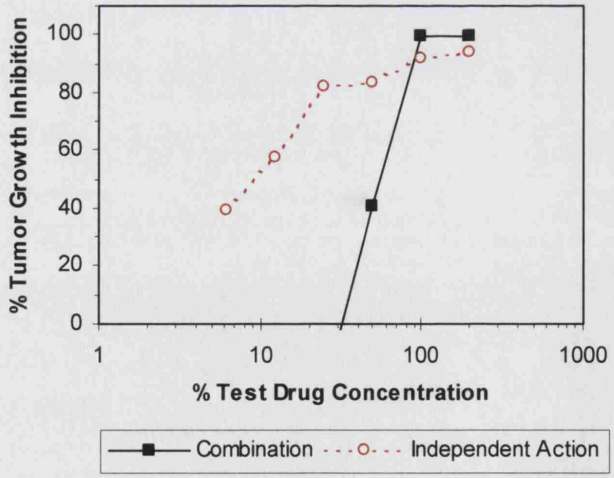


Figure 4.10c. In this example 5FU is very active as a single agent, and celecoxib not at all. Although the combination reaches >90% inhibition at 100% TDC, the area under the curve is much less than that for 5FU alone. On the Poch graph, the continuous line is below and to the right of the dotted line, indicating that the combination has achieved less growth inhibition than that expected by the addition of the two drugs.

4.6 Discussion

4.6.1 Tumour Specimens and Evaluability

The evaluability of colorectal samples using the ATP-TCA was 82% which is similar to evaluability rates achieved in other "cleaner" tumour types using this assay (Andreotti *et al.*, 1995; Cree *et al.*, 1999; Neale *et al.*, 1999). Other *in vitro* studies of colorectal cancer cells, including the use of the MTT assay and histoculture drug response assay, have produced similar evaluability rates (Furukawa *et al.*, 1995; Araki *et al.*, 1999). The ATP-Tumour Chemosensitivity Assay has been shown to be more sensitive than these assays and to have technical advantages over the MTT and clonogenic assays (Cree *et al.*, 1995; Petty *et al.*, 1995).

In general, the colorectal samples were quite fibrous and therefore did not dissociate in the enzymatic solution particularly well. It was necessary to increase the concentration of collagenase from 1 mg/ml to 1.5 mg/ml for better tumour dissociation. In five samples there were no live tumour cells after dissociation, and nine samples only yielded enough cells for one test plate. There are two reasons for lack of yield of cells. In the smaller specimens only a small sample was resected to avoid interference with histopathological margins, and thus few cells were obtained. Some of the larger specimens only yielded a small number of cells because of the fibrotic nature of the sample. Two rectal samples obtained early in the study had undergone preoperative chemoradiotherapy. Irradiation of rectal tumours may produce so much shrinkage that little or no tumour remains macroscopically, and what is left may be very fibrotic. It was therefore decided to exclude any further patients who had undergone radiation therapy from the study.

4.6.2 *Ex vivo* Chemosensitivity

None of the samples were from patients who had received neoadjuvant chemotherapy, apart from the two rectal cancers that had undergone neoadjuvant chemoradiotherapy. The results show the marked heterogeneity of chemosensitivity of colorectal cancer to both single agent and combinations of cytotoxic drugs. This is

in keeping with the heterogeneity seen in other tumour types (Kurbacher *et al.*, 1996; Cree *et al.*, 1999; Mercer *et al.*, 2003) and clinical heterogeneity in response to treatment.

(i) Single Agents

All the single agents tested, with the exception of oxaliplatin, show a range of *ex vivo* responses. Only 2% of the samples tested with oxaliplatin had an $Index_{SUM}$ value below 300, the threshold used to indicate sensitivity in the assay. This is in keeping with clinical experience, as single agent oxaliplatin has a low objective response rate of 10-18% (Machover *et al.*, 1996; Bécouran *et al.*, 1998; Diaz-Rubio *et al.*, 1998).

Gemcitabine showed activity in 44% of tumours tested. It is licensed for use in pancreatic and non-small-cell lung cancers, and has also shown preclinical and clinical activity in several other solid tumours, including ovarian, head and neck and breast cancers (Carmichael *et al.*, 1995; Markman, 2002). However, phase I/II trials of single-agent gemcitabine have not demonstrated any activity in advanced colorectal and gastric cancers (Moore *et al.*, 1992; Christman *et al.*, 1994; Mani *et al.*, 1998).

Mitomycin C (MMC) has been used in the treatment of gastrointestinal tumours for many years. As a single agent it has produced response rates of up to 23% in colorectal cancer (Moertel *et al.*, 1968; Moore *et al.*, 1968). In the ATP-TCA 52% of samples appeared sensitive to single agent MMC.

Single agent 5-FU and irinotecan showed activity in 57% and 47% of samples respectively. Both these agents have been shown to improve response rate and overall survival in colorectal cancer. For the past 50 years 5-FU has been the main chemotherapeutic agent used in colorectal cancer, and has increased survival. As a single agent, irinotecan has been found to improve survival after fluorouracil failure in patients with metastatic CRC (Cunningham *et al.*, 1998; Rougier *et al.*, 1998).

(ii) Drug Combinations

The proportion of samples that responded strongly to combinations was greater than the proportion that responded to single agents. For most samples showing sensitivity to combinations, the effect was greater than expected by the addition of the action of the drugs as single agents, i.e. supra-additive. It is possible that the cut-off point for sensitivity ($Index_{SUM}$ <300) is too high for drug combinations, and an $Index_{SUM}$ of <200 may be more suitable.

The combination of Gemcitabine + MMC (GeM) was the most active, with all but one sample showing sensitivity. This combination is not currently in clinical practice for treatment of colorectal cancer but has recently been used in a phase II trial in advanced pancreatic cancer (Tuinmann *et al.*, 2004). For comments on the combination of GeM see chapter 5 where this combination has been investigated in more detail.

5-FU + irinotecan is a drug combination that has clinically been shown to improve survival compared to 5-FU/FA alone (Douillard *et al.*, 2000; Saltz *et al.*, 2000), and is currently in practice, particularly in the USA. The mechanism of synergism remains unclear. However, 85% of samples showed sensitivity to this combination in the ATP-TCA.

The combination of MMC and 5-FU has shown synergistic growth inhibition of cell lines (Sartorelli and Booth, 1965), including colorectal cell lines (Russello *et al.*, 1989). Clinically the combination has increased response rates to 54% with improved survival at 2 years (Ross *et al.*, 1997; Price *et al.*, 1999).

Combinations of oxaliplatin with 5-FU and gemcitabine were investigated. These combinations demonstrated sensitivity in 81% and 76% of samples respectively. However, only 41% and 29% 90% inhibition rates were achieved for oxaliplatin + 5-FU and oxaliplatin + gemcitabine respectively.

As a single agent, oxaliplatin has a poor response rate, but combined with other cytotoxic agents it demonstrates an enhanced effect. Many clinical studies have

shown that oxaliplatin with 5-FU has a response rate benefit, particularly in the down-staging of metastatic liver disease (de Gramont *et al.*, 2000; Giacchetti *et al.*, 2000; Grothey *et al.*, 2002).

In the laboratory setting, oxaliplatin has been combined with a number of other drugs, with the additive effect often being sequence dependent, which may vary with drug combination. A synergistic effect has been found when gemcitabine is followed by oxaliplatin (Faivre *et al.*, 1999), whereas when combined with topoisomerase I inhibitors, the synergistic effect is seen when oxaliplatin is administered first (Goldwasser *et al.*, 1999; Zeghari-Squalli *et al.*, 1999).

4.6.5 Multi-drug resistance

Two samples were found to be resistant to all single agent and drug combinations tested. Five samples showed sensitivity to only one drug/combination. As most samples were resistant to single agent oxaliplatin, and gemcitabine has a very shallow dose response curve, the sensitivity to 5-FU, irinotecan, and MMC were compared in 48 samples. These drugs all act in different ways, as previously described in chapter 1. In this subgroup, 19% of samples were sensitive to all three agents, whereas 23% were resistant to all three, showing multi-drug resistance. Resistance is often multifactorial and several other mechanisms may be important, which may explain the heterogeneity of individual tumours.

4.6.6 Celecoxib

Cyclo-oxygenase-2 inhibitors are not cytotoxic agents, and celecoxib did not demonstrate any inhibition of cell growth in the assay. However, both COX-1 and COX-2 inhibitors have been found to inhibit cell survival of COX-2 expressing and COX-2 deficient cell lines, and to inhibit G0/G1 to S phase transition by decreasing the expression of cyclins and increasing the expression of the cell cycle inhibitory proteins p21 and p27, by mechanisms unrelated to COX-2 expression (Grösch *et al.*, 2001).

Chen *et al.* found that simultaneous exposure to 5-FU and etodalac (COX-2 inhibitor) only produced an additive effect, which became supra-additive when

administered sequentially (Chen *et al.*, 2004). They found similar results with the combination of SN38 and etodalac. There are two explanations for the differences found between our results and those of the group of Chen. The first is that they used colon cancer cell lines rather than tumour-derived cells, which show considerable heterogeneity of response to drugs. An additive, and indeed supra-additive, effect was seen with the combination in a number of individual samples, but due to the heterogeneity between samples this result was not seen with median figures. Second, we exposed the cells to both drugs at the same time. Thus sequential exposure may have demonstrated increased activity, which is a factor to consider in future work.

CHAPTER 5

Combination of Mitomycin C and Gemcitabine

5.1 Introduction

From the heterogeneity data (Chapter 4), the combination of MMC and gemcitabine was found to be the most sensitive combination tested, and therefore was investigated further.

5.2 Methods

The ATP-TCA was carried out as previously described in Chapter 2. 48 samples were tested with single agent MMC and gemcitabine and 46 samples were tested with the combination. Mitomycin C (Kyowa, London, UK) and gemcitabine (Eli Lilly, Basingstoke, UK) were obtained as vials for injection and made up according to the manufacturer's instructions. The 100% TDC of mitomycin C was 0.7 μg/ml (2.0 μM) and the 100% TDC of gemcitabine was 12.5 μg/ml (40 μM).

Data Analysis

Data from each assay were transferred directly from the luminometer to an Excel 2000 spreadsheet (Microsoft®) and the IC50, IC90, concentration-inhibition curve ($Index_{AUC}$) and sensitivity index ($Index_{SUM}$) were calculated, as well as the percentage of tumours achieving 90% inhibition.

Assessment of Combination Effects

The effect of drug combinations compared to the constituent single agents was analysed in the Excel spreadsheet using the method determined by Poch *et al.* (1995). This method has been shown to be better suited to the data produced by the ATP-TCA compared to methods such as Chou and Talalay (1984) (see chapter 2).

Time Schedule Experiments

In order to assess any schedule dependency the combination of MMC and gemcitabine was tested by adding the drugs at different time intervals as follows: (i) MMC + Gemcitabine at 0 hours, (ii) MMC 0 hours + Gemcitabine 6 hours, (iii)

MMC 0 hours + Gemcitabine 24 hours, and (iv) Gemcitabine 0 hours + MMC 24 hours.

In order to achieve a final concentration of 200% TDC in row B, higher concentrations of the drugs had to be used to take into account the dilution (table 5.1). A 96 well plate was then made up as in figure 5.1.

Table 5.1. Drug concentrations used for schedule experiments

Time (hrs)	Drug TDC	CAM (ml)	MMC (µl)	Gem (µl)
0	800% MMC + Gem	5	28	12
0	1200% MMC	2.5	21	
0	1200% Gem	5		18
6 and 24	1600% MMC + Gem	2.5	28	12

	1	2	3	4	5	6	7	8	9	10	11	12
A	100µl MI	100µl MI	100µl MI	50µl MI	50µl MI	50µl MI	50µl MI	50µl MI	50µl MI	50µl MI	50µl MI	50µl MI
B	**DRUG A**			**DRUG B**			**DRUG C**			**DRUG D**		
C	**MMC + Gem 0hrs**			**MMC 0hrs Gem 6hrs**			**MMC 0hrs Gem 24hrs**			**Gem 0hrs MMC 24hrs**		
D												
E	100µl CAM	100µl CAM	100µl CAM	50µl CAM	50µl CAM	50µl CAM	50µl CAM	50µl CAM	50µl CAM	50µl CAM	50µl CAM	50µl CAM
F	100µl CAM	100µl CAM	100µl CAM	50µl CAM	50µl CAM	50µl CAM	50µl CAM	50µl CAM	50µl CAM	50µl CAM	50µl CAM	50µl CAM
G	100µl CAM	100µl CAM	100µl CAM	50µl CAM	50µl CAM	50µl CAM	50µl CAM	50µl CAM	50µl CAM	50µl CAM	50µl CAM	50µl CAM
H	100µl MO	100µl MO	100µl MO	50µl MO	50µl MO	50µl MO	50µl MO	50µl MO	50µl MO	50µl MO	50µl MO	50µl MO

Figure 5.1. Plate layout for combination of mitomycin C and gemcitabine schedule experiment

Drug A at 0 hours

Columns 1-3 were made up as in the normal TCA. 100 µl MI was added to row A, and 100 µl CAM to rows B-H. 100 µl of 800% TDC of MMC and Gemcitabine was added to row B and double diluted to row G. 100 µl cell suspension was added to rows A-H.

Drugs B, C and D at 0 hours

50 µl of MI was added to row A and 50 µl of CAM to rows B-H of columns 4-12. 50 µl of 1200% Mitomycin C or Gemcitabine (whichever drug added at 0 hours) was added to row B and double diluted to row G. 100 µl cell suspension was added to rows A-H.

After addition of the cells, the plates were incubated at 37°C in humidified air and 5% CO_2 until the time when the second drug was added. After addition of the second drug the plates were then replaced into the incubator and processed in the usual manner.

Drugs B, C and D at 6 and 24 hours

A second plate was made up of drug dilutions at 6 and 24 hours. 100 µl MI was added to row A and 100 µl CAM to rows B-H (figure 5.2). 100 µl of 1600% TDC MMC and gemcitabine was added to row B and double diluted to row G. At the appropriate time, 50 µl was removed from rows A-H of the experimental plate and added to the appropriate wells in the experimental plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A				100µl MI	100µl MI	100µl MI	100µl MI	100µl MI	100µl MI	100µl MI	100µl MI	100µl MI
B				**6 hours**			**24 hours**			**24 hours**		
C				**1600% TDC Gemcitabine + 1600% TDC MMC**			**1600% TDC Gemcitabine + 1600% TDC MMC**			**1600% TDC Gemcitabine + 1600% TDC MMC**		
D												
E				100µl CAM	100µl CAM	100µl CAM	100µl CAM	100µl CAM	100µl CAM	100µl CAM	100µl CAM	100µl CAM
F				100µl CAM	100µl CAM	100µl CAM	100µl CAM	100µl CAM	100µl CAM	100µl CAM	100µl CAM	100µl CAM
G				100µl CAM	100µl CAM	100µl CAM	100µl CAM	100µl CAM	100µl CAM	100µl CAM	100µl CAM	100µl CAM
H				100µl CAM	100µl CAM	100µl CAM	100µl CAM	100µl CAM	100µl CAM	100µl CAM	100µl CAM	100µl CAM

Figure 5.2. Drug dilution plate for schedule experiments

5.3 Results

The sensitivity data for MMC, gemcitabine and the combination of MMC and gemcitabine (GeM) are shown in table 5.2 and figure 5.3. Both single agents showed sensitivity (i.e. $Index_{SUM}$ <300) in about 50% of samples although there was heterogeneity between individual tumours. MMC demonstrated sensitivity in 52% (25/48), and gemcitabine in 44% (21/48) of samples tested. Despite these apparently encouraging results, 90% inhibition was only achieved in 21% of samples tested with MMC alone (10/48) and 8% (4/48) tested with single agent gemcitabine.

In contrast to the single agent results, gemcitabine in combination with mitomycin C (GeM) showed activity in 45/46 (98%) of samples and achieved >90% inhibition at clinically achievable concentrations in 83% of samples (38/46). Figure 5.3 shows this as a shift in activity towards the lower concentrations tested. The addition of gemcitabine decreased the median MMC IC90 from 3.73 μM to 0.96 μM. Using the Chou and Talalay method for assessing drug combinations, this equates to a combination index of 0.44, indicating synergism.

Table 5.2. Summary of sensitivity data for tumour-derived cells tested with mitomycin C, gemcitabine and the combination mitomycin C + gemcitabine (using $Index_{SUM}$ < 300 to indicate sensitivity)

Drug	**No. assessed**	**No. sensitive**	**Sensitivity (%)**	**>90% Inhibition**
MMC	48	25	52	10 (21%)
Gemcitabine	48	21	44	4 (8%)
MMC + Gemcitabine	46	45	98	38 (83%)

Figure 5.4 is an example of a sample showing the advantage of the GeM combination over the individual agents in terms of inhibition. When analysed by the method of Poch *et al.* (1995), by which the observed effect at each concentration tested is compared with that expected, the effect is greater than additive (figure 5.4b). TORC02-0356 was the only sample to show resistance to the GeM combination

(figure 5.5). As can be seen on the Poch graph (figure 5.5b), the achieved inhibition is less than that estimated for the addition of the independent action of the two drugs.

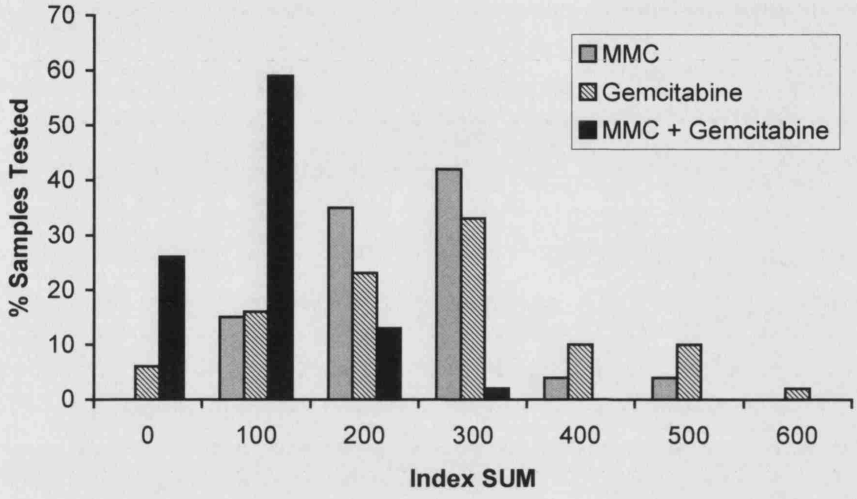
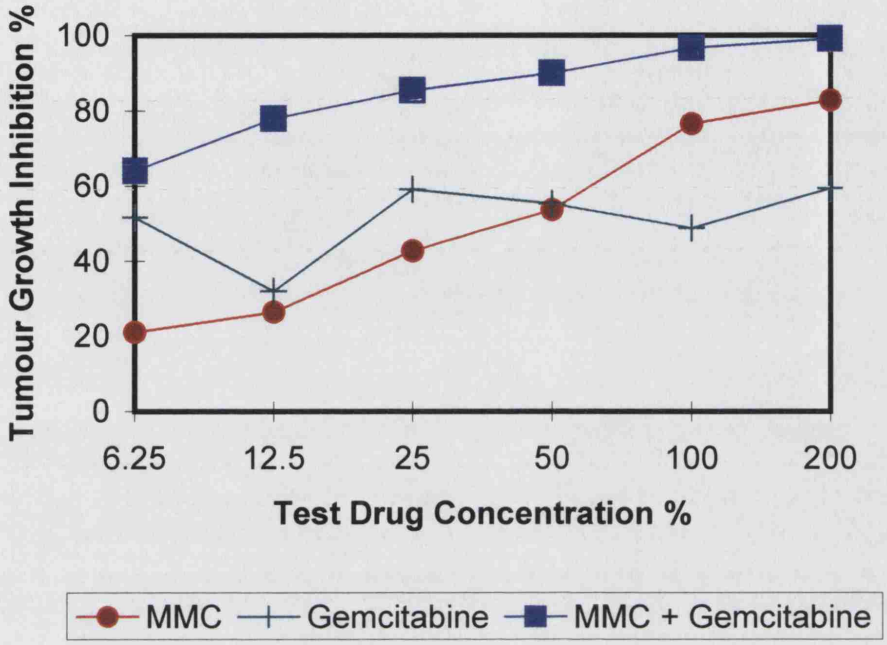


Figure 5.3. Histogram depicting the percentage of samples sensitive to MMC, gemcitabine and the combination of the two drugs. The combination shifts the bars to the left (lower $Index_{SUM}$) indicating greater sensitivity.

Schedule experiments of gemcitabine and mitomycin C are shown in figure 5.6. There was no apparent difference in inhibition between the different schedules (93.84-95.84% tumour growth inhibition at 100% TDC), although gemcitabine added to mitomycin C at 24 hours did produce the lowest growth inhibition of 93.84%).

a)



b)

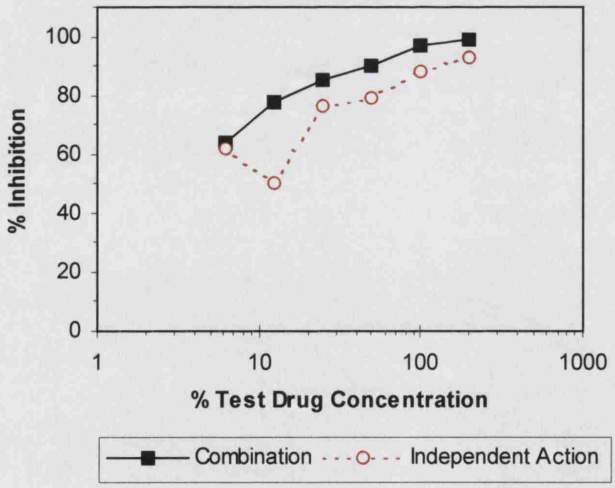
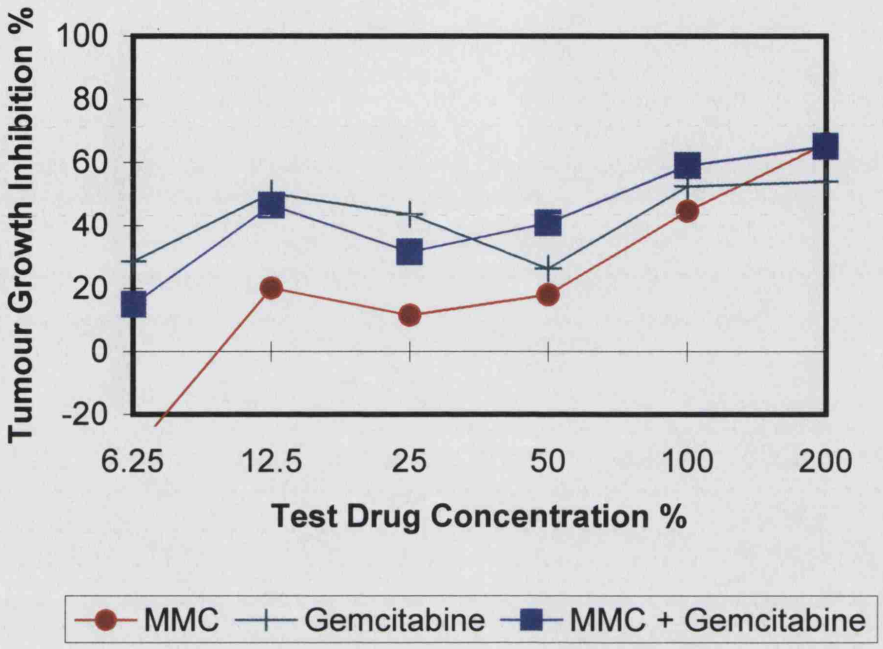


Figure 5.4. Results for TORC02-0206, a sample sensitive to MMC, gemcitabine and the combination (GeM). a) ATP-TCA concentration-inhibition curve. GeM clearly achieves greater tumour growth inhibition and reaches 90% inhibition. b) The Poch graph shows the curve produced from GeM is above and to the left of that predicted by the addition of the independent action of mitomycin C and gemcitabine, indicating a greater than additive effect, i.e. synergism

a)



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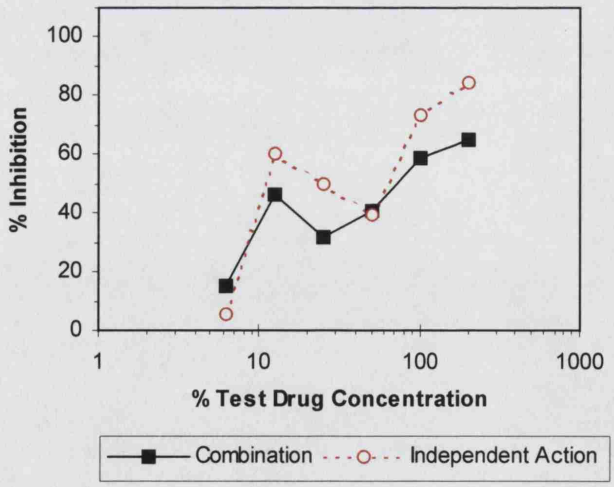


Figure 5.5. Results for TORC02-0356, the only sample tested showing resistance to GeM. a) The combination only achieves 60% growth inhibition on the ATP-TCA concentration-inhibition curve. b). The Poch graph shows the curve produced from GeM is below and to the right of that predicted by the addition of the independent action of mitomycin C and gemcitabine, indicating less than the additive effect expected, i.e. antagonism.

a)

Expt A	AUC	IC 90	IC 50	Index
MMC + Gem 0hrs	17948	37	5	87
MMC 0hrs + Gem 6hrs	18221	22	5	70
MMC 0hrs + Gem 24hrs	17851	34	5	93
Gem 0hrs + MMC 24hrs	18033	23	4	68
Expt B				
MMC + Gem 0hrs	18107	23	5	74
MMC 0hrs + Gem 6hrs	18292	20	4	64
MMC 0hrs + Gem 24hrs	17907	29	5	87
Gem 0hrs + MMC 24hrs	17938	33	5	77
Expt C				
MMC + Gem 0hrs	15932	35	4	79
MMC 0hrs + Gem 6hrs	17833	35	5	81
MMC 0hrs + Gem 24hrs	18470	29	4	73
Gem 0hrs + MMC 24hrs	18139	33	5	86

b)

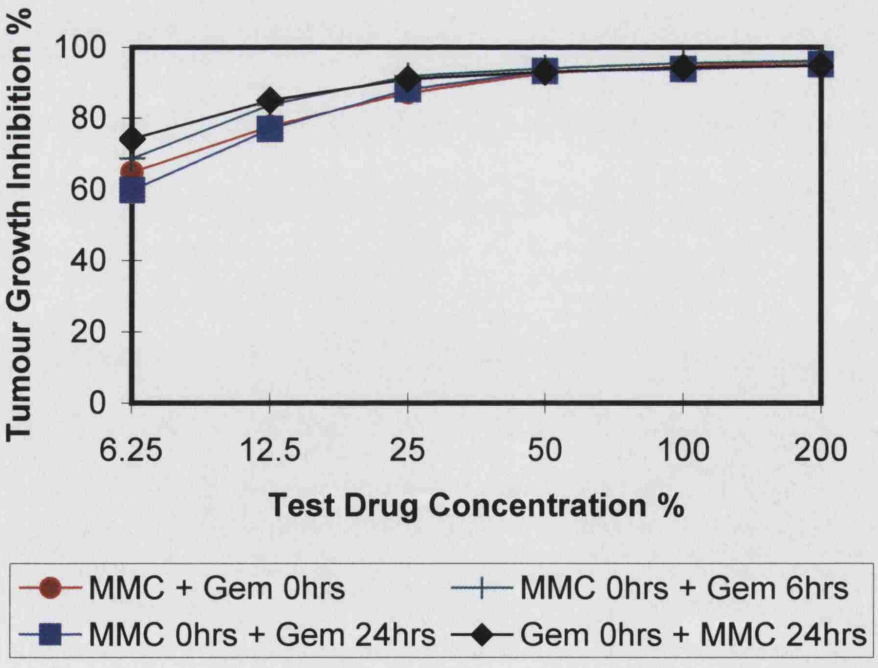


Figure 5.6. Schedule experiments of the combination of MMC and gemcitabine. a) sensitivity data for time course experiments performed in triplicate. b) ATP-TCA concentration-inhibition curves for time-course experiments of MMC and gemcitabine, showing no difference in sensitivity.

5.4 Discussion

The results presented here and in chapter four demonstrate the feasibility of using the ATP-TCA as a tool in the development of new drugs and regimens in colorectal cancer. While this data may not of course translate into clinical efficacy of similar magnitude, it does provide a basis for the clinical investigation of this combination in gastrointestinal tumours.

There is no doubt that cells derived from tumour samples have different sensitivities to a number of chemotherapeutic agents compared to established cells lines (Andreotti *et al.*, 1994; Cree and Andreotti, 1997), and thus ideally the pre-clinical selection of drugs should be carried out on tumour samples. The ATP-TCA has previously been used to assist new drug and regimen development (Neale *et al.*, 1999; Di Nicolantonio *et al.*, 2002; Cree, 2003), some of which are now in current clinical practice.

Some experimental regimens not currently used in clinical practice were tested (e.g. gemcitabine + MMC (GeM) and oxaliplatin + gemcitabine). Both these combinations contain gemcitabine which showed some activity as a single agent in 50% of samples. GeM was the most sensitive combination tested overall, with all but one sample showing sensitivity using the $Index_{SUM}$ <300 threshold.

The exact mechanism for the synergistic effect seen between mitomycin C and gemcitabine has not been investigated in this thesis. Gemcitabine possesses a number of mechanisms of cytotoxicity, all of which may contribute to the synergism seen. These include inhibition of DNA polymerase, the direct incorporation of the drug into DNA and RNA, and the alteration of cellular dNTP pools (Peters *et al.*, 1996). It has been shown to modulate the activity of a wide range of DNA-damaging agents, including platinum (Peters *et al.*, 1995; Sandler *et al.*, 2000) and alkylating agents (Neale *et al.*, 1999) probably due to inhibition of repair of alkylating agent induced DNA adducts. Studies of gemcitabine in combination with cisplatin are based on this mechanism of action (Cardenal *et al.*, 1999).

There are very few *in vitro* studies of the combination of gemcitabine and MMC. The combination was found to be synergistic after 4 hours on a Lewis lung cancer cell line, without any increase in DNA double-strand breaks (van Moorsel *et al.*, 1997). Similarly, MMC and gemcitabine had a synergistic effect, when administered concurrently but not sequentially, on the HT29 human colon cancer cell line (Aung *et al.*, 2000), suggesting gemcitabine could be beneficial in the treatment of cancers sensitive to MMC.

Clinical studies are also few in number: intravenous and intra-arterial locoregional treatment with MMC and gemcitabine has been found to be highly effective with improved response rates in pancreatic cancer (Klapdor *et al.*, 2000; Tuinmann *et al.*, 2004)). This drug combination (median total dose MMC 32mg m^{-2}) has been also administered together with radiotherapy with tolerable toxicity (Korneck *et al.*, 2001).

This study has not demonstrated any schedule-specific alterations in chemosensitivity. A study of mitomycin C and gemcitabine on the HT29 human colon cancer cell line showed simultaneous exposure to be necessary to demonstrate synergism (Aung *et al.*, 2000). The effect of gemcitabine in combination with other alkylating and platinum agents has also been shown to be time-dependent (Braakhuis *et al.*, 1995; Faivre *et al.*, 1999; Neale *et al.*, 1999; van Moorsel *et al.*, 2000). Since simultaneous administration is generally preferable to patients and oncology units, it would probably be reasonable to give both drugs together in future clinical studies.

These results demonstrate that gemcitabine + mitomycin C (GeM) is effective *ex vivo* against CRC tumour-derived cells. There is little evidence of schedule dependency and simultaneous administration should be feasible. These results have encouraged us to explore the GeM regimen further in a phase I clinical trial to establish its safety and efficacy in metastatic gastrointestinal cancer.

CHAPTER 6

Molecular determinants of chemosensitivity in colorectal cancer

6.1 Introduction

Currently several molecular markers are under investigation as predictors of outcome for specific drug treatments. Methods include immunohistochemical staining and detection of up- or down-regulation of proteins by RT-PCR. Table 1.15 in chapter 1 summarises some of the molecular markers under investigation as predictors of outcome to treatment in CRC. With these in mind, the expression of several molecular determinants was investigated, using immunohistochemistry and qRT-PCR, and expression correlated with the chemosensitivity data from chapter 4. Using qRT-PCR, the effect of short-term 5-FU and irinotecan exposure on the expression of mRNA of some molecular determinants was also investigated.

6.2 Methods

Immunohistochemistry was carried out as described in chapter 2.3 and the results compared to ATP-TCA results performed as described in chapter 2.2. The H-score (an index derived by multiplication of the percentage area stained and intensity of staining) was used. The H-score varies between 0 and 300, with an H-score of 100 or more regarded as positive. qRT-PCR was performed on 10 samples as described in chapter 2.4. The amount of mRNA of the genes of interest are expressed relative to the expression of the housekeeping genes. Appropriate statistical analysis was performed using InStat (GraphPad ®, San Diego, USA) and StatsDirect (Camcode, Herts, UK).

6.3 Results

6.3.1 IHC expression of molecular determinants of chemosensitivity and correlation with the ATP-TCA

The number and percentage of samples staining positive for each antibody is shown in table 6.1. Immunocytochemistry for the microsatellite inhibition markers hMLH1,

hMSH2 and hMSH6 were graded positive or negative on nuclear staining. Most samples showed positive nuclear staining; 40/47 (85%) for hMLH1, 46/47 (98%) for hMSH2, and 43/45 (96%) for hMSH6. All the samples tested stained positively for BCRP (100%), and none were positive for TP (0%). The number of samples staining positively for COX-2, TOPO I, and TS were 28/48 (58%), 39/49 (80%) and 43/49 (88%) respectively. Photographic examples of slides staining positive and negative for each antibody can be seen in figures 6.1-6.8.

Table 6.1. Number and percentage of samples staining positive with a panel of antibodies

Antibody	**No. samples tested**	**No. samples staining positive**	**Percentage samples staining positive**
BCRP	47	47	100
COX-2	48	28	58
hMLH1	47	40	85
hMSH2	47	46	98
hMSH6	45	43	96
TOPO I	49	39	80
TP	48	0	0
TS	49	43	88

The immunohistochemical expression of TS was compared with sensitivity in the ATP-TCA to 5-FU, and the expression of TOPO I was compared with sensitivity to irinotecan. COX-2 expression was compared with sensitivity to both irinotecan and 5-FU.

The median 5-FU IC90 values for TS negative and positive samples were 197.00 and 170.90 μM respectively (p=NS, Mann-Whitney U test), while the median IC50 values were 35.5 and 38.29 μM respectively (p=NS, Mann-Whitney U).

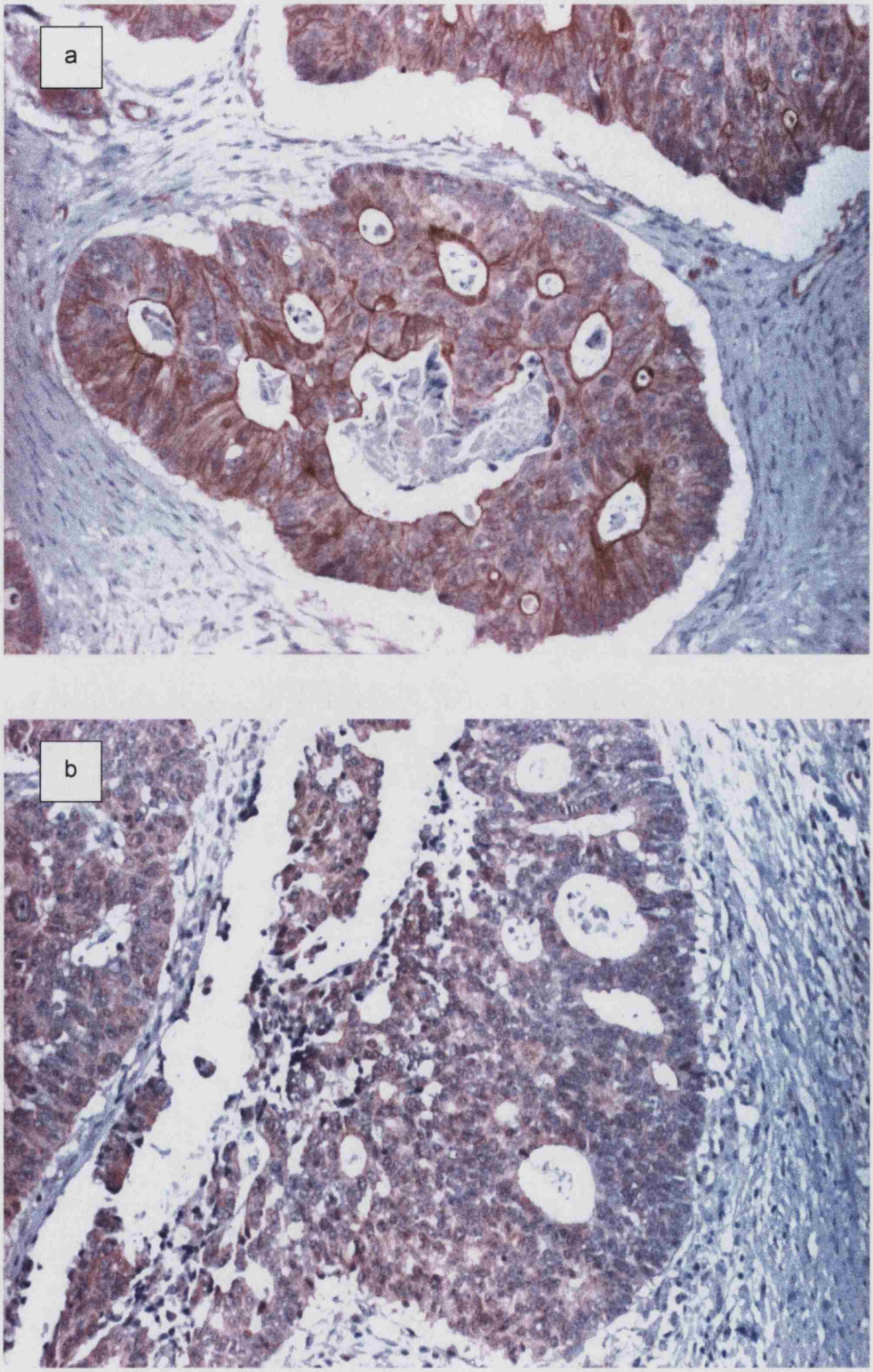


Figure 6.1. Immunohistochemical staining for BCRP (cytoplasmic) where a) stains positive and b) is negative.

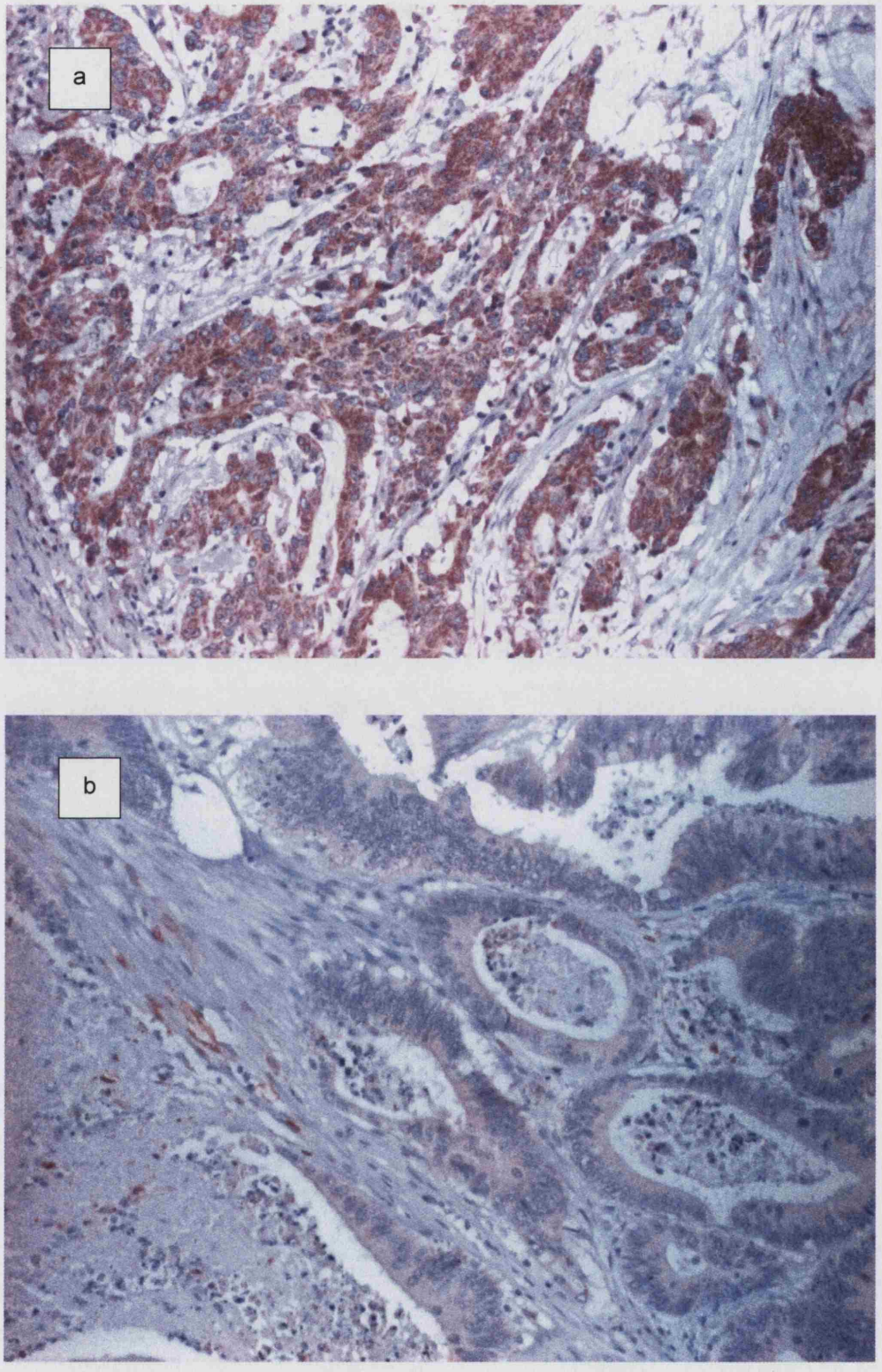


Figure 6.2. Immunohistochemical staining for COX-2 (cytoplasmic) where a) stains positive and b) is negative.

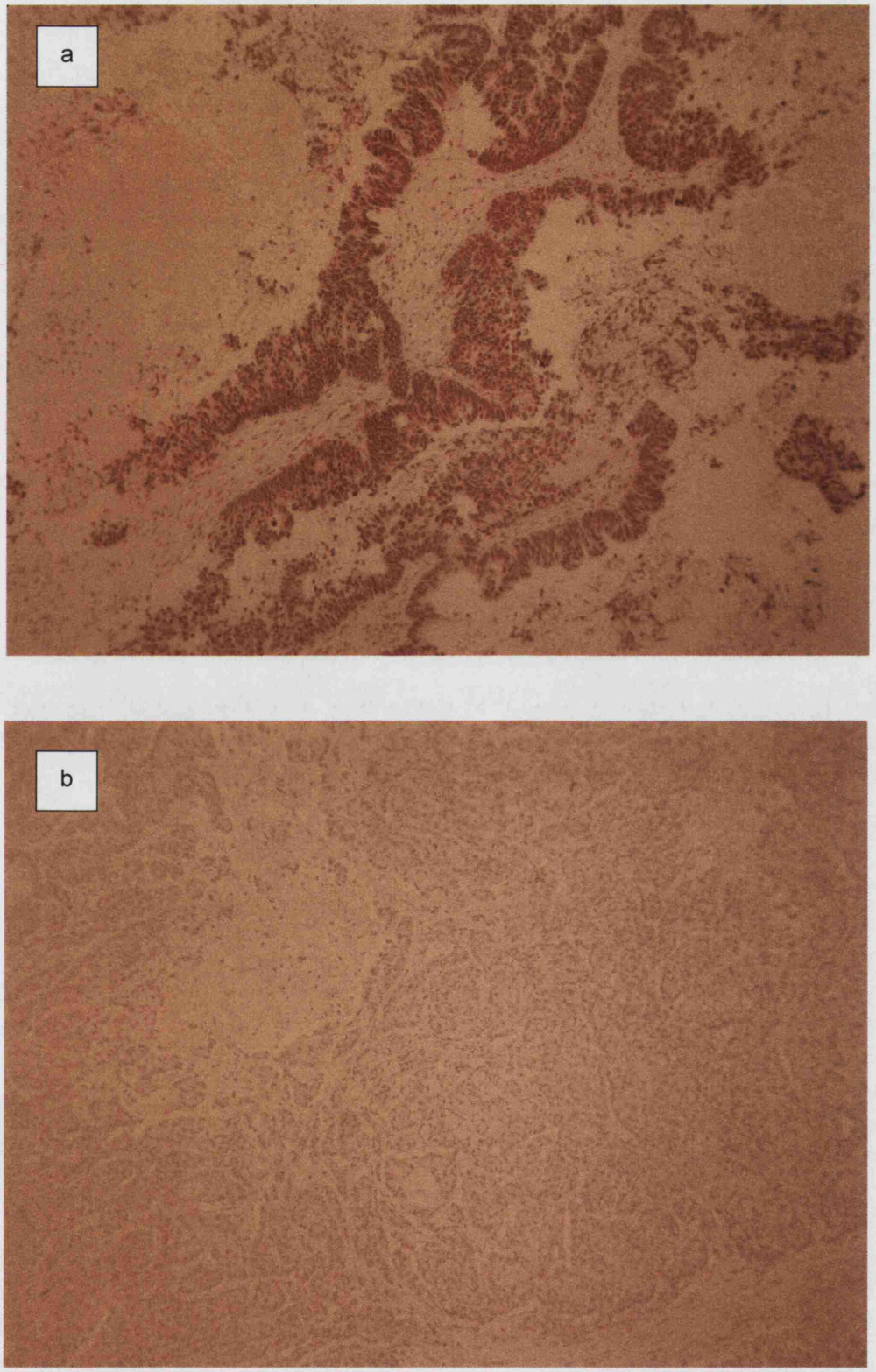


Figure 6.3. Immunohistochemical staining for hMLH-1 (nuclear) where a) stains positive and b) is negative.

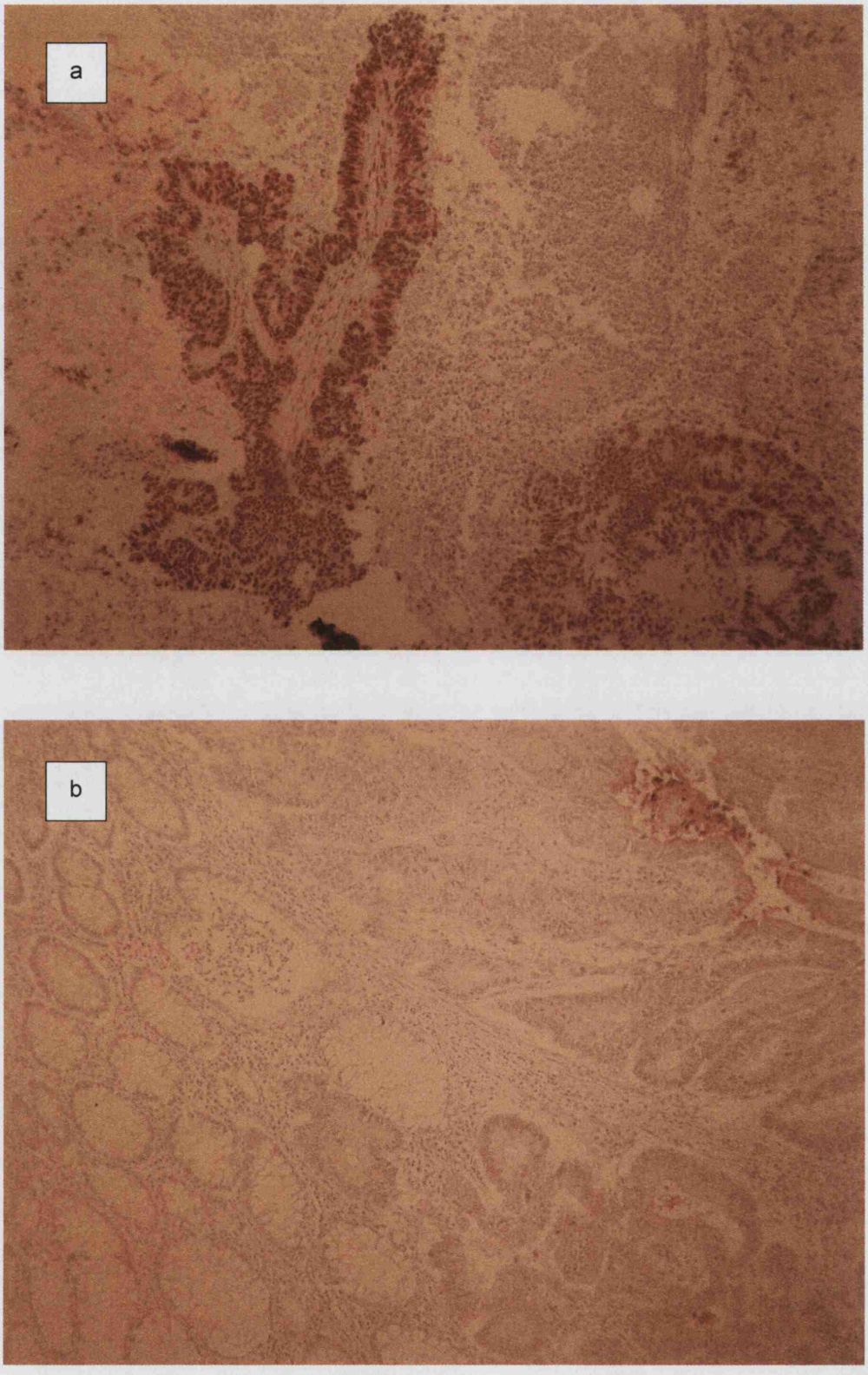


Figure 6.4. Immunohistochemical staining for hMSH-2 (nuclear) where a) stains positive and b) is negative.

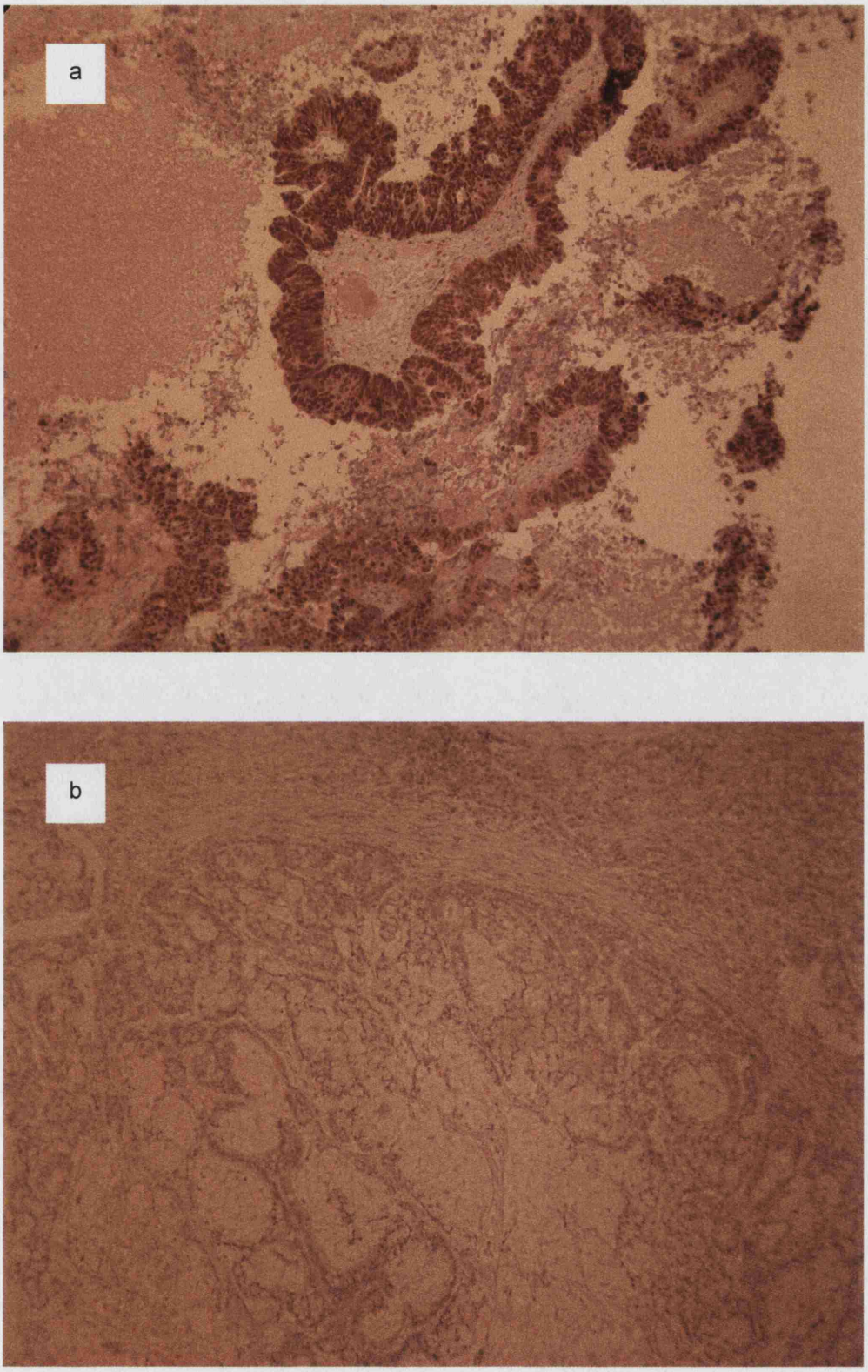


Figure 6.5. Immunohistochemical staining for hMSH-6 (nuclear) where a) stains positive and b) is negative.

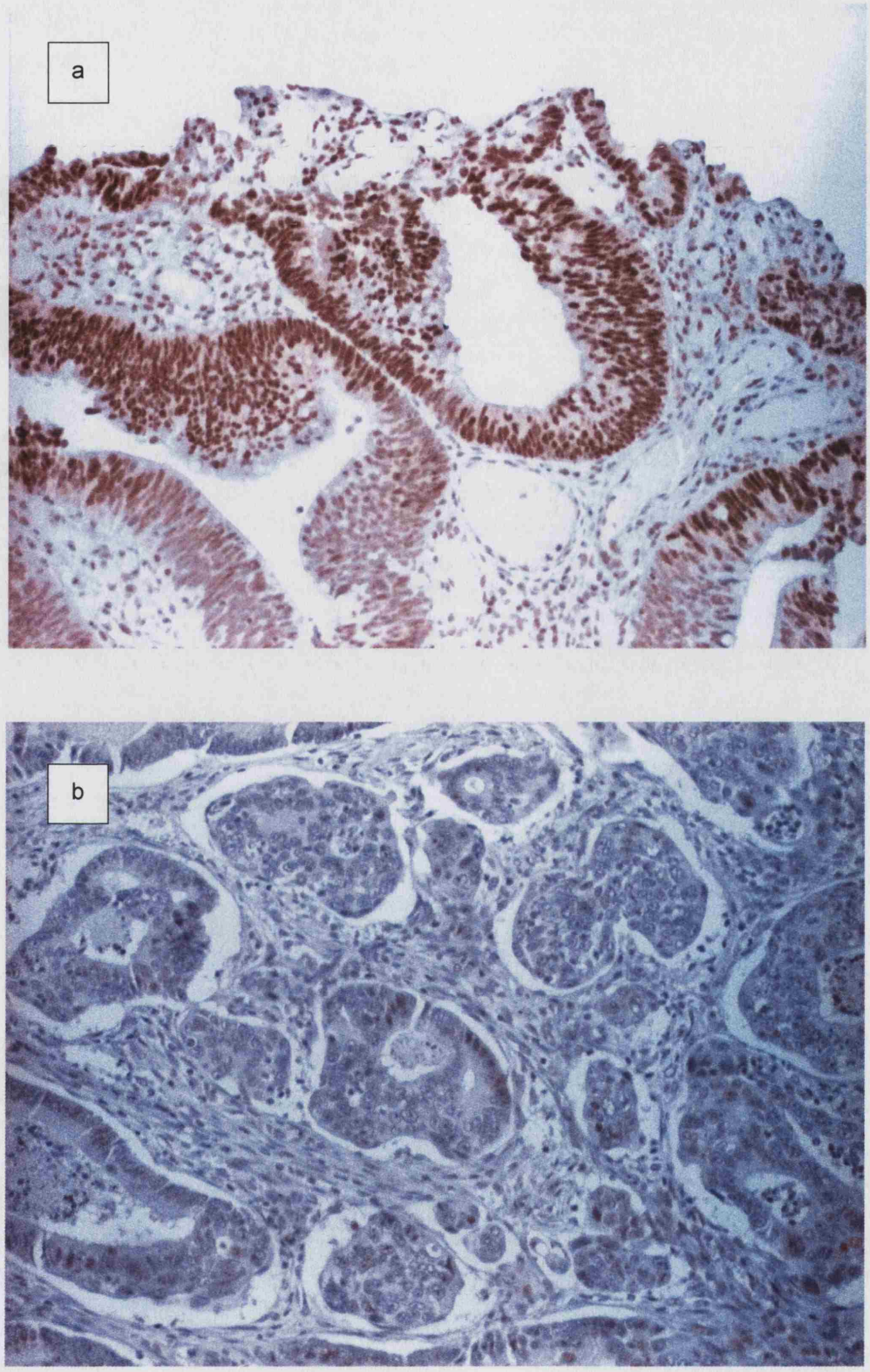


Figure 6.6. Immunohistochemical staining for Topoisomerase-I (nuclear) where a) stains positive and b) is negative.

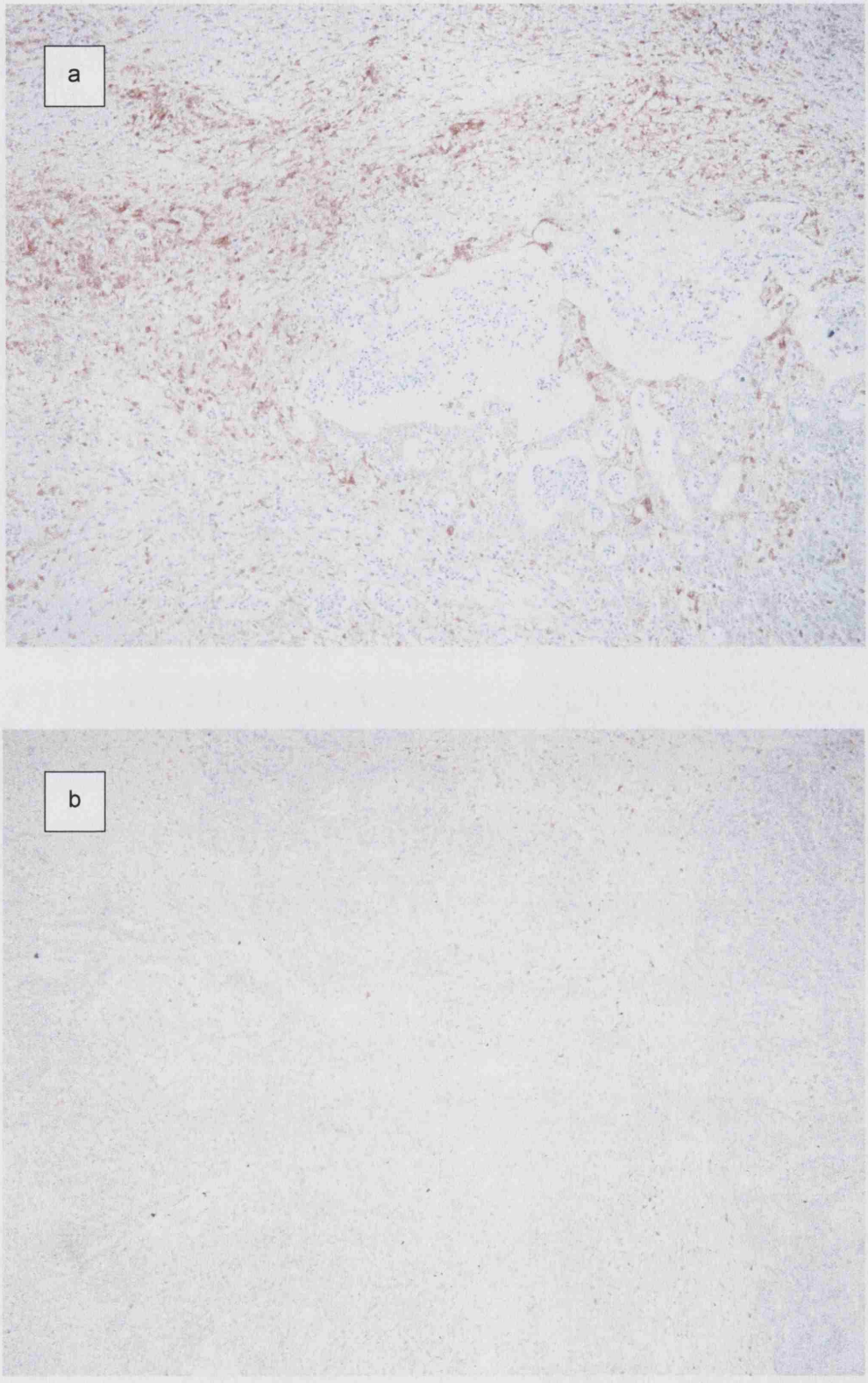


Figure 6.7. Immunohistochemical staining for Thymidine Phosphorylase (TP) (nuclear) where a) stains positive and b) is negative. None of the colorectal samples stained positive for TP. The example shown here is an oesophageal sample from the same batch of staining using the same antibody preparation that was used for the colorectal samples.

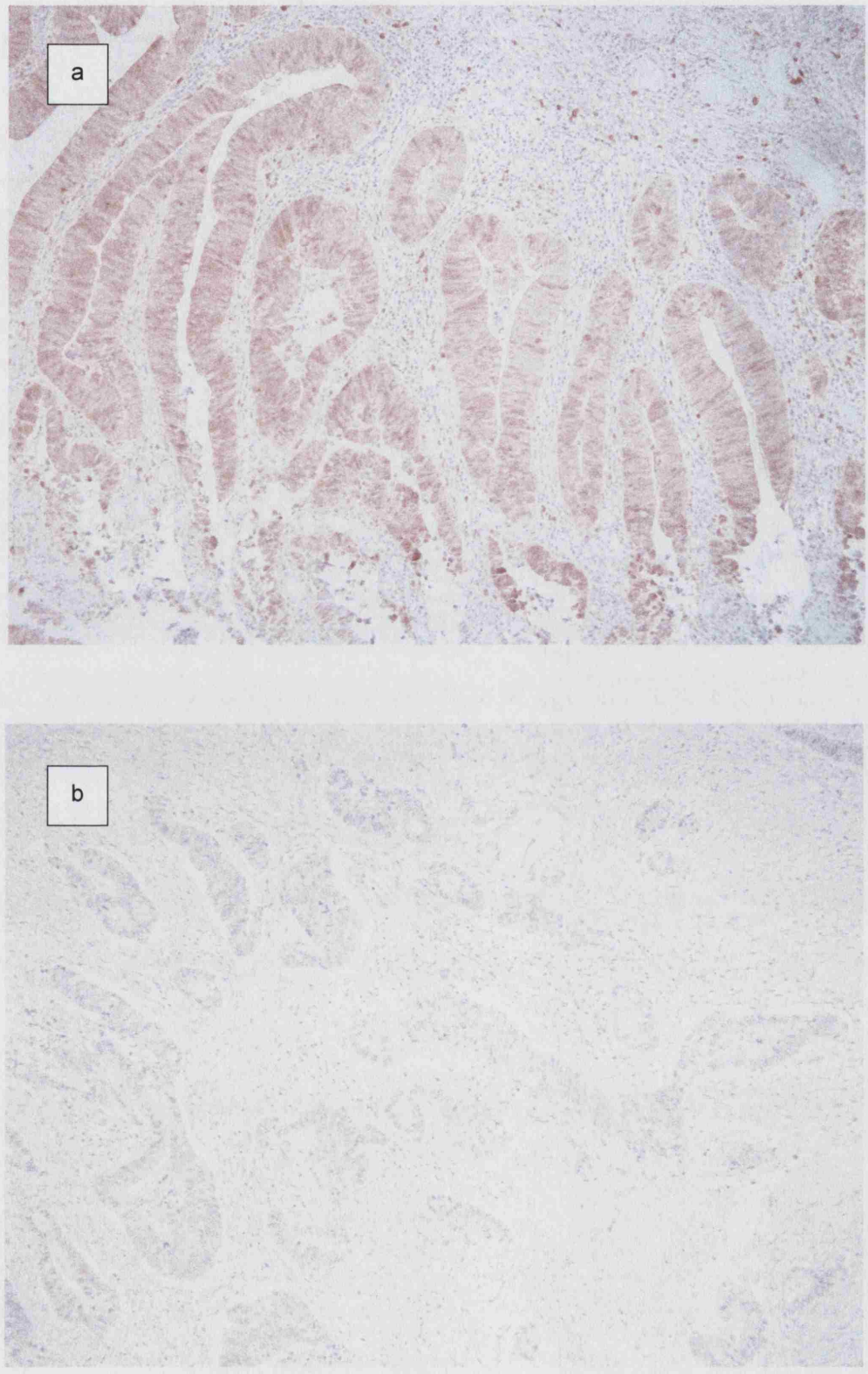


Figure 6.8. Immunohistochemical staining for Thymidylate Synthase (nuclear) where a) stains positive and b) is negative.

No significant difference was found between staining for COX-2 and 5-FU IC50, IC90 and $Index_{SUM}$ (table 6.2). Similarly, there was no significant difference in median irinotecan IC50 and IC90 values between samples staining positive or negative for COX-2 (table 6.3).

Table 6.2. Median IC50, IC90 and $Index_{SUM}$ of 5-FU for samples staining positive or negative for TS and COX-2

	IC50 (μM)	**IC90 (μM)**	**$Index_{SUM}$**
TS +ve (n=43)	38.29	170.90	286.30
TS –ve (n=6)	35.50 (p=0.8428)	197.00 (p=0.3931)	285.5 (p=0.9881)
COX-2 +ve (n=28)	40.64	173.79	282.15
COX-2 –ve (n=20)	34.15 (p=0.5723)	174.70 (p=0.5168)	259.08 (p=0.3922)

Table 6.3. Median IC50, IC90 and $Index_{SUM}$ of irinotecan for samples staining positive or negative for TOPO I and COX-2

	IC50 (μM)	**IC90 (μM)**	**$Index_{SUM}$**
TOPO I +ve (n=39)	42.8	151.00	329.03
TOPO I –ve (n=10)	33.00 **(p=0.0256)**	92.00 **(p=0.0256)**	328.55 (p=0.7129)
COX-2 +ve (n=31)	42.81	135.13	302.00
COX-2 –ve (n=17)	40.17 (p=0.9927)	128.28 (p=0.8115)	310.00 (p=0.8257)

Immunostaining for TOPO I was positive in 39/49 (80%) of samples tested with irinotecan. The median irinotecan IC50 values for TOPO I negative and positive samples were 33.00 and 42.8 μM respectively (p=0.0256, Mann-Whitney U test). The median irinotecan IC90 values were 92.00 and 151.00 μM respectively (p=0.0256, Mann-Whitney U test). No correlation was found between irinotecan IC50 or IC90 and staining for TOPO I using linear regression analysis or non-parametric Spearman correlation (R=0.2043 and p=0.1462 for IC50; R=0.1279 and p=0.3661 for IC90). TOPO I positivity shifts the concentration-inhibition curve of irinotecan to the right (figure 6.9).

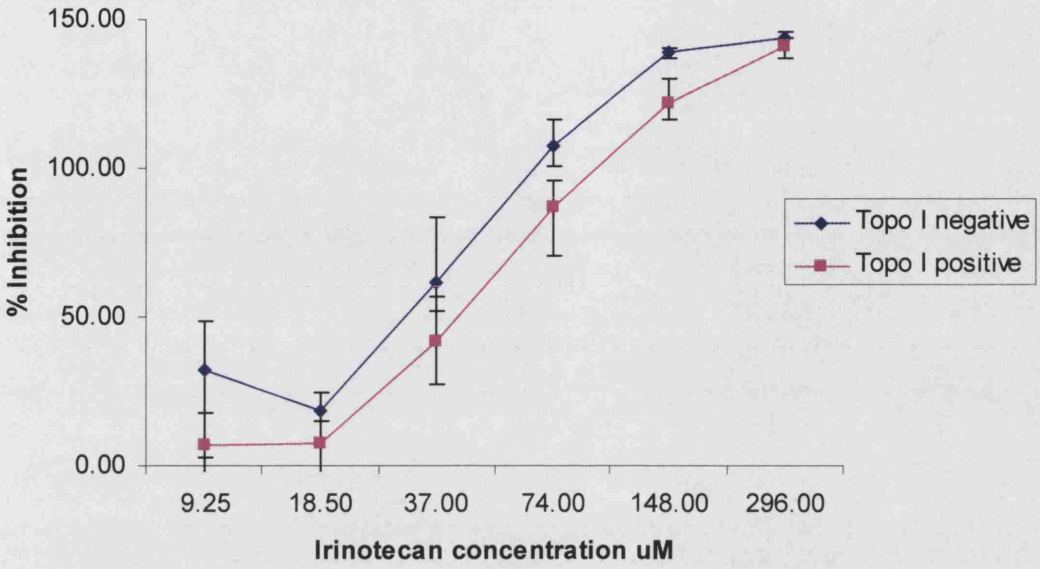


Figure 6.9. Concentration-inhibition curves for irinotecan in TOPO I positive (n=39) and negative (n=10) samples. The error bars show the interquartile range.

6.3.2 mRNA expression of molecular determinants of chemosensitivity and correlation with the ATP-TCA

qRT-PCR required considerable numbers of cells and was therefore performed on just 10 samples. The mean relative mRNA levels for a panel of genes are shown in table 6.4 and are expressed in relation to the expression of at least two housekeeping genes.

Univariate and multivariate analyses were performed to correlate the results obtained in the ATP-TCA with the gene expression profile measured in tumour-derived cells by qRT-PCR. There were no statistically significant correlations found between the mRNA levels of COX-2, DPD, TP or TS and the IC50 or IC90 of 5-FU. Similarly, no correlation was found between BCRP, COX-2 or TOPO I and the IC50 or IC90 of irinotecan.

Table 6.4. Expression of mRNA levels (ΔCt), where the mean RNA expression of MO wells in ATP-TCA compared to mean RNA expression in at least 2 housekeeping (HK) genes (n=10)

Target Gene	Mean Ct HK	Ct	ΔCt
BCRP	25.82	29.81	+3.99
COX-2	25.75	19.35	-6.40
DPD	25.76	26.67	+0.91
ERCC-1	26.10	26.09	-0.01
TOPO I	25.99	27.59	+1.6
TOPO IIα	26.31	31.60	+5.29
TOPO IIβ	25.73	26.82	+1.09
TP	25.76	23.67	-2.09
TS	25.76	24.38	-1.38

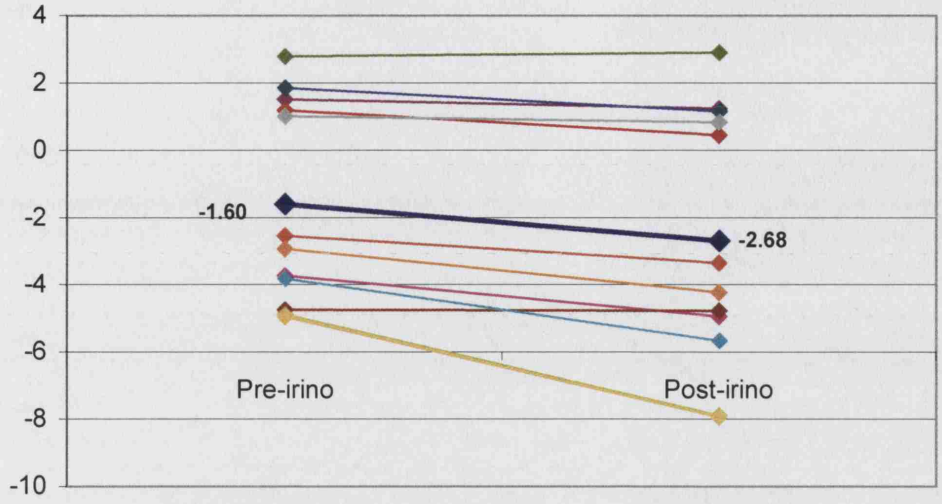
6.3.3 Gene expression changes induced by irinotecan in short-term cell culture

Irinotecan acts via TOPO I inhibition, and the level of the target enzyme is documented above. A trend towards down-regulation of TOPO I was found in treated cells, with exposure to irinotecan decreasing TOPO I levels >2-fold in 3/7 tumour samples (figure 6.10, table 6.5). This was accompanied by a reduction of TOPO IIα expression, which was particularly pronounced (>4-fold) in 6/7 samples. However, statistical significance was not reached.

No significant changes were observed in expression of the drug efflux molecules MDR1 and BCRP (figure 6.11, table 6.5), although there was considerable heterogeneity. ERCC-1 expression was up-regulated in all 7 samples, although the modest increase did not reach significance (p=0.016, Wilcoxon; NS with Bonferroni correction).

COX-2 was down-regulated in all 7 samples (figure 6.12), with the median level decreasing from 105.17 to 38.49 units (p=0.0156, Wilcoxon matched pairs test; NS with Bonferroni correction).

(a)



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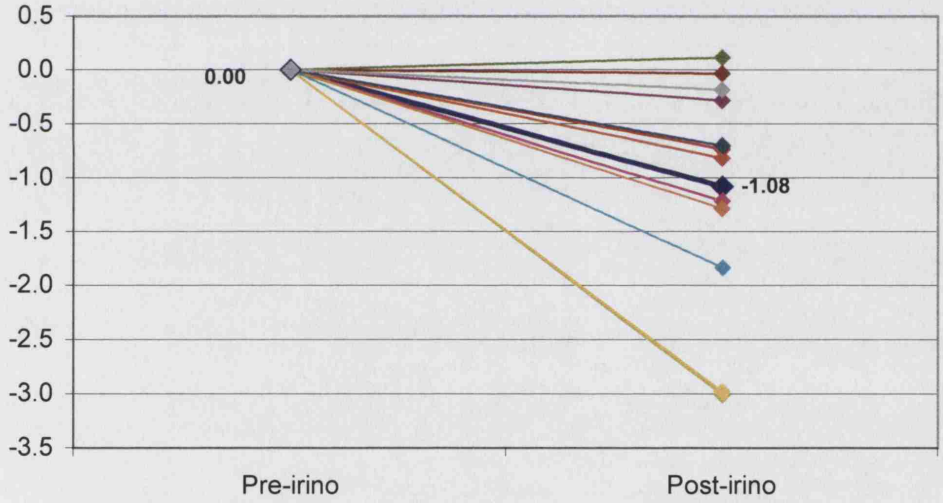
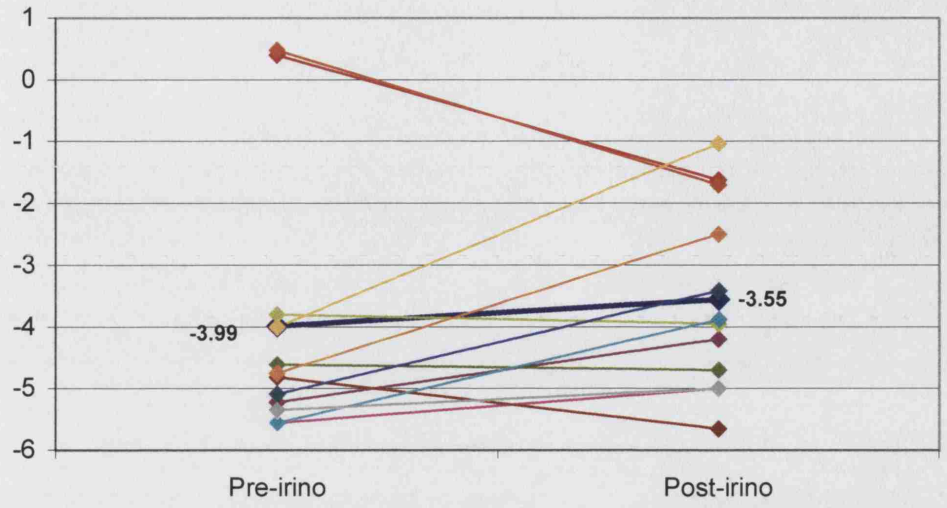


Figure 6.10. a) Relative expression of TOPO I pre- and post- ex vivo treatment with irinotecan given on a natural logarithmic scale: $2^{-\Delta Ct}$, where Ct is the difference in threshold cycle (Ct) between the gene of interest and the mean of at least two reference genes. b) The pre- treatment level of TOPO I has been zeroed and the relative up or down regulation for each sample can be seen.

(a)



(b)

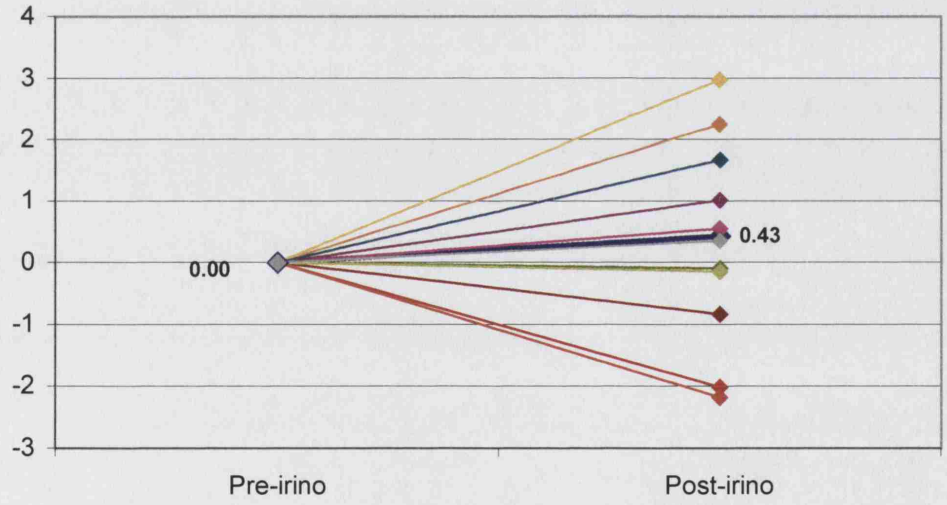
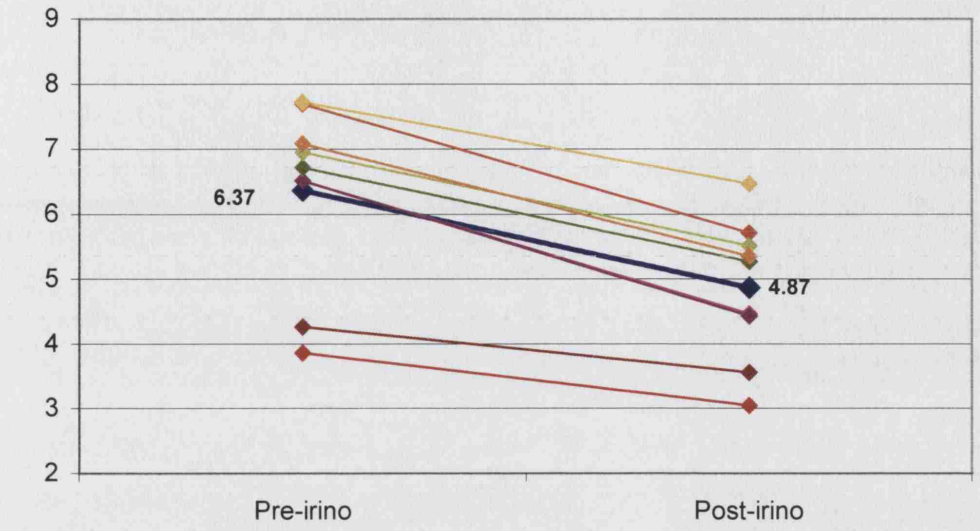


Figure 6.11. a) Relative expression of BCRP pre- and post- ex vivo treatment with irinotecan given on a natural logarithmic scale: $2^{-\Delta Ct}$, where Ct is the difference in threshold cycle (Ct) between the gene of interest and the mean of at least two reference genes. b) The pre- treatment level of BCRP has been zeroed and the relative up or down regulation for each sample can be seen.

(a)



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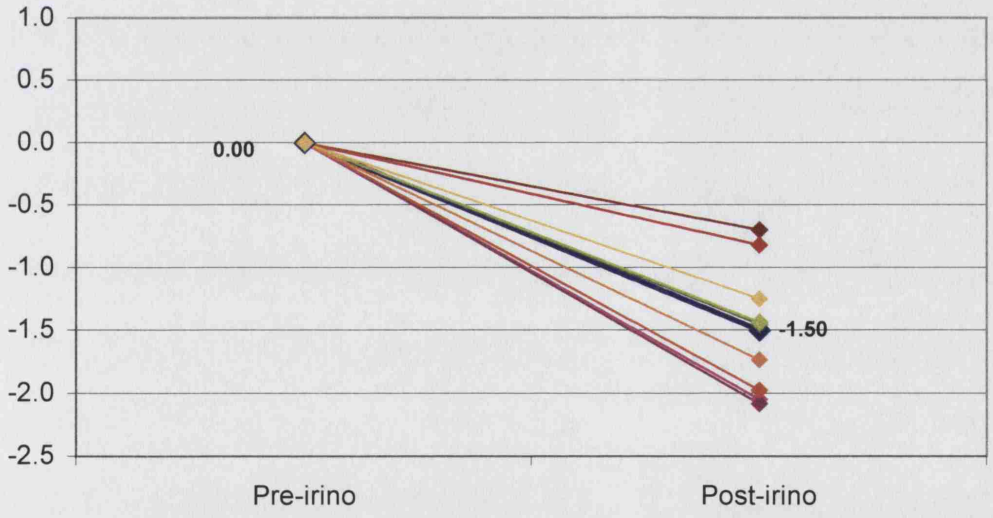


Figure 6.12. a) Relative expression of COX-2 pre- and post- ex vivo treatment with irinotecan given on a natural logarithmic scale: $2^{-\Delta Ct}$, where Ct is the difference in threshold cycle (Ct) between the gene of interest and the mean of at least two reference genes. b) The pre- treatment level of COX-2 has been zeroed and the relative up or down regulation for each sample can be seen.

Table 6.5. Relative expression of mRNA levels $2^{-\Delta Ct}$ in tumour samples after *ex-vivo* exposure to irinotecan (n=7 except for TOPO IIα where n=6). The p values have been calculated using non-parametric statistics (Wilcoxon matched pairs test for paired samples with a Bonferroni correction; statistical significance was taken at $p<0.005$).

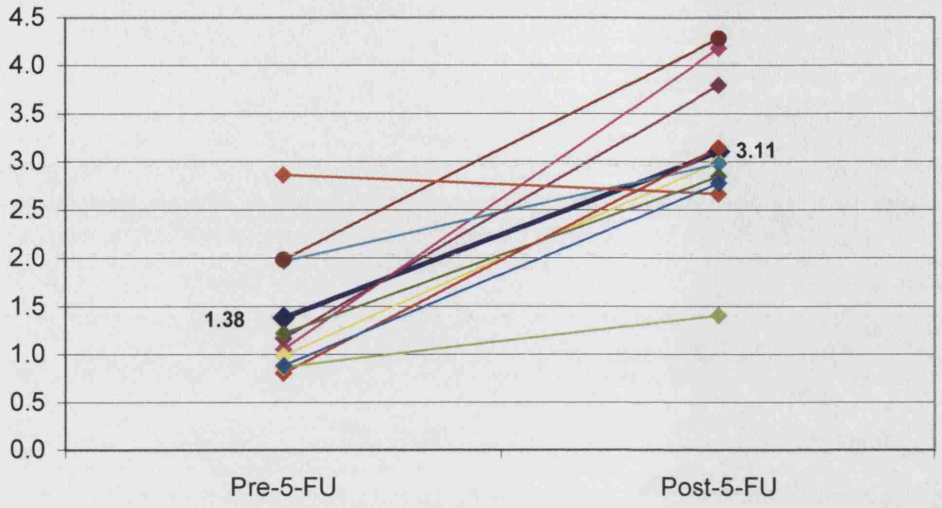
Target gene	Control	Irinotecan	P
BCRP	0.0354	0.0515	0.688
COX-2	105.17	38.49	0.016
ERCC1	1.403	2.469	0.016
TOPOI	1.468	1.119	0.109
TOPO IIα	0.2847	0.0733	0.219
TOPO IIβ	0.7405	0.5058	0.031

6.3.4 Gene expression changes induced by 5-FU in short-term cell culture

The genes well known to be involved in 5-FU metabolism include DPD, TP and TS. After *ex-vivo* exposure to 5-FU we found an increase in TS levels in all samples (p=0.002, Wilcoxon matched pairs test). There was a general trend towards down-regulation of DPD (6/10 samples), although there was a >2-fold up-regulation in 3/10 samples. Considerable heterogeneity was found in the expression of TP, which increased in 6/10 samples, decreased in 2 and remained unchanged in 2 (figures 6.13-6.15, table 6.6).

There was significant up-regulation of expression of COX-2 after exposure to 5-FU, from 121.6 to 391.7 units ($p<0.004$, Wilcoxon matched pairs test). This increase was more than 2-fold in 9 of 10 samples (figure 6.16).

(a)



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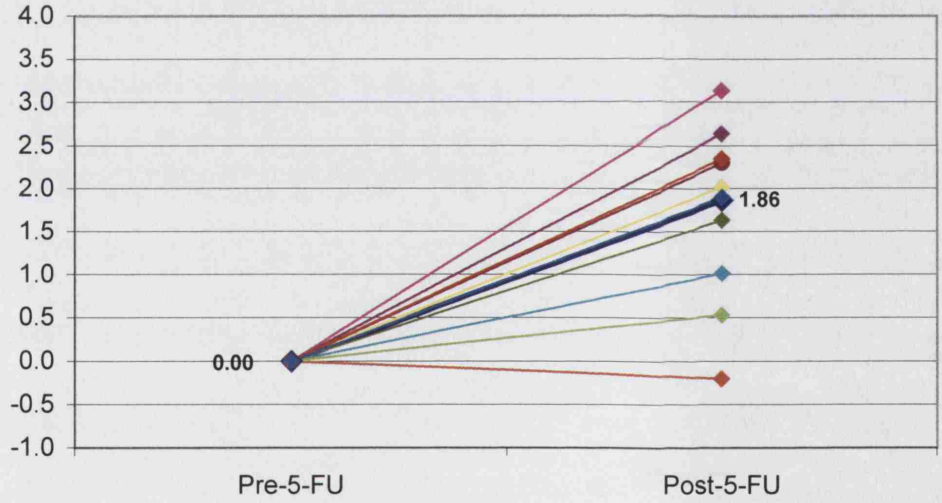
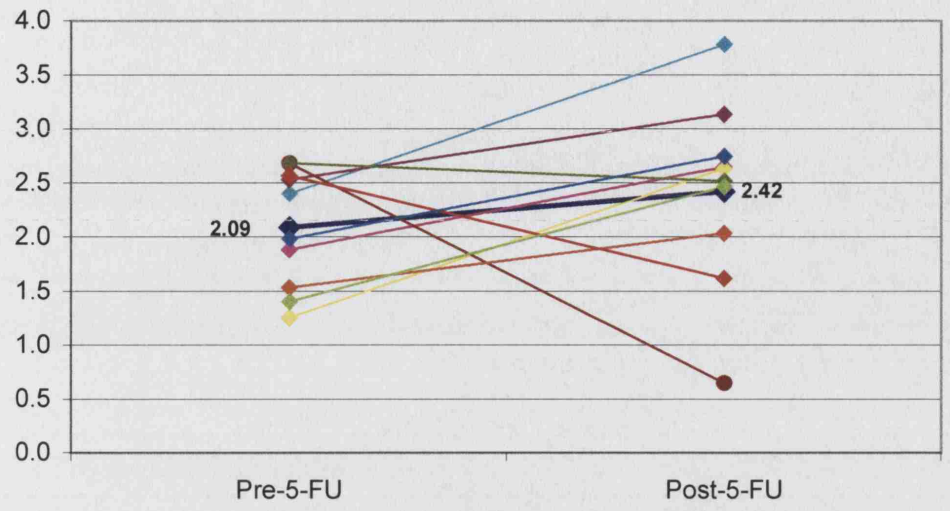


Figure 6.13. a) Relative expression of TS pre- and post- ex vivo treatment with 5FU given on a natural logarithmic scale: $2^{-\Delta Ct}$, where Ct is the difference in threshold cycle (Ct) between the gene of interest and the mean of at least two reference genes. b) The pre- treatment level of TS has been zeroed and the relative up or down regulation for each sample can be seen.

(a)



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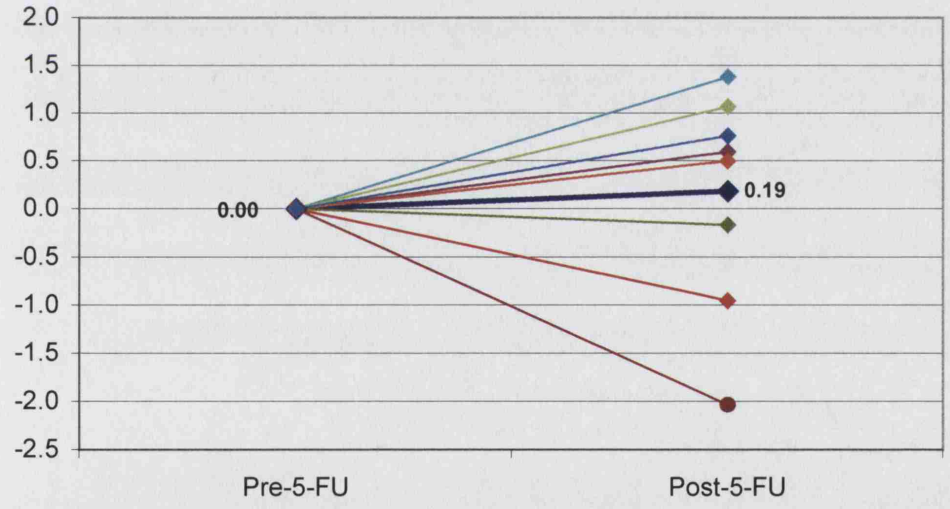
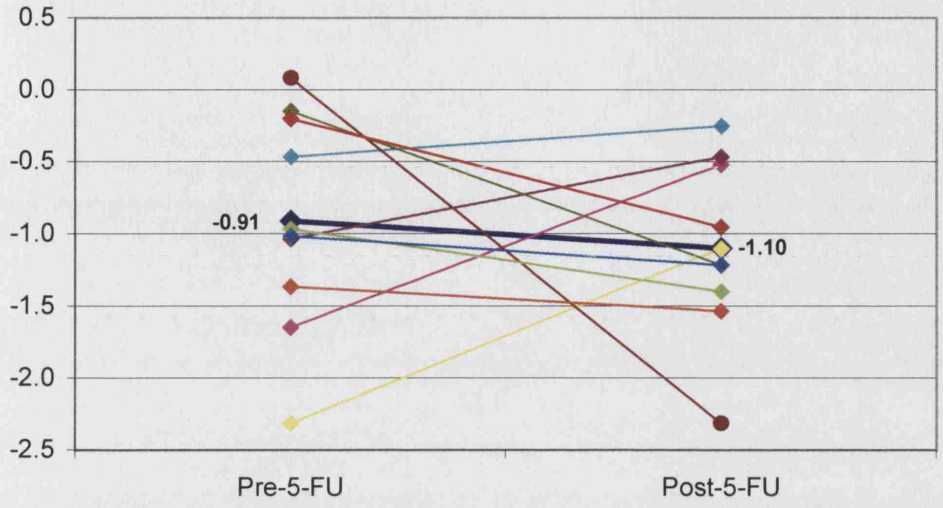


Figure 6.14. a) Relative expression of TP pre- and post- ex vivo treatment with 5FU given on a natural logarithmic scale: $2^{-\Delta Ct}$, where Ct is the difference in threshold cycle (Ct) between the gene of interest and the mean of at least two reference genes. b) The pre- treatment level of TP has been zeroed and the relative up or down regulation for each sample can be seen.

(a)



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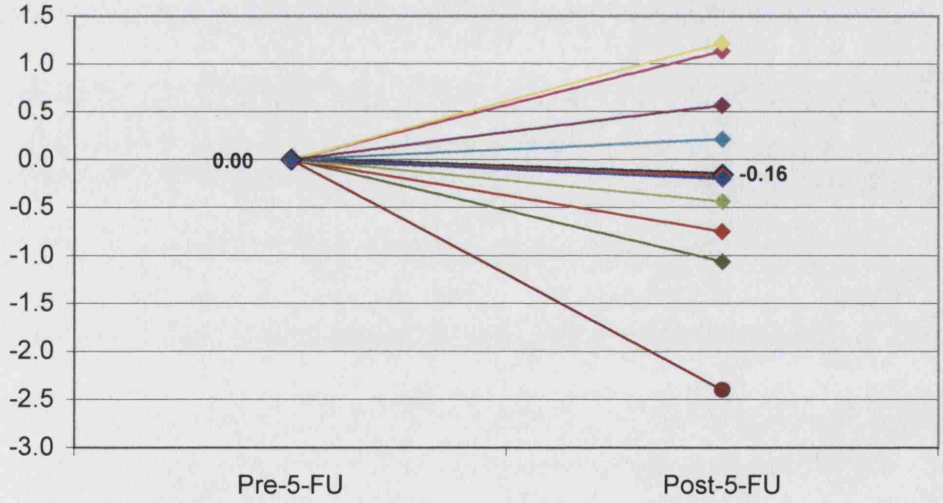
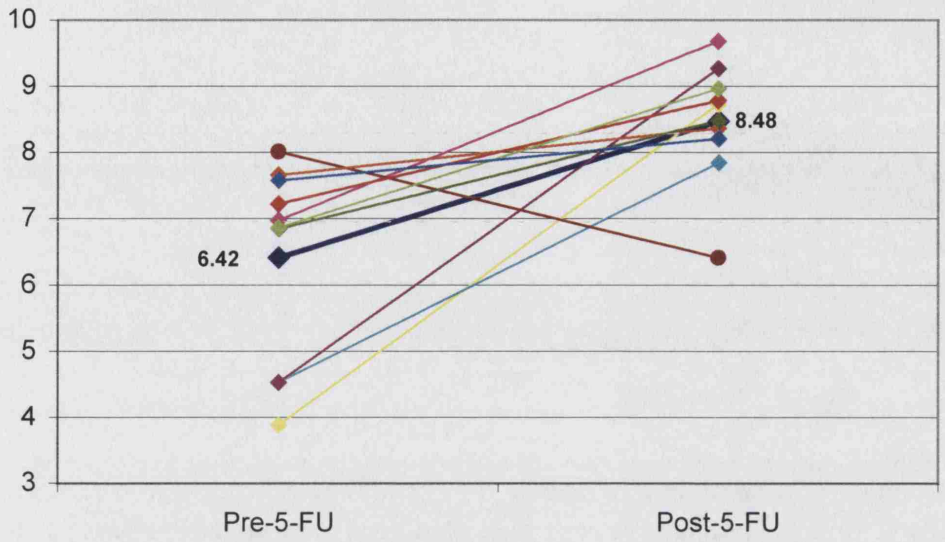


Figure 6.15. a) Relative expression of DPD pre- and post- ex vivo treatment with 5FU given on a natural logarithmic scale: $2^{-\Delta Ct}$, where Ct is the difference in threshold cycle (Ct) between the gene of interest and the mean of at least two reference genes. b) The pre- treatment level of DPD has been zeroed and the relative up or down regulation for each sample can be seen.

(a)



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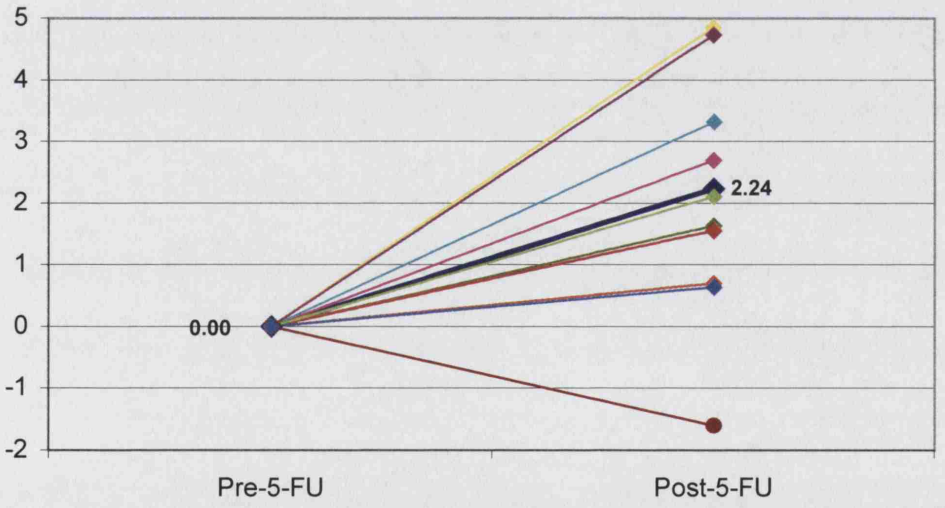

Figure 6.16. a) Relative expression of COX-2 pre- and post- ex vivo treatment with 5FU given on a natural logarithmic scale: $2^{-\Delta Ct}$, where Ct is the difference in threshold cycle (Ct) between the gene of interest and the mean of at least two reference genes. b) The pre- treatment level of COX-2 has been zeroed and the relative up or down regulation for each sample can be seen.

Table 6.6. Relative expression of mRNA levels ($2^{-\Delta Ct}$) in tumour samples after *ex-vivo* exposure to 5-FU (n=10). The p values have been calculated using non-parametric statistics (Wilcoxon matched pairs test for paired samples with a Bonferroni correction; statistical significance was taken at $p<0.005$).

Target gene	Control	5-FU	p
COX-2	121.61	391.73	**0.0039**
DPD	0.5029	0.4484	0.4922
TP	4.616	5.963	0.084
TS	2.157	7.954	**0.002**

6.4 Discussion

The heterogeneity of chemosensitivity of colorectal tumours demonstrated in chapter 4 has been further investigated in this chapter. The correlation of expression of a number of genes, determined by both immunohistochemistry and qRT-PCR, to sensitivity to 5-FU and irinotecan has been performed. There appears to be a relationship between the degree of staining for TOPO I, the target gene of irinotecan and chemosensitivity to irinotecan (IC50 and IC90) in the ATP-TCA. In addition, the qRT-PCR work revealed rapid adaptation to chemotherapy in tumour-derived cells demonstrated by up- or down-regulation of genes.

When investigating the correlation between immunohistochemistry and the ATP-TCA only TS expression was compared to sensitivity of 5-FU, TOPO I expression to sensitivity of irinotecan, and COX-2 expression to sensitivity of both single agents. This was because TP was not expressed by any samples, while BCRP and the microsatellite instability markers, hMLH1, hMSH2 and hMSH6, were expressed by all or nearly all of the samples. The molecular markers most widely investigated as predictors of response to 5-FU are TS, TP and DPD. For antibodies where almost the entire group stained positively or negatively, analysis would be futile. It was not possible to obtain a reliable DPD antibody for use with formalin fixed samples, and therefore DPD was not tested by immunohistochemistry. The H-score was used for all but the MSI markers, and a score of 100 or more used to indicate positivity. The

positive group was not sub-divided into strong or weak staining, which may have resulted in more diverse results for statistical analysis. The IHC was performed by one technician and slides inspected by one consultant histopathologist in order to reduce observer variability.

Expression of TS, TP and DPD, and combination indices for all three genes, has been found to correlate with response in gastrointestinal cancers. High levels of TS in pre-treatment samples have identified tumours that are non-responders to 5-FU therapy (Leichman *et al.*, 1997). Johnston *et al.* found that low expression of TS was significantly associated with Dukes' staging of primary rectal cancers, with a higher TS level being found in advanced Dukes' stage tumours. Expression of TS was also found to be an independent prognosticator of disease-free and overall survival, with a statistically significant survival benefit with low TS. However, adjuvant 5-FU therapy was found to significantly improve survival in the high TS group but with no difference found in the low TS group (Johnston *et al.*, 1994). In contrast, no correlation between mRNA expression of TS and response has been found in some studies, although the same increased benefit of fluorouracil treatments was found in the high TS group (Sugiyama *et al.*, 2002; Ichikawa *et al.*, 2003). Recently a systematic review and meta-analysis of TS expression has shown that colorectal tumours expressing high levels of TS appear to have a poorer overall survival compared to tumours expressing low levels (Popat *et al.*, 2004).

Johnston *et al.* later performed a retrospective analysis and found the level of TS expression in primary tumours did not correlate with survival in patients with metastatic or recurrent CRC (Johnston *et al.*, 2003). This is likely to be due to an altered molecular phenotype and clinical behaviour in the disease at the metastatic site and has been reported by others (Findlay *et al.*, 1997). However, it is also thought that TS gene polymorphisms may be important.

TP expression in cell culture and xenograft models has been shown to increase sensitivity to 5-FU and its prodrugs (Kato *et al.*, 1997, Evrard *et al.*, 1999). However, retrospective analysis of TP mRNA expression in CRC indicates that tumours with high TP levels were less likely to respond to 5-FU (Metzger *et al.*, 1998).

DPD is the rate-limiting step in 5-FU catabolism and patients deficient in the enzyme experience profound systemic toxicity to 5-FU. High levels of DPD mRNA have been shown to correlate significantly with resistance to 5-FU chemotherapy (Salonga *et al.*, 2000; Ichikawa *et al.*, 2003).

TS and DPD gene expression are independently regulated, thus combining the expression of both genes improves the prognostic value (Ishikawa *et al.*, 1999). For example, Ichikawa *et al.* found no responders in a group with high TS and high DPD levels, compared to a 75% response rate in the low TS and low DPD group, median survival 8.4 and 16.3 months respectively (Ichikawa *et al.*, 2003). Patients with low expression of all three genes, TS, TP and DPD, have been found to survive longer than patients with a high expression of any one gene (Salonga *et al.*, 2000)

ERCC-1 has been found to be an independent predictive marker of survival for 5-FU and oxaliplatin combination therapy (Shirota *et al.*, 2001). IHC was not performed for ERCC-1, and there was no correlation between mRNA levels and sensitivity to single agent 5-FU or irinotecan. As previously mentioned, no molecular results were compared with sensitivity to single agent oxaliplatin, as the sensitivity rate was only 2%. However, a general trend of down-regulation of ERCC-1 after exposure to irinotecan was found, although this did not reach statistical significance ($p=0.016$).

The IC50 and IC90 of irinotecan was statistically significantly higher in samples staining positive for TOPO I, i.e. high expression of TOPO I was associated with less sensitivity to irinotecan. This is in contrast to published data in which mutations, decreased expression and/or activity of TOPO I have been associated with camptothecin resisitance in cell lines (Saleem *et al.*, 2000; Chang *et al.*, 2002). Jansen *et al.* found neither expression of TOPO I mRNA or cellular carboxylesterase activity was predictive of the antiproliferative effects of CPT-11 or SN38 in 5 human colon cancer cell lines. However there was a correlation between DNA TOPO I activity, measured using a DNA relaxation assay, and sensitivity to CPT-11 and SN38 (Jansen *et al.*, 1997). It has therefore been suggested that gene expression levels of TOPO I may be predictive of response to therapy with irinotecan in CRC

(Iqbal and Lenz, 2001), with high expression associated with response (Paradiso *et al.*, 2001).

BCRP mRNA expression in human lung cancer cells has been found to be in proportion to the degree of resistance to SN38, with BCRP being directly involved in SN38 resistance by efflux transport of SN38 (Kawabata *et al.*, 2001). No such correlation was found here.

From these results short term *in vitro* exposure to 5-FU resulted in a significant increase in both TS and COX-2 mRNA. TP mRNA was also increased, but this did not reach statistical significance. Short-term exposure to irinotecan did not result in statistically significant changes in expression of any of the genes, although the decrease in COX-2 and the increase in ERCC-1 was marked and almost reached significance.

Immunohistochemical staining for COX-2 was not found to be an indicator of sensitivity to 5-FU or irinotecan. However, of considerable interest, *in vitro* exposure to 5-FU caused a 3-fold upregulation of COX-2 mRNA, whilst exposure to irinotecan decreased expression of COX-2 in all samples. COX-2 is expressed by 75-85% of colorectal cancers, and COX-2 inhibitors are currently under investigation for prevention and treatment for CRC. However, little is currently known about the effect exposure to cytotoxic agents has on COX-2 expression.

Forced COX-2 expression has been found to inhibit apoptosis by 5-FU, mainly via inhibition of the cytochrome c-dependent apoptotic pathway and independently of COX-2 activity (Sun *et al.*, 2002). Celecoxib, a COX-2 specific inhibitor, has been found to induce cell cycle arrest and apoptosis in cultured CRC cells independent of expression of COX-2 (Grösch *et al.*, 2001). The results in chapter 4 do not show that celecoxib alone had any anti-tumour activity.

SN38 (found to be less active than irinotecan in this thesis, chapter 3) has recently been shown to inhibit phorbol ester (PMA)-mediated induction of COX-2 in human GI cancer cell lines (Yamaguchi *et al.*, 2003). Although a direct effect of

camptothecin derivatives on COX-2 levels has not been reported, a possible link between TOPO I and COX-2 has been suggested.

Other drugs have been found to affect COX-2 expression in other cell types. Microtubule-interfering agents, in particular docetaxel, have been found to stimulate COX-2 transcription and stabilise COX-2 mRNA in human breast cells (Subbaramaiah *et al.*, 2000; Subbaramaiah *et al.*, 2003). Subsequently a selective COX-2 inhibitor has been found to enhance the efficacy of docetaxel in experimental lung cancer (Hida *et al.*, 2002). However, all these investigations used cell lines, and the expression of COX-2 was stimulated or forced.

The effect on the tumour gene expression profile in patients during the actual period of drug exposure has been investigated and reported by Clarke *et al.* (2003). Microarray gene profiling of rectal biopsies taken before and during treatment with MMC/5-FU found an inhibition of TS. Alterations in genes encoding products involved in RNA and protein synthesis and processing and cellular metabolism were also found.

These results, showing the up- and down-regulation of certain genes after short-term exposure to 5-FU or irinotecan, may have important implications for the use of sequential therapy in the treatment of cancers. It may, therefore, be possible to enhance sensitivity to second-line treatment or maintenance therapy by careful selection of first-line therapy (Kurata *et al.*, 2004). In addition, gene expression profiling may provide a mechanism basis for combination therapy of established and novel agents (Clarke *et al.*, 2003). The finding that COX-2 expression is altered by exposure to 5-FU and irinotecan is of particular interest and provides a molecular rationale for the observed efficacy of combinations of topoisomerase I inhibitors with 5-FU in gastrointestinal cancers.

Chapter 7

General Discussion and Future Work

7.1 General Discussion

The ATP-TCA has provided a system with which to investigate the differences in sensitivity and resistance of colorectal adenocarcinoma samples to a range of chemotherapeutic agents. Technical development and modification of the ATP-TCA was necessary at the start of the project in order to render it suitable for use with this tumour type. The mechanisms of resistance to cytotoxic agents are not covered in this thesis apart for a few drugs where possible mechanisms have been studied. Nevertheless, the molecular work with qRT-PCR has highlighted areas of interest that are being further investigated by members of TORC.

The results show the marked heterogeneity of chemosensitivity of colorectal cancer to both single agent and combinations of cytotoxic drugs. The drugs and combinations found effective in this assay are similar to those found to be active in clinical trials, suggesting that the ATP-TCA is able to predict sensitivity and resistance to chemotherapy in individual patients.

7.2 Technical Development

The main difficulty in applying primary cell assays in the routine pathology department is microbial contamination. This is particularly true of cells derived from colorectal samples, and is one reason why such cell-based assays have not become popular. Although most culture media contain some antibiotics, most commonly penicillin and streptomycin, a number of antibiotics, including amphotericin B, are known to affect the cytotoxicity of cytotoxic drugs. However, in this study the addition of 2.5 μg/ml amphotericin B and 1 μg/ml metronidazole did not affect the cytotoxicity of a panel of cytotoxic agents. This enabled the ATP-TCA to be performed on colorectal samples with minimal problems due to infection. This is encouraging for the development of further studies using CRC tumour-derived cells.

Most laboratory experiments substitute irinotecan with its active metabolite SN38. SN38 appeared inactive in the ATP-TCA compared to irinotecan, as has been found by other groups (Jonsson *et al.*, 2000).

7.3 Heterogeneity of Colorectal Chemosensitivity

This thesis has confirmed previous findings that considerable heterogeneity of chemosensitivity exists between different tumours of the same tumour-type (Cree *et al.*, 1999; Andreotti *et al.*, 2003). Cross-resistance to drugs with unrelated mechanisms of action may be identified using fresh tumour cells for chemosensitivity testing.

The method used for comparing sensitivity of drugs, the $Index_{SUM}$, has received some criticism, but is particularly suitable for comparison of results from these studies. Early in the history of the ATP-TCA, a considerable amount of work was done to find the best way of comparing drugs of different types between the same and different samples (Andreotti et al., 1995). The IC50 and IC90 are known to have their own limitations, as they fail to take the shape of the concentration-inhibition curve into account. Nevertheless, the IC90 remains useful as maximal inhibition less that this is unlikely to produce clinical responses according to logarithmic kill models (Goldie and Coldman, 1979).

Use of an $Index_{SUM}$ threshold of 300 to indicate sensitivity may over-estimate the sensitivity of some drugs, particularly drug combinations. It may, therefore, be more suitable to reduce the $Index_{SUM}$ threshold for drug combinations, perhaps using a cut-off of 200. The Poch or Chou and Talalay methods are useful to determine whether the effect seen is more than that expected when two drugs are added together. It must be remembered that the additive or synergistic effect of two drugs may be reflected in their side effects. Thus, if one of the drugs in a combination only offers a small increase in cytotoxicity it may be better to use the most active drug as a single agent.

7.4 Development of new drug regimens

The ATP-TCA has previously been used to assist drug and regimen development in different tumour types. The results from chapter 5 confirm that it is possible to use the ATP-TCA for drug development using *ex vivo* colorectal cancer cells. It would,

therefore, be possible to test new drugs against a range of tumour types using tumour-derived cells rather than cell lines.

Ideally all pre-clinical selection of potential drugs should be carried out on tumour-derived cells rather than cell lines because of the many differences known to exist between them (Andreotti *et al.*, 1994; Cree, 2003). However, this is not practical at the early stages of drug development as it is possible to test a much greater number of drugs against cell lines. However, once initial screening has been performed, further selection of drugs could be made using the ATP-TCA. This may streamline drugs put forward for phase I trials by eliminating inactive drugs early on and ensuring drugs are targeted towards the right tumour type. The aim is to reduce time taken for new drug development and the cost involved.

In this study, gemcitabine was found to have a synergistic effect on MMC and oxaliplatin activity. It has a low toxicity profile and is known to have some clinical activity against a number of solid malignancies, making it an attractive drug for use in combination therapy. It is probable that gemcitabine, or other drugs acting in a similar manner, may be useful as combination therapy with new or existing cytotoxic agents.

7.5 Molecular Studies

qRT-PCR and immunohistochemistry have been used to assess the expression of a number of genes. The IC50 and IC90 of irinotecan appeared to correlate with immunohistochemical expression of TOPO I. No other correlations were found between expression of genes, using IHC or qRT-PCR, and sensitivity to drugs in the ATP-TCA.

The qRT-PCR results indicate that up- or down-regulation of genes occurs rapidly after short term *ex vivo* exposure to drugs. 5-FU was found to increase COX-2 expression, a gene recently shown to be implicated in tumour cell growth. In contrast, irinotecan was found to reduce COX-2 expression. These findings have important implications in the use of sequential therapy and suggest that sensitivity to

second-line treatment may be enhanced by careful selection of first-line drugs. This concept of manipulating gene exposure and thus chemosensitivity is being further developed by members of the TORC staff.

7.6 Future work

This thesis can be looked upon as forming the basis for many further studies – its aim was to prove that the ATP-TCA can reliably be used with colorectal cancer cells with a good evaluability rate, and this has been found to be feasible. There are many directions in which further work could be undertaken, ranging from molecular to clinical studies. A few examples are suggested here, many of which are already under investigation by my colleagues and successors in TORC.

1. In the course of this thesis a drug combination has proved so effective *ex vivo* that it is currently being investigated in a phase I trial. There are many other drugs already in existence that could be tried in combination.

2. This is a very exciting time in drug development with many new agents available with novel mechanisms of action. These include drugs such as EGFR inhibitors, tyrosine kinase inhibitors and anti-angiogenic drugs. The use of these drugs in combination could be studied using the cellular and molecular mechanisms used in this thesis.

3. Cyclo-oxygenase 2 (COX-2) is already being used as a target for maintenance therapy. However, adding COX-2 inhibitors to chemotherapy may turn cells off proliferation, resulting in an antagonistic effect – this potentially useful drug therefore needs further investigation, particularly in combination with other drugs.

4. In order to determine whether the ATP-TCA can predict sensitivity or resistance to certain drugs, correlation with patient outcome is required. A number of the patients whose samples were tested in this study have received chemotherapy, but so far the number is too small to make a comparison. Because of the nature of disease in this tumour type, it is expected to be several years until correlation data is

available. The aim is to eventually perform a randomised trial of ATP-TCA versus physician choice therapy.

5. Metastases appear to have a different molecular phenotype to the primary tumour, and indeed the phenotype appears to change after exposure to chemotherapy. This means that chemosensitivity results from the primary tumour are likely to be less useful in treating relapsed disease, and it would be valuable to access metastatic material for future studies to examine this issue.

REFERENCES

- ABI PRISM 7700 Sequence Detection System User Bulletin #2. Relative Quantitation of Gene Expression (P/N 4303859B). 1997 (10/2001 update); 11-15
- ABPI Compendium 1996 pp 856-857
- ABPI Compendium 1996 pp 1135-6
- Adam R, Avisar E, Ariche A, Giachetti S, Azoulay D, Castaing D, Kunstlinger, Levi F, Bismuth F. Five-year survival following hepatic resection after neoadjuvant therapy for nonresectable colorectal (liver) metastases. Ann Surg Oncol 2001; 8(4):347-353
- Adlard JW, Richman SD, Seymour MT, Quirke P. Prediction of the response of colorectal cancer to systemic therapy. Lancet Oncol 2002; 3:75-82
- Advanced Colorectal Cancer Meta-Analysis Project. Modulation of Fluorouracil by Leucovorin in patients with advanced colorectal cancer: Evidence in terms of response rate. J Clin Oncol 1992; 10(6):896-903
- Ahnen DJ, Feigl P, Quan G, Fenoglio-Preiser C, Lovato LC, Bunn PA Jr, Stemmerman G, Wells JD, Macdonald JS, Meyskens FL Jr. Ki-ras mutation and p53 overexpression predict the clinical behaviour of colorectal cancer: a Southwest Oncology Group study. Cancer Res 1998; 58:1149-58
- Alberts DS, Chen HSG. Tabular summary of pharmacokinetic parameters relevant to *in vitro* drug assay. In: S Salmon (ed.), Human Tumour Stem Cells, pp.351-59. New York: Alan R Liss, 1980
- Andre T, Boni C, Mounedji-Boudiaf L, Navarro M, Tabernero J, Hickish T, Topham C, Zaninelli M, Clingan P, Bridgewater J, Tabah-Fisch I, de Gramont A; Multicenter International Study of Oxaliplatin/5-Fluorouracil/Leucovorin in the Adjuvant Treatment of Colon Cancer (MOSAIC) Investigators. Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. N Engl J Med 2004; 350:2343-51
- Andreotti PE, Linder D, Hartmann DM, Cree IA, Pazzagli M, Bruckner HW. TCA-100 Tumour Chemosensitivity Assay: Differences in sensitivity between cultured tumour cell lines and clinical studies. J Biolumin Chemilumin 1994; 9:373-378
- Andreotti PE, Cree IA, Kurbacher CM, Hartmann DM, Linder D, Harel G, Gleiberman I, Caruso PA, Ricks SH, Untch M, Sartori C, Bruckner HW. Chemosensitivity testing of human tumours using a microplate adenosine triphosphate luminescence assay: clinical correlation for cisplatin resistance of ovarian carcinoma. Cancer Res 1995; 55:5276-5282

- Andreotti PE, Caceres G, Zamkina R, Dauphinee M. Luminescence applications for chemotherapeutic drug development. Recent Results Cancer Res 2003; 161:3012
- Araki Y, Isomoto H, Matsumoto A, Kaibara A, Yasunaga M, Hayashi K, Yatsugi H, Yamauchi K. An in vitro chemosensitivity test for colorectal cancer using collagen-gel droplet embedded cultures. Kurume Med J 1999; 46(3-4):163-6
- Asano TK, McLeod RS. Dietary fibre for the prevention of colorectal adenomas and carcinomas (Cochrane Review). In: The Cochrane Library, Issue 1 2003. Oxford: Update Software
- Aschele C, Debernardis D, Casazza S, Antonelli G, Baldo C, Lionetto R, Maley F, Sobero A. Immunohistochemical quantitation of thymidylate synthase expression in colorectal cancer metastases predicts for clinical outcome to fluorouracil-based chemotherapy. J Clin Oncol 1999; 17:1760-1767
- Assem M, Bonvalot S, Beltramo JL, Garrido C, Dimanche-Boitrel MT, Genne P, Rebibou JM, Caillot D, Chauffert B. Deleterious effect of serum proteins on the amphotericin B-induced potentiation of cisplatin in human colon cancer cells. Br J Cancer 1994; 70:631-5
- Astler VB, Coller FA. The prognostic significance of direct extension of carcinoma of the colon and rectum. Ann Surg 1954; 139:846-51
- Atkin WS, Saunders BP. Surveillance guidelines after removal of colorectal adenomatous polyps. Gut 2002; 51(Suppl V):v6-v9
- Aung TT, Davis MA, Ensminger WD, Lawrence TS. Interaction between gemcitabine and mitomycin-C in vitro. Cancer Chemother Pharmacol 2000; 45:38-42
- Bardakji Z, Jolivet J, Langelier Y, Besner JG, Ayoub J. 5-Fluorouracil-metronidazole combination therapy in metastatic colorectal cancer. Clinical, pharmacokinetic and in vitro cytotoxicity studies. Cancer Chemother Pharmacol 1986; 18(2):140-144
- Bardou M, Montembault S, Giraud V, Balian A, Borotto E, Houdayer C, Capron F, Chaput J-C, Naveau S. Excessive alcohol consumption favours high risk polyp or colorectal cancer occurrence among patients with adenomas: a case control study. Gut 2002; 50:38-42
- Baron JA, Beach M, Mandel JS, Van Stolk RU, Haile RW, Sandler RS, Rothstein R, Summers RW, Snover DC, Beck GJ, Bond JH, Greenberg ER. Calcium supplements for the prevention of colorectal adenomas. N Eng J Med 1999; 340:101-7

- Barone LR, Tew KD. Glutathione and Glutathione-associated enzymes in multidrug resistance. Multidrug resistance in cancer cells. Ed Gupta S & Tsururo T. Wiley & Sons Ltd. 1996;151-176
- Beahrs OH, Meyers MD. American Joint Committee on Cancer. Manual for Staging of Cancer, 2nd edn. Philadelphia, PA: Lippincott, 1983
- Becerra CR, Frenkel EP, Ashfaq R, Gaynor RB. Increased toxicity and lack of efficacy of rofecoxib in combination with chemotherapy for treatment of metastatic colorectal cancer: A phase II study. Int J Cancer 2003; 105:868-872
- Bécouran Y, Ychou M, Ducreux M, Borel C, Bertheault-Cvitovic F, Seitz JF, Nasca S, Nguyen TD, Paillot B, Raoul JL, Duffour J, Fandi A, Dupont-Andre G, Rougier P. Phase II trial of oxaliplatin as first-line chemotherapy in metastatic colorectal cancer patients. J Clin Oncol 1998; 16:2739-2744
- Bécouran Y, Gamelin E, Coudert B, Négrier S, Pierga J-Y, Raoul J-L, Provençal J, Rixe O, Krisch C, Germa C, Bekradda M, Mignard D, Mousseau M. Randomized multicentre phase II study comparing a combination of fluorouracil and folinic acid and alternating irinotecan and oxaliplatin with oxaliplatin and irinotecan in fluorouracil-pretreated metastatic colorectal cancer patients. J Clin Oncol 2001; 19(22):4195-4201
- Benson AB 3rd, Schrag D, Somerfield MR, Cohen AM, Figueredo AT, Flynn PJ, Krzyzanowska MK, Maroun J, McAllister P, Van Cutsem E, Brouwers M, Charette M, Haller DG. American Society of Clinical Oncology recommendations on adjuvant chemotherapy for stage II colon cancer. J Clin Oncol 2004; 22(16):3408-19
- Bellamy WT. Prediction of response to drug therapy of cancer. A review of in vitro assays. Drugs 1992; 44:690-708
- Birbeck KF, Macklin CP, Tiffin NJ, Parsons W, Dixon MF, Mapstone NP, Abbott CR, Scott N, Finan PJ, Johnston D, Quirke P. Rates of circumferential resection margin involvement vary between surgeons and predict outcomes in rectal cancer surgery. Ann Surg 2002; 235:449-57
- Blumberg D, Paty PB, Guillem JG, Picon AI, Minsky BD, Wong WD, Cohen AM. All patients with small intramural rectal cancers are at risk for lymph node metastases. Dis Colon Rectum 1999; 42:881-885
- Bodmer WF, Bailey CJ, Bodmer J, Bussey HJR, Ellis A, Gorman P, Lucibello FC, Murday VA, Rider SH, Scambler P, Sheer D, Solomon E, Spurr NK. Localization of

the gene for familial adenomatous polposis on chromosome 5. Nature 1987; 328:614-6-6

- Bombardier C, Laine L, Reicin A, et al. Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis. VIGOR Study Group. N Engl J Med 2000; 343:1520-8
- Bosanquet AG. In vitro sensitivity testing for the individual patient: An ideal adjunct to current methods of treatment choice. Clin Oncol 1993; 5:195-197
- Boyum A. Isolation of mononuclear cells and granulocytes from human blood. Scand J Clin Lab Invest Suppl. 1968; 97:77-89
- Braakhuis BJ, Ruiz van Haperen VW, Welters MJ, Peters GJ. Schedule-dependent therapeutic efficacy of the combination of gemcitabine and cisplatin in head and neck cancer xenografts. Eur J Cancer 1995; 31A:2335-40
- Brower M, Ashbury R, Kramer Z, Pandya K, Bennet M. Adjuvant chemotherapy for colorectal cancer in the elderly: population-based experience. Proc ASCO 1993; #195
- Buess G. Review: Transanal endoscopic microsurgery (TEM). J R Coll. Surg. Edinb. 1993; 38:239-245
- Burgess DS, Hastings RW, Summers KK, Hardin TC, Rinaldi MG. Pharmacodynamics of fuconazole, itraconazole, and ampotericin B against Candida albicans. Diagn Microbiol Infect Dis 2000; 36:13-18
- Burkitt DP. Possible relationships between bowel cancer and dietary habits. Proc R Soc Med 1971; 64:964-5
- Buyse M, Thirion P, Carlson RW, Burzykowski T, Molenberghs G, Piedbois P. Relation between tumour response to first-line chemotherapy and survival in advanced colorectal cancer: a meta-analysis. Meta-Analysis Group in Cancer. Lancet 2000; 356:373-78
- Buyse M, Piedbois P. Should Dukes' B patients receive adjuvant therapy? A statistical perspective. Semin Oncol 2001; 28(Suppl 1):20-4
- Cardenal F, Lopez-Cabrerizo MP, Anton A, Alberola V, Massuti B, Carrato A, Barneto I, Lomas M, Garcia M, Lianes P, Montalar J, Vadell C, Gonzalez-Larriba JL, Nguyen B, Artal A, Rosell R. Randomized phase III study of gemcitabine-cisplatin versus etoposide-cisplatin in the treatment of locally advanced or metastatic non-small-cell lung cancer. J Clin Oncol 1999; 17(1):12-8

- Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB. Evaluation of tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. Cancer Res 1987; 47(4):936-42
- Carmichael J, Possinger K, Phillip P, Beykirch M, Kerr H, Walling J, Harris AL. Advanced breast cancer: a phase II trial with gemcitabine. J Clin Oncol 1995; 13(11):2731-6
- Carmichael J, Popiela T, Radstone D, Falk S, Borner M, Oza A, Skovsgaard T, Munier S, Martin C. Randomized comparative study of tegafur/uracil and oral leucovorin versus parenteral fluorouracil and leucovorin in patients with previously untreated metastatic colorectal cancer. J Clin Oncol 2002; 20(17):3617-27
- Cassidy J, Scheithauer W, McKendrick J, Kröning H, Nowacki MP, Seitz JF, Twelves C, Van Hazel G, Wong A, Díaz-Rubio E, On Behalf Of X-act Study Investigators. Capecitabine (X) vs bolus 5-FU/leucovorin (LV) as adjuvant therapy for colon cancer (the X-ACT study): efficacy results of a phase III trial (abstract). J Clin Oncol 2004; 22(14S): #3509
- Chang J-Y, Liu J-F, Juang S-H, Liu T-W, Chen L-T. Novel mutation of topoisomerase I in rendering cells resistant to camptothecin. Cancer Res 2002; 62:3716-3721
- Chen W-S, Lui J-H, Liu JM, Lin J-K. Sequence-dependent effect of a cyclooxygenase-2 inhibitor on topoisomerase I inhibitor and 5-fluorouracil-induced cytotoxicity of colon cancer cells. Anti-Cancer Drugs 2004; 15:287-294
- Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 1984; 22:27-55
- Christman K, Kelson D, Saltz L, Tarassoff PG. Phase I trial of gemcitabine in patients with advanced gastric cancer. Cancer 1994; 73:5-7
- Colorectal Cancer Collaborative Group. Palliative chemotherapy for advanced colorectal cancer: systematic review and meta-analysis. BMJ 2000; 321:531-535
- Cree IA, Pazzagli M, Mini E, Mazzei T, Hunter EM, Sutherland LA, Pinzani P, Gerli A, Andreotti PE. Methotrexate Chemosensitivity by ATP luminescence in human leukaemia cell lines and in breast cancer primary cultures: comparison of the TCA-100 assay with a clonigenic assay. Anti-Cancer Drugs 1995; 6:398-404
- Cree IA, Kurbacher CM, Untch M, Sutherland LA, Hunter EMM, Subedi AMC, James EA, Dewar JA, Preece PE, Andreotti PE, Bruckner HW. Correlation of the

clinical response to chemotherapy in breast cancer with ex vivo chemosensitivity testing. Anti-Cancer Drugs 1996; 7:630-635

- Cree IA and Andreotti PE. Measurement of cytotoxicity by ATP-based luminescence assay in primary cell cultures and cell lines. Toxicology in Vitro 1997; 11:553-556
- Cree IA, Kurbacher CM. Individualizing chemotherapy for solid tumours – is there any alternative? Anti-Cancer Drugs 1997; 8:541-8
- Cree IA, Neale MH, Myatt NE, de Takats PG, Hall P, Grant J, Kurbacher CM, Reinhold U, Neuber K, MacKie RM, Chana J, Weaver PC, Khoury GG, Satori C, Andreotti PE. Heterogeneity of chemosensitivity of metastatic cutaneous melanoma. Anti-Cancer Drugs 1999; 10:437-444
- Cree IA, Knight L, Di Nicolantonio F, Sharma S, Gulliford T. Chemosensitization of solid tumours by modulation of resistance mechanisms. Curr Opin Invest Drugs 2002; 3(4):634-640
- Cree IA. Chemosensitivity testing as an aid to anti-cancer drug and regimen development. Recent Results Cancer Res 2003; 161:119-25
- Cromheecke M, de Jong KP, Hoekstra HJ. Current treatment for colorectal cancer metastatic to the liver. Eur J Surg Oncol 1999; 25:451-463
- Crouch SP, Kazlowski R, Slater KJ, Fletcher J. The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. J Immunol. Methods 1993; 160:81-8
- Cunningham D, Pyrhonen S, James RD, Punt CJA, Hicklish TF, Heikkila R, Johannesen B, Starkhammer H, Topham CA, Awad L, Jacques C, Herait P. Randomised trial of Irinotecan plus supportive care versus supportive care alone after fluorouracil failure for patients with metastatic colorectal cancer. Lancet 1998; 352:1418-8
- Cunningham D, Zalcberg J, Maroun J, James R, Clarke S, Maughan TS, Vincent M, Schulz J, Gonzalez Baron M, Facchini T. Efficacy, tolerability and management of raltitrexed (Tomudex) monotherapy in patients with advanced colorectal cancer. A review of phase II/III trials. Eur J Cancer 2002; 38(4):478-86
- Cunningham D, Humblet Y, Siena S, Khayat D, Bleiberg H, Santoro A, Bets D, Mueser M, Harstrick A, Verslype C, Chau I, Van Cutsem E. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. N Engl J Med 2004; 351(4):337-45

- Curley SA Izzo F, Delrio P, Ellis LM, Granchi J, Vallone P, Fiore F, Pignata S, Daniele B, Cremona F. Radiofrequency ablation of unresectable primary and metastatic hepatic malignancies. Ann Surg 1999; 1:1-8
- Danenberg K, Salonga D, Park JM, Leichman CG, Leichman L, Johnson M, Diasio R, Danenberg P. Dihydropyrimidine dehydrogenase (DPD) and thymidylate synthase (TS) gene expressions identify a high percentage of colorectal tumours responding to 5-fluorouracil (5-FU). Proc ASCO 1998; #992
- Danks MK, Morton CL, Pawlik CA, Potter PM. Overexpression of a rabbit liver carboxylesterase sensitises human tumour cells to CPT-11. Cancer Res 1998; 58:20-22
- de Graaf EJR, Doornebosch PG, Stassen LPS, Debets JMH, Tetteroo GWM, Hop WCK. Transanal endoscopic microsurgery for rectal cancer. Eur J Cancer 2002; 38:904-910
- de Gramont A, Bosset JF, Milan C, Rougier P, Bouche O, Etienne PL, Morvan F, Louvet C, Guillot T, Francois E, Bedenne L. Randomized trial comparing monthly low-dose leucovorin and fluorouracil bolus with bimonthly high-dose leucovorin and fluorouracil bolus plus continuous infusion for advanced colorectal cancer: a French intergroup study. J Clin Oncol 1997; 15(2):808-15
- de Gramont A, Figer A, Seymour M, Homerin M, Hmissi A, Cassidy J, Boni C, Cortes Funes H, Cervantes A, Freyer G, Papamichael D, Le Bail N, Louvet C, Hendler D, de Braud F, Wilson C, Morvan F, Bonetti A. Leucovorin and fluroruracil with or without Oxaliplatin as first-line treatment in advanced colorectal cancer. J Clin Oncol 2000; 18:2938-2947
- Del Poeta M, Chen S-F, Von Hoff D, Dykstra CC, Wani MC,Manikumar G, Heitman J, Wall ME, Perfect JR. Comparison of in vitro activities of Camptothecin and nitidine derivatives against fungal and cancer cells. Antimicrob Agents Chemother 1999; 43(12):2862-2868
- Denoix P. French Ministry of Public Health National Institute of Hygeine Monograph No. 4. Paris, 1954
- Diaz-Rubio E, Sastre J, Zaniboni A, Labianca R, Cortes-Funes H, de Braud F, Boni C, Benavides M, Dallavalle G, Homerin M. Oxaliplatin as a single agent in previously untreated colorectal carcinoma patients: A phase II multicentre study. Ann Oncol 1998; 9:105-108

- Di Nicolantonio F, Neale MH, Knight LA, Lamont A, Skailes GE, Osborne RJ, Allerton R, Kurbacher CM, Cree IA. Use of an ATP-based chemosensitivity assay to design new combinations of high-concentration Doxorubicin with other drugs for recurrent ovarian cancer. Anti-Cancer Drugs 2002; 13:625-630
- Di Nicolantonio F, Neale M, Onadim Z, Hungerford JL, Kingston JL, Cree IA. The chemosensitivity profile of retinoblastoma. Recent Results Cancer Res 2003; 161:73-80
- Di Nicolantonio F, Knight LA, Di Palma S, Sharma S, Whitehouse PA, Mercer SJ, Charlton PA, Norris D, Cree IA. *Ex vivo* characterization of XR11576 (MLN576) against ovarian cancer and other solid tumors. Anti-Cancer Drugs 2004a; 15(9):849-60
- Di Nicolantonio F, Knight LA, Glaysher S, Whitehouse PA, Mercer SJ, Sharma S, Mills L, Prin A, Johnson P, Charlton PA, Norris D,Cree IA. *Ex vivo* reversal of chemoresistance by tariquidar (XR9576). Anti-Cancer Drugs 2004b; 15(9):861-9
- Di Nicolantonio F, Knight LA, Whitehouse PA, Mercer SJ, Sharma S, Charlton PA, Norris D, Cree IA. The ex vivo characterization of XR5944 (MLN944) against a panel of human clinical tumor samples. Mol Cancer Ther. 2004c: 3(12):1631-7
- Dohmoto M. New method – endoscopic implantation of rectal stent in palliative treatment of malignant stenosis. Endoscopica Digestiva 1991; 3:1507-12
- Douglass HO Jr, Moertel CG, Mayer RJ, Thomas PR, Lindblad AS, Mittleman A, Stablein DM, Bruckner HW. Survival after postoperative combination treatment of rectal cancer. New Engl J Med 1986; 315:1294-1299
- Douillard JY, Cunningham D, Roth AD, Navarro M, James RD, Karasek P, Jandik P, Iveson T, Carmichael J, Alakl M, Gruia G, Awad L, Rougier P. Irinotecan combined with 5-Fluorouracil compared with 5-Fluorouracil alone as first line treatment for metastatic colorectal cancer. A multicentre randomised trial. Lancet 2000; 355:1041-7
- Douillard JY, Hoff PM, Skillings JR, Eisenberg P, Davidson N, Harper P, Vincent MD, Lembersky BC, Thompson S, Maniero A, Benner SE. Multicenter phase III study of uracil/tegafur and oral leucovorin versus fluorouracil and leucovorin in patients with previously untreated metastatic colorectal cancer. J Clin Oncol 2002; 20(17):3605-16

- Dukes C. The classification of cancer or the rectum. J Pathol Bacteriol 1932; 35:323-32
- Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, Dubois RN. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenocarcinomas and in azoxymethane induced colonic tumours in rats. Gut 1994; 107(4):1183-8
- Elsaleh H, Powell B, McCaul K, Grieu F, Grant R, Joseph D, Iacopetta B. P53 alteration and microsatellite instability have predictive value for survival benefit from chemotherapy in stage III colorectal carcinoma. Clin Cancer Res 2001; 7:1343-9
- Eltabbakh GH. Piver MS, Hempling RE, Recio FO, Lele SB, Marchetti DL, Baker TR, Blumenson LE. Correlation between extreme drug resistance assay and response to primary paclitaxel and cisplatin in patients with epithelial ovarian cancer. Gynecol Oncol 1998; 70(3):392-7
- Evrard A, Cuq P, Ciccolini J, Vian L, Cano JP. Increased cytotoxicity and bystander effect of 5-fluorouracil and 5-deoxy-5-fluorouridine in human colorectal cancer cells transfected with thymidine phosphorylase. Br J Cancer 1999; 80:1726-1733
- Fain SN, Patin CS, Morgenstern L. Use of mechanical suturing apparatus in low colorectal anastomosis. Archives of Surg 1975; 110(9):1079-82
- Faivre S, Raymond E, Woynarowski JM, Cvitkovic E. Supraadditive effect of 2',2'-difluorodeoxycytidine (gemcitabine) in combination with oxaliplatin in human cancer cell lines. Cancer Chemother Pharmacol 1999; 44:117-123
- Fata F, Mirza A, Wood GC, Nair S, Law A, Gallagher J, Ellison N, Bernath A. Efficacy and toxicity of adjuvant chemotherapy in elderly patients with colon carcinoma. Cancer 2002; 94:1931-8
- Ferguson PJ, Currie C, Vincent MD. Enhancement of platinum-drug cytotoxicity in a human head and neck squamous cell carcinoma line and its platinum-resistant variant by liposomal Amphotericin B and phospholipase A2-II. Drug Met and Dis 1999; 27:1399-1405
- Figueras J, Valls C, Rafecas A, Fabregat J, Ramos E, Jaurieta E. Resection rate and effect of postoperative chemotherapy on survival after surgery for colorectal liver metastases. Br J Surgery 2001; 88:980-985

- Findlay MPN, Cunningham D, Morgan G, Clinton S, Hardcastle A, Aherne GW. Lack of correlation between thymidylate synthase levels in primary colorectal tumours and subsequent response to chemotherapy. Br J Cancer 1997; 75(6):903-909
- Fink D, Nebel S, Aebi S, Zheng H, Cenni B, Nehme A, Christen RD, Howell SB The role of DNA mismatch repair in platinum drug resistance. Cancer Res 1996; 56(21):4881-6
- Fisher B, Wolmark N, Rockette H, Redmond C, Deutsch M, Wickerham DL, Fisher ER, Caplan R, Jones J, Lerner H, Gordon P, Feldman M, Cruz A, Legault-Poisson S, Wexler M, Lawrence W, Robidoux A, Other NSABP Investigators. Postoperative adjuvant chemotherapy or radiation therapy for rectal cancer: results from NSABP protocol R-01. J Natl Cancer Inst 1988; 80:21-29
- Fodde R, Smits R, Clevers H. APC, signal transduction and genetic instability in colorectal cancer. Nature Reviews (Cancer) 2001; 1:55-67
- Fong Y, Fortner J, Sun RL, Brennan MF, Blumgart LH. Clinical score for predicting recurrence after hepatic resection for metastatic colorectal cancer. Analysis of 1001 consecutive cases. Ann Surg 1999; 230:309-321
- Fridborg H, Nygren P, Dhar S, Csoka K, Kristensen J, Larsson R. *In vitro* evaluation of new anticancer drugs, exemplified by vinorelbine, using the fluorometric microculture cytotoxicity assay on human tumour cell lines and patient biopsy cells. J Exp Ther Oncol 1996; 1(5):286-95
- Fruehauf JP, Bosanquet AG. In vitro determination of drug response: A discussion of clinical applications. Principles & Practice of Oncology 1993; 7(12):1-16
- Fruehauf J. Prospective analysis of association between in vitro extreme drug resistance assay results and clinical outcomes for ovarian cancer patients. Proc ASCO 2001; #2529
- Fuchs CS, Giovannucci EL, Colditz GA, Hunter DJ, Stampfer MJ, Rosner B, Speizer FE, Willett WC. Dietary Fiber and the risk of colorectal cancer and adenoma in women. New Eng J Med 1999; 340:169-176
- Furukawa T, Kubota T, Hoffman RM. Clinical applications of the histoculture drug response assay. Clin Cancer Res 1995; 1:305-11
- Ganu UK, Khandalekar DD, Gokhale SV. Inability of Amphotericin B (Fungizone) or of Sodium Deoxycholate to alter natural drug resistance or sensitivity of transplanted murine leukaemias. Ind J Physiol Pharmac 1984; 28:21-26

- Gasparini G, Longon R, Sarmiento R, Morabito A. Inhibitors of cyclo-oxygenase 2: a new class of anticancer agents? Lancet Oncol 2003; 4:605-15
- Gastrointestinal Tumour Study Group. Prolongation of the disease-free interval in surgically treated rectal carcinoma. N Eng J Med 1985; 312:1465-72
- Gastrointestinal Tumour Study Group (GITSG): Radiation therapy and flurouracil with or without semustine for the treatment of patients with surgical adjuvant adenocarcinoma of the rectum. J Clin Oncol 1992; 10:549-557
- Ghadirian P, Lacroix A, Maisonneuve P, Perret C, Potvin C, Gravel D, Bernard D, Boyle P. Nutritional factors and colon carcinoma: a case-control study involving French Canadians in Montreal, Quebec, Canada. Cancer 1997; 80(5):858-64
- Giacchetti S, Itzhaki M, Adam R, Zidani R, Kunstlinger F, Brienza S, Alafaci E, Bertheault-Cvitkovic F, Jasmin C, Reynes M, Bismuth F, Misset JL, Levi F. Long-term survival of patients with unresectable colorectal cancer liver metastases following infusional chemotherapy with 5-fluorouracil, Leucovorin, Oxaliplatin and surgery. Annals of Oncology 1999; 10:663-9
- Giacchetti S, Perpoint B, Zidani R, Le Bail N, Faggiuolo R, Focan C, Chollet P, Llory JF, Letourneau Y, Coudert B, Bertheaut-Cvitkovic F, Larregain-Fournier D, Le Rol A, Walter S, Adam R, Misset JL, Leévi F. Phase III multicentre randomised trial of oxaliplatin added to chronomodulated Fluorouracil-Leucovorin as first-line treatment of metastatic colorectal cancer. J Clin Oncol 2000; 18:136-147
- Giovannucci E. An updated review on the epidemiological evidence that cigarette smoking increases risk of colorectal cancer. Cancer Epidemiol Biomark Prev 2001; 10:725-31
- Goldberg RM, Sargent DJ, Morton RF, Fuchs CS, Ramanathan RK, Williamson SK, Findlay BP, Pitot HC, Alberts SR. A randomized controlled trial of fluorouracil plus leucovorin, irinotecan, and oxaliplatin combinations in patients with previously untreated metastatic colorectal cancer. J Clin Oncol 2004; 22(1):23-30
- Goldie JH, Coldman AJ. A mathematic model for relating the drug sensitivity of tumours to their spontaneous mutation rate. Cancer treatment Reports 1979; 63:1727-1733
- Goldwasser F, Bozec L, Zeghari-Squalli N, Misset JL. Cellular pharmacology of the combination of oxaliplatin with topotecan in the IGROV-1 human ovarian cancer cell line. Anti-Cancer Drugs 1999; 10:195-201

- Goodman LS, Wintrobe MM, Dameshek W, Goodman MJ, Gilman A, McLennan MT. Nitrogen Mustard Therapy. JAMA 1946; 21:126-132
- Gray RG, Barnwell J, Hills R, McConkey C, Williams N, Kerr D, for the QUASAR Collaborative Group. QUASAR: A randomized study of adjuvant chemotherapy (CT) *vs* observation including 3238 colorectal cancer patients. J Clin Oncol 2004; 22(14S): #3501
- Greco WR, Bravo G, Parsons JC. The search for synergy: a critical review from a response surface perspective. Pharmacol Rev 1995; 47(2):331-85
- Grinnell RS. The grading and prognosis of carcinoma of the colon and rectum. Ann Surg 1939; 109:500-33
- Grösch S, Tegeder I, Niederberger E, Brautigam L, Geisslinger G. COX-2 independent induction of cell cycle arrest and apoptosis in colon cancer cells by the selective COX-2 inhibitor celecoxib. FASEB J 2001; 15:2742-2744
- Grothey A, Deschler B, Kroening H, Ridwelski K, Reichardt P, Kretzschmar A, Clemens M, Hirschmann W, Lorenz M, Asperger W, Buechele T, Schmoll H-J. Phase III study of bolus 5-fluorouracil (5-FU)/folinic acid (FA) (Mayo) vs weekly high-dose 24h 5-FU infusion/FA + oxaliplatin (OXA) (FUFOX) in advanced colorectal cancer (ACRC). Proc ASCO 2002; #512
- Gundersen GG, Cook TA. Microtubules and signal transduction. Curr Opin Cell Biol 1999; 11(1):81-94
- Haas CE, Kaufman DC, DiCenzo RC. Effects of metronidazole on hepatic CY3A4 activity. Pharmacotherapy 2001; 21(10):1192-5
- Haezenel W and Kurihara M. Studies of Japanese migrants. 1. Mortality from cancer and other diseases among Japanese in the United States. J Nat Cancer Inst 1968; 40:43-68
- Haller DG, Catalano PJ, Macdonald JS, Mayer RJ. Fluorouracil, leucovorin and levamisole adjuvant therapy for colon cancer. Five year final report of Int-0089. Proc ASCO 1998; #982
- Hamburger A, Salmon SE. Primary bioassay of human myeloma stem cells. J Clin Invest 1997; 70(4):846-54

- Hamm JT, Allegra JC. Hormonal therapy. In: Moosa AR, Schimpff SC, Robson MC, eds. Comprehensive textbook of oncology, 2nd edn. Baltimore: Williams & Wilkins 1991: 597-600
- Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000; 100:57-70
- Harrison DJ. Molecular Mechanisms of drug resistance in tumours. J Pathol 1995; 175:7-12
- Heald R. The mesorectum in rectal cancer surgery – the clue to pelvic recurrence? Br J Surgery 1982; 69:613-16
- Heriot AG, Karanjia ND. A review of techniques for liver resection. Ann R Coll Surg Eng 2002; 84:371-380
- Hida T, Kozaki K-I, Ito H, Miyaishi O, Tatematsu Y, Suzuki T, Matsuo K, Sugiura T, Ogawa M, Takahashi T, Takahashi T. Significant growth inhibition of human lung cancer cells both *in Vitro* and *in Vivo* by the combined use of selective cyclooxygenase 2 inhibitor, JTE-522, and conventional anticancer agents. Clin Cancer Res 2002; 8:2443-2447
- Hoff PM, Ansari R, Batist G, Cox J, Kocha W, Kuperminc M, Maroun J, Walde D, Weaver C, Harrison E, Burger HU, Osterwalder B, Wong AO, Wong R. Comparison of oral Capecitabine versus intravenous Fluorouracil plus Leucovorin as first-line treatment in 605 patients with metastatic colorectal cancer: Results of a randomised phase III study. J Clin Oncol 2001; 19(8):2282-2292
- Hrycyna CA, Zhang S, Ramachandra M, Ni B, Pastan I, Gottesman MM. Functional and molecular characterization of the Human Multidrug Transporter. In Multidrug Resistance. Multidrug resistance in cancer cells, Wiley & Sons Ltd, 1996; 29-38
- Hunter EM, Sutherland LA, Cree IA, Dewar JA, Preece PE, Wood RA, Linder D, Andreotti PE. Heterogeneity of chemosensitivity in human breast carcinoma: use of an adenosine triphosphate (ATP) chemiluminescence assay. Eur J Surg Oncol 1993; 19:242-249
- Hunter EM, Sutherland LA, Cree IA, Subedi AMC, Hartmann D and Andreotti PE. The influence of storage on cytotoxic drug activity in an ATP-based chemosensitivity assay. Anti-Cancer Drugs 1994; 5:171-176
- Ichikawa W, Uetake H, Shirota H, Yamada H, Nishei Z, Sugihara K, Hirayama R. Combination of dihydropyrimidine dehydrogenase and thymidylate synthase gene expressions in primary tumours as predictive parameters for the efficacy of

fluoropyrimidine-based chemotherapy for metastatic colorectal cancer. Clin Cancer Res 2003; 9(2):786-791

- Iida N, Takara K, Ohmoto N, Nakamura T, Kimura T, Wada A, Hirai M, Sakaeda T, Okumura K. Reversal effects of antifungal drugs on multidrug resistance in MDR1-overexpressing HeLa cells. Biol Pharm Bull 2001; 24(9):1032-6
- IMPACT. Efficacy of adjuvant Fluorouracil and folinic acid in colon cancer. Lancet 1995; 345:939-44
- IMPACT B2 Investigators. Efficacy of adjuvant Fluorouracil and Folinic Acid in B2 colon cancer. J Clin Oncol 1999; 17:1356-1363
- Inaba M, Tashiro T, Sato S, Ohnishi Y, Tanisaka K, Kobayashi H, Koezuka M. *In vitro-in-vivo* correlation in anticancer drug sensitivity testing using AUC-based concentrations and collage gel droplet embedded culture. Oncology 1996; 53:250-257
- Iqbal S, Lenz HJ. Determinants of prognosis and response to therapy in colorectal cancer. Curr Oncol Rep 2001; 3(2):102-8
- Ishikawa Y, Kubota T, Otani Y, Watanabe M, Teramoto T, Kumai K, Takechi T, Okabe H, Fukushima M, Kitajima M. Thymidylate synthetase and dihydropyrimidine dehydrogenase levels in gastric cancer. Anticancer Res 1999; 19(6C):5635-40
- Isshi K, Sakuyama T, Gen T, Nakamura Y, Kuroda T, Katuyama T, Maekawa Y. Predicting 5-FU sensitivity using human colorectal cancer specimens: comparison of tumour dihydropyrimidine dehydrogenase and oratate phosphoribosyl transferase activities with in vitro chemosensitivity to 5-FU. Int J Clin Oncol 2002; 7(6):335-42
- Jansen WJM, Zwart B, Hulscher STA, Giaccone G, Pinedo HM, Boven E. CPT-11 in human colon-cancer cell lines and xenografts: characterization of cellular sensitivity determinants. Int J Cancer 1997; 70:335-340
- Jarvinen R, Knekt P, Hakulinen T, Rissanen H, Heliovaara M. Dietary fat, cholesterol and colorectal cancer in a prospective study. Br J Cancer 2001; 85(3):357-61
- Jason Y. Sustained growth and three-dimensional organization of primary mammary tumour epithelial cells embedded in collagen gels. Proc Natl Acad Sci USA 1979; 76:3401-3405

- Johns LE, Houlston RS. A systematic review and meta-analysis of familial colorectal cancer risk. Am J Gastroenterol 2001; 96:2992-3003
- Johnston PG, Fisher ER, Rockette HE, Fisher B, Wolmark N, Drake JC, Chabner BA, Allegra CJ. The role of thymidylate synthase expression in prognosis and outcome of adjuvant chemotherapy in patients with rectal cancer. J Clin Oncol 1994; 12:2640-2647
- Johnston PG, Benson AB, Catalano P, Rao S, O'Dwyer PJ, Allegra CJ. Thymidylate synthase protein expression in primary colorectal cacner: lack of correlation with outcome and response to fluorouracil in metastatic disease sites. J Clin Oncol 2003; 21:815-819
- Jonsson E, Fridborg H, Nygren P, Larsson R. Synergistic interactions of combinations of topotecan with standard drugs in primary cultures of human tumor cells from patients. Eur J Clin Pharmacol 1998; 54(7):509-14
- Jonsson E, Dhar S, Jonsson B, Nygren P, Graf W, Larsson R. Differential activity of Topotecan, Irinotecan and SN-38 in fresh human tumour cell but not in cell lines. Eur J Cancer 2000; 36:2120-2127
- Kabeshima Y, Kubota T, Watanabe M, Hasegawa H, Furukawa T, Kitajima M. Clinical usefulness of chemosensitivity test for advanced colorectal cancer. Anticancer Res 2002; 22:3033-7
- Kaltenbach JP, Kaltenbach MH Lyons WB. Nigrosin as a dye for differentiating live and dead ascites cells. Experimental Cell Research 1958; 15:112-117
- Kangas L, Gronroos M, Nieminen AL. Bioluminescence of cellular ATP: a new method for evaluating cytotoxic agents *in vitro*. Med Biol 1984; 62:338-43
- Kanzawa F, Sugimoto Y, Minato K, Kasahara K, Bungo M, Nakagawa K, Fujiwara Y, Liu LF, Saijo N. Establishment of a camptothecin analogy (CPT-11)-resistant cell line of human non-small cell lung cancer: characterization and mechanism of resistance. Cancer Res 1990; 50(18):5919024
- Karanjia ND, Corder AP, Bearn P, Heald RJ. Leakage from stapled low anastomosis after total mesorectal excision for carcinoma of the rectum. Br J Surgery 1994; 81:1224-1226
- Kato Y, Matsukawa S, Muraoka R, Tanigawa N. Enhancement of drug sensitivity and a bystander effect in PC-9 cells transfected with a platelet-derived endothelial cell growth factor thymidine phosphorylase cDNA. Br J Cancer 1997; 75:506-11

- Kawabata S, Oka M, Shiozawa K, Tsukamoto K, Nakato K, Soda H, Fukuda M, Ikegami Y, Sugahara K, Yamada Y, Kamihara S, Doyle LA, Ross DD, Kohno S. Breast cacner resistance protein directly confers SN-38 resistance of lung cancer cells. Biochem and Biophys Res Comm 2001; 280:1216-1223
- Kawato Y,Furuta T, Aonuma M, Yasuoka M, Yokokura T, Matsumoto K. Antitumour activity of a camtothecin derivative, CPT-11, against human tumour xenograpfts in nude mice. Cancer Chemother Pharmacol 1991; 28:192-198
- Keepers YP, Pizao PE, Peters GJ, van Ark-Otte J, Winograd B, Pinedo HM. Comparison of the sulforhodamine B protein and tetrazolium (MTT) assays for in vitro chemosensitivity testing. Eur J Cancer 1991; 21(7):897-900
- Kern DH, Weisenthal LM. Highly specific prediction of antineoplastic drug resistance with an in vitro assay using suprapharmacologic drug exposures. J Natl Cancer Inst 1990; 82(7):582-8
- Khot UP, Wenk Lang A, Murali K, Parker MC. Systemic review of the efficacy and safety of colorectal stents. Br J Surgery 2002; 89:1096-1102
- Kikkawa F, Kojima M, Oguchi H, Maeda O, Ishikawa H, Tamakoshi K, Mizuno K, Kawai M, Suganuma N, Tomoda Y. Potentiating effect of Amphotericin B on five platinum anticancer drugs in human cis-diamminedichloroplatinum (II) sensitive and resistant ovarian carcinoma cells. Anticancer Res 1993; 13:891-6
- Klapdor R, Muller C, Seutter R, Bahlo M, Peters W, Fenner C. Improvement of survival by efficacy orientated polychemotherapy of exocrine pancreatic cancer. Anticancer Res 2000; 20:5201-5207
- Klapdor R, Bahlo M, Babinsky A. Further evidence for prolongation of survival of pancreatic cancer patients by efficacy orientated sequential polychemotherapy (EOSPC) based on serial tumor marker determinations (CA 19-9/CEA). Anticancer Res 2005; 25(3A):1687-91
- Knudson AG. Hereditary Cancer, Oncogenes, and Antioncogenes. Cancer Research 1985; 45:1437-43
- Kobayashi H. Development of a new in vitro chemosensitivity test using collagen gel droplet embedded culture and image analysis for clinical usefulness. Recent Res Cancer Res 2003; 161:48-61

- Konecny G, Crohns C, Pegram M, Felber M, Lude S, Kurbacher C, Cree IA, Hepp H, Untch M. Correlation of drug response with the ATP tumorchemosensitivity assay in primary FIGO stage III ovarian cancer. Gynecol Oncol 2000; 77:258-63.
- Kornech GV, Potter R, Selzer E, Schratter A, Ulrich-Pur H, Rogy M, Kraus G, Scheithauer W. Combined radiochemotherapy of locally advanced unresectable pancreatic adenocarcinoma with Mitomycin C plus 24-hour continuous infusional Gemcitabine. Int J Radiat Oncol Biol Phys 2001; 49:665-71
- Krook JE, Moertel CG, Gunderson LL, Wiennd HS, Collins RT, Beart RW, Kubista TP, Poon MA, Meyers WC, Mailliard JA, Twito DI, Morton RF, Veeder MH, Witzig TE, Cha S, Vidyarthi SC. Effective surgical adjuvant therapy for high-risk rectal carcinoma. N Eng J Med 1991; 324:709-15
- Kuo T, Cho CD, Halsey J, Wakelee HA, Advani RH, Ford JM, Fisher GA, Sikic BI. Phase II study of gefitinib, fluorouracil, leucovorin, and oxaliplatin therapy in previously treated patients with metastatic colorectal cancer. J Clin Oncol 2005; 23(24):5613-9
- Kurata T, Tamura K, Kaneda T, Nogami H, Uejima H, Asai G, Nakagawa K, Fukuoka M. Effect of re-treatment with gefitinib ('Iressa', ZD1839) after acquisition of resistance. Ann Oncol 2004; 15:173-4
- Kurbacher CM, Cree IA, Brenne U, Bruckner HW, Kurbacher JA, Mallmann PK, Andreotti PE, Krebs D. Heterogeneity of in vitro chemosensitivity in perioperative breast cancer cells to mitoxantrone versus Doxorubicin evaluated by a microplate ATP bioluminescence assay. Breast Cancer Res Treat 1996; 41:161-70
- Kurbacher CM, Untch M, Cree IA. A randomised trial of chemotherapy directed by a tumour chemosensitivity assay versus physician's choice in patients with recurrent platinum-resistant ovarian adenocarcinoma. Lancet website, Protocol 97PRT/1
- Kurbacher CM, Cree IA, Bruckner HW, Brenne U, Kurbacher JA, Muller K, Ackermann T, Gilster TJ, Wilhelm LM, Engel H, Mallmann PK, Andreotti PE. Use of an ex vivo ATP luminescence assay to direct chemotherapy for recurrent ovarian cancer. Anti-Cancer Drugs 1998; 9:51-57
- Larsson R. Nygren P. Prediction of individual patient response to chemotherapy by the fluorometric micoculture cytotoxicitiy assay (FMCA) using drug specific cut-off limits and a Bayesian model. Anticancer Res 1993; 13(5C):1825-9

- Lehander Martling A, Holm T, Rutqvist L-E, Moran BJ, Heald RJ, Cedermark B, for the Stockholm Colorectal Cancer Study Group and the Basingstoke Bowel Cancer Research Project. Lancet 2000; 356:93-96
- Leichman L, Lenz HJ, Leichman CG, Danenberg KD, Baranda J, Groshen S, Boswell W, Metzger R, Tan M, Danenberg PV. Quantitation of intratumoral thymidylate synthase expression predicts for disseminated colorectal response and resistance to protracted infusion of fluorouracil and weekly leucovorin. J Clin Oncol 1997; 15:3223-3229
- Leppert M, Dobbs M, Scambler P, O'Connell P, Nakamura Y, Stauffer D, Woodward S, Burt R, Hughes J, Gardener *et al.* The gene for familial polyposis coli maps to the long arm of chromosome 5. Science 1987; 238:1411-3
- Levi F, Zidani R, Misset JL. Randomised multicentre trial of chronotherapy with oxaliplatin, fluorouracil, and folinic acid in metastatic colorectal cancer. International Organization for cancer chronotherapy. Lancet 1997; 350:681-6
- Li FP, Fraumeni JF,Mulvihill JJ, Blattner WA, Dreyfus MG, Tucker MA, Miller RW. A cancer family syndrome in twenty-four kindreds. Cancer Res 1988; 48:5358-5362
- Li MC & Ross ST. Chemoprophylaxis for patients with colorectal cancer. Prospective study with five year follow-up. JAMA 1976; 235:2825-8
- Lundin A, Hasenson M, Persson J, Pousette A. Estimation of biomass in growing cell lines by adenosine triphosphate assay. Methods in Enzymology 1986;133:27-42
- Lynch HT, Rozen P, Schuelke GS. Hereditary colon cancer: polyposis and nonpolyposis variants. CA Cancer J Clin 1985; 35:95-114
- MacFarlane, Rydall RD, Heald RJ. Mesorectal excision for rectal cancer. Lancet 1993; 341;457-60
- Machover D, Diaz-Rubio E, de Gramont A, Schilf A, Gastiaburu JJ, Brienza S, Itzhaki M, Metzger G, N'Daw D, Vignoud J, Abad A, Francois E, Gamelin E, Marty M, Sastre J, Seitz JF, Ychou M. Two consecutive phase II studies of oxaliplatin (L-OHP) for treatment of patients with advanced colorectal carcinoma who were resistant to previous treatment with fluoropyrimidines. Ann Oncol 1996; 7:95-98
- Mainprize KS, Mortensen NJMcC and Warren BF. Dukes' staging is poorly understood by doctors managing colorectal cancer. Ann R Coll Surg Eng 2002; 84:23-25

- Mamounas E, Wieand S, Wolmark N, Bear HD, Atkins JN, Song K, Jones J, Rockette H. Comparative efficacy of adjuvant chemotherapy in patients with Dukes' B versus Dukes' C Colon Cancer: Results from four National Surgical Adjuvant Breast and Bowel Project Adjuvant Studies (C-01, C-02, C-03 and C-04). J Clin Oncol 1999; 17:1349-1355
- Mani S, Kugler JW, Knost JA, Sciortino DF, Gibbons J, Garcia JC, Ansari RH, Schilsky RL, Vokes EE. Phase II trial of 150-minute weekly infusion of gemcitabine in advanced colorectal cancer: minimal activity in colorectal cancer. Invest New Drugs 1998; 16:275-8
- Marijnen CAM and Glimelius B. The role of radiotherapy in rectal cancer. Eur J Cancer 2002; 38:943-952
- Markman M. Second-line treatment of ovarian cancer with single-agent gemcitabine. Semin Oncol 2002; 29(1 Suppl 1):9-10
- Martinez ME, Heddens DL, Bogert CL, Roe D, Einspahr J, Marshall JR, Alberts DS. Physical activity, body mass index, and prostaglandin E2 levels in rectal mucosa. J Natl Cancer Inst 1999: 91(11);950-3
- Martling AL, Holm T, Rutqvist LE, Moran BJ, Heald RJ, Cedemark B. Effect of a surgical training programme on outcome of rectal cancer in the County of Stockholm. Stockholm Colorectal Cancer Study Group, Basingstoke Bowel Cancer Research Project. Lancet 2000; 256(9224):93-6
- McKay JA, Lloret C, Murray GI, Johnston PG, Bicknell R, Ahmed FY, Cassidy J, McLeod HL. Application of the enrichment approach to identify purative markers of response to 5-fluorouracil therapy in advanced colorectal carcinomas. Int J Oncol 2000; 17:153-58
- Mehta RS, Bornstein R, Yu I-R, Parker RJ, McClarenm CE, Nguyen KP, Fruehauf JP. Breast cancer survival and *in vitro* tumour response in the extreme drug resistance assay. Breast Cancer Res Treat 2001; 66:225-37
- Mercer SJ, Somers SS, Knight LA, Whitehouse PA, Sharma S, Di Nicolantonio F, Glaysher S, Simon Toh S, Cree IA for the Portsmouth Upper GI Cancer Multi-Disciplinary Team. Heterogeneity of chemosensitivity of oesophageal and gastric carcinoma. Anti-Cancer Drugs 2003; 14:397-403

- Merkel S, Wein A, Gunther K, Papadopoulos T, Hohenberger W, Hermanek P. High-Risk groups of patients with stage II colon carcinoma. Cancer 2001; 92:1435-43
- Meta-analysis Group in Cancer. Efficacy of Intravenous Continuous Infusion of Fluorouracil compared with Bolus administration in Advanced Colorectal Cancer. J Clin Onc 1998; 16:301-308
- Metzger R, Danenberg K, Leichman GC, Salonga D, Schwartz EL, Wadler S, Lenz HJ, Grogher S, Leichman L, Danenberg PV. High basal level gene expression of thymidine phosphorylase (platelet-derivd endothelial cell growth factor) in colorectal tumours is associated with non-response to 5-fluorouracil. Clin Cancer Res 1998; 4:2371-2376
- Miles DW. Update on HER-2 as a target for cancer therapy Herceptin in the clinical setting. Breast Cancer Res 2001; 3:380-384
- Million Women Study Collaborators. Breast cancer and hormone-replacement therapy in the Million Women Study. Lancet 2003; 362:419-27
- Mistry P, Stewart A, Dangerfield W, Okiji S, Liddle C, Bootle D, Plumb JA, Templeton D, Charlton P. In vitro and in vivo reversal of P-Glycoprotein-mediated multidrug resistance by a novel potent modulator, XR9576. Cancer Res 2001; 61:749-758
- Moertel C, Reitemeier R, Hahn R. Mitomycin C therapy in advanced gastrointestinal cancer. JAMA 1968; 204:1045-1048
- Moertel CG, Fleming TR, Macdonald JS, Haller DG, Laurie JA, Tangen CM, Ungerleider JS, Emerson WA, Tormey DC, Glick JH, et al. Intergroup study of fluorouracil plus levamisole as adjuvant therapy for stage II/Dukes' B2 colon cancer. J Clin Oncol 1995; 13(12):2936-43
- Möllgård L, Tidefelt U, Sundman-Engberg B, Lofgren C, Paul C. In vitro chemosensitivity testing in acute non lymphocytic leukaemia using the bioluminescence ATP assay. Leuk Res 2000; 24(5):445-52
- Monks A, Scudiero D, Skehan P, Shoemaker R, Paull K, Vistica A, Hose C, Langley J, Cronise P, Vaigro-Wolff A. Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumour cell lines. J Natl Cancer Inst 1991; 83(11):757-66

- Moore DF, Pazdur R, Daugherty K Tarassoff P, Abbruzzese JL. Phase II study of gemcitabine in advanced colorectal adenocarcinoma. Invest New Drugs 1992; 10:323-325
- Moore GE, Bross DJ, Ausman R, Nadler S, Jones R, Slack N, Rimm AA. Effects of Mitomycin C (NSC-26980) in 346 patients with advanced cancer. Cancer Chem Reports 1968; 52(6):675-684
- Morikage T, Ohmori T, Nishio K, Fujuwara Y, Takeda Y, Saijo N. Modulation of Cisplatin sensitivity and accumulation by Amohotericin B in Cisplatin-resistant Human lung cancer cell lines. Cancer Res 1993; 53:3302-7
- Mostafa G, Matthews BD, Norton HJ, Kercher KW, Sing RF, Heniford BT. Influences of demographics on colorectal cancer. Am Surg 2004; 70(3):259-64
- Mulier S, Ni Y, Jamart J, Ruers T, Marchal G, Michel L. Local recurrence after hepatic radiofrequency coagulation: multivariate meta-analysis and review of contributing factors. Ann Surg 2005; 242(2):158-71
- Myatt NE, Cree IA, Kurbacher AM, Foss AJE, Hungerford JL, Plowman PN, The ex vivo chemosensitivity profile of choroidal melanoma. Anti-Cancer Drugs 1997; 8:756-762
- Nagtegaal ID, Marijnen CA, Kranenbarg EK, van de Velde CJ, van Krieken JH; Pathology Review Committee; Cooperative Clinical Investigators. Am J Surg Pathol 2002; 26(3):350-7
- Neale MH, Myatt N, Cree IA, Kurbacher CM, Foss AJE, Hungerford JL, Plowman PN. Combination chemotherapy for choroidal melanoma: ex vivo sensitivity to treosulfan with gemcitabine or cytosine arabinoside. Br J Cancer 1999; 79:1487-93
- Neale MH, Charlton PA, Cree IA. Ex vivo acticity of XR5000 against solid tumours. Anti-Cancer Drugs 2000; 11(6):471-8
- NICE. Guidance on the use of Irinotecan, Oxaliplatin and Raltitrexed for the treatment of advanced colorectal cancer – Technology Appraisal Guidance No.33. National Institute for Clinical Excellence, 2002
- NICE. Irinotecan, oxaliplatin and raltitrexed for the treatment of advanced colorectal cancer; Review of Technology Appraisal 33 – Technology Appraisal Guidance No. 93. National Institute for Clinical Excellence, 2005

- NIH consensus conference. Adjuvant therapy for patients with colon and rectal cancer. JAMA 1990; 264:1444-50
- Nishimura G, Ninomiya I, Fujimura T, Ohta T, Miwa K. Thymidine phosphorylase (TP) and dihydrogenase (DPD) levels in primary colorectal cancer correlated to response to doxifluridine (5'-DFUR) in adjuvant chemotherapy. Proc ASCO 2001; #598
- Norat T, Lukanova A, Ferrari P, Riboli E. Meat consumption and colorectal cancer risk: dose-response meta-analysis of epidemiological studies. Int J Cancer 2002; 98(2):241-56
- Nordic Gastrointestinal Tumour Adjuvant Therapy Group. Expectancy or primary chemotherapy in patients with advanced asymptomatic colorectal cancer: a randomised trial. J Clin Oncol 1992; 10(6):904-11
- Nordlinger B, Guiguet M, Vaillant JC, Balladur P, Boudjema K, Bachellier P, Jaeck D. Surgical resection of colorectal carcinoma metastases to the liver. A prognostic scoring system to improve case selection, based on 1568 patients. Association Francaise de Chirurgie. Cancer 1996; 77:1254-62
- Nygren P, Csoka K, Larsson R, Busch C, Wester K, Malmstrom PU. Activity of standard and investigational cytotoxic drugs in primary cultures of tumour cells from patients with kidney and urinary bladder carcinomas. J Urol 1999; 162(6):2200-4
- O'Connell JB, Maggard MA, Ko CY. Colon cancer survival rates with the new American Joint Committee on Cancer sixth edition staging. J Natl Cancer Inst 2004; 96(19):1420-5
- O'Connell MJ, Laurie JA, Kahn M, Fitzgibbons, RJ, Erlichman C, Shepherd L, Moertel CG, Kocha WI, Pazdur R, Wieand S, Rubin J, Vokov AM, Donohue JH, Krook JE, Figueredo A. Prospectively randomised trial of postoperative adjuvant chemotherapy in patients with high-risk colon cancer. J Clin Oncol 1998; 16:295-300
- Office for National Statistics. Mortality Statistics: cause: review of the Registrar General on deaths by cause, sex and age, in England and Wales, 1998. (DH2; no.25). London: Stationary Office, 2002
- Paradiso AV, Maiello EM, Ranieri G, Galetta D, Mangia A, Giuliani F, Silvestris N, Lorusso V, Zito A, Montemurro S, Johnston P, Collucci G. Topoisomerase-1 (Topo-I) and thymidylate synthase (TS) primary tumour expression are predictive of

response to CPT-11 in advanced colorectal cancer (CRC) patients. Proc ASCO 2001; #599

- Park DJ, Stoehlmacher J, Zhang W, Groshen S, Lenz H-J. Human thymidylate synthase gene polymorphism determines response to capecitabine chemotherapy in advanced colorectal cancer. Proc ASCO 2001; #514
- Parkin DM, Pisani P, Ferlay J. Estimates of the worldwide incidence of 25 major cancers in 1990. Int J Cancer 1999; 80:827-841
- Peters GJ, Ruiz van Haperen VW, Bergman AM, Veerman G, Smitskamp-Wilms E, van Moorsel CJ, Kuiper CM, Braakhuis BJ. Preclinical combination therapy with gemcitabine and mechanisms of resistance. Semin Oncol 1996; 23(5 Suppl 10):16-24
- Peters GJ, Bergman AM, Ruiz van Haperen VW, Veerman G, Kuiper CM, Braakhuis BJ. Interaction between cisplatin and gemcitabine in vitro and in vivo. Semin Oncol 1995; 22(4 Suppl 11):72-9
- Petersen VC, Baxter KJ, Love SB, Shepherd NA. Identification of objective pathological prognostic determinants and models of prognosis in Dukes' B colon cancer. Gut 2002; 51:65-69
- Petty RD, Sutherland LA, Hunter EM, Cree IA. Comparison of the MTT and ATP-Based assays for the measurement of viable cell number. J Biolumin Chemilumin 1995; 10:29-34
- Poch G, Reiffenstein RJ, Kock P, Pancheva SN. Uniform characterization of potentiation in simple and complex situations when agents bind to different molecular sites. Can J Physiol Pharmacol 1995; 73:1574-81
- Popat S, Matakidou A, Houlston RS. Thymidylate synthase expression and prognosis in colorectal cancer: A systematic review and meta-analysis. J Clin Oncol 2004; 22(3):529-536
- Popescu RA, Norman A, Ross PJ, Parikh B, Cunningham D. Adjuvant or Palliative chemotherapy for colorectal cancer in patients 70 years or older. J Clin Oncol 1999; 17:2412-2418
- Poulain L, Sichel F, Crouet H, Bureau F,Gauduchon P, Gignoux M, Le Talaer J-Y. Potentiation of cisplatin and carboplatin cytotoxicity by amphotericin B in different human ovarian carcinoma and malignant peritoneal mesothelioma cells. Cancer Chemother Pharmacol 1997; 40:385-90

- Presant CA, Metter GE, Multhauf P, Bertrand M, Chang F-F, Klein L, Rappaport D, Kendregan B, Mackie A. Effects of amphotericin B with combination chemotherapy on response rates and on survival in non-small cell carcinoma of the lung. Cancer Treat Rep 1984; 68:651-654
- Price T, Cunningham D, Hickish T, Tait D, Norman A, Ross PJ, Middleton G, Ford HE, Sumpter K. Phase III study of Mitomycin-C wth protracted venous infusion 5FU or circadian timed infusion 5FU in advanced colorectal cancer. Proc ASCO 1999; #1008
- Primrose JN. Treatment of colorectal metastases: surgery, cryotherapy, or radiofrequency ablation. Gut 2002; 50:1-5
- QUASAR Collaborative Group. Comparison of fluorouracil with additional levamisole, higher dose folinic acid, or both, as adjuvant chemotherapy for colorectal cancer: a randomised trial. Lancet 2000; 355:1588-95
- Quirke P, Dixon MF, Durdey P, Williams NS. Local recurrence of rectal carcinoma due to inadequate surgical resection. Lancet 1986; ii:996-99
- Rao S, Cunningham D, de Gramont A, Scheithauer W, Smakal M, Humblet Y, Kourteva G, Iveson T, Andre T, Dostalova J, Illes A, Belly R, Perez-Ruixo JJ, Park YC, Palmer PA. Phase III double-blind placebo-controlled study of farnesyl transferase inhibitor R115777 in patients with refractory advanced colorectal cancer. J Clin Oncol 2004; 22(19):3950-7
- Rees M, Plant G, Bygrave S. Late results justify resection for multiple hepatic metastases from colorectal cancer. Br J Surgery 1997; 84:1136-1140
- Rivory LP, Riou JF, Haaz MC, Sable S, Vuilhorgne M, Commercon A, Pond SM, Robert J. Identification and properties of a major plasma metabolite of irinotecan (CPT-11) isolated from the plasma of patients. Cancer Res 1996; 56(16):3689-94
- Ross P, Norman A, Cunningham D, Webb A, Iveson T, Padhani A, Prendiville J, Watson M, Massey A, Popescu R, Oates J. A prospective randomised trial of protracted venous infusion 5-fluorouracil with or without mitomycin C in advanced colorectal cancer. Ann Oncol 1997; 8(10):995-1001
- Rothenberg ML, Oza AM, Bigelow RH, Berlin JD, Marshall JL, Ramanathan RK, Hart LL, Gupta S, Garay CA, Burger BG, Le Bail N, Haller DG. Superiority of oxaliplatin and fluorouracil-leucovorin compared with either therapy alone in

patients with progressive colorectal cancer after irinotecan and fluorouracil-leucovorin: interim results of a phase III trial. J Clin Oncol 2003;21(11):2059-69

- Rougier P, Van Cutsem E, Bajetta E, Niederle N, Possinger K, Labianca R, Navarro M, Morant R, Bleiberg H, Wils J, Awad L, Herait P, Jaques C. Randomised trial of irinotecan versus fluorouracil by continuous infusion after fluorouracil failure in patients with metastatic colorectal cancer. Lancet 1998; 352:1407-12
- Rowinsky EK, Grochow LB, Ettinger DS, Sartorius SE, Lubejko BG, Chen TL, Rock MK, Donehower RC. Phase I and pharmacological study of the novel topoisomerase I inhibitor 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin (CPT-11) administered as a ninety-minute infusion every 3 weeks. Cancer Res 1994; 54:427-36
- Rusello O, Romanini A, Civalleri D, Rosso R, Nicolin A, Sobrero A. Time-dependent interactions between 5-fluorouracil and mitomycin C on a human cancer cell line, HCT-8, in vitro. Eur J Cancer Clin Oncol 1989; 25:571-2
- Saleem A, Edwards TK, Rasheed Z, Rubin EH. Mechanisms of resistance to camptothecins. Ann NY Acad Sci 2000; 922:46-55
- Salonga D, Danenberg KD, Johnson M, Metzger R, Groshen S, Tsao-Wei DD, Lenz H-J, Leichman CG, Leichman L, Diasio RB, Danenberg PV. Colorectal tumours responding to 5-Fluorouracil have low gene expression levels of Dihydropyrimidine Dehydrogenase, Thymidylate Synthase, and Thymidine Phosphorylase. Clin Cancer Res 2000; 6:1322-27
- Saltz LB, Cox JV, Blanke C, Rosen LS, Fehrenbacher L, Moore MJ, Maroun JA, Ackland SP, Locker PK, Pirotta N, Elfring GL, Miller LL. Irinotecan plus Fluorouracil and Leucovorin for metastatic colorectal cancer. Irinotecan Study Group. N Engl J Med 2000; 343:905-914
- Sandler AB, Nemunatis J, Denham C, von Pawel J, Cormier Y, Gatzemeier U, Mattson K, Manegold Ch, Palmer MC, Gregor A, Nguyen B, Niyikiza C, Einhorn KH. Phase III trial of Gemcitabine plus Cisplatin versus Cisplatin alone in patients with locally advanced or metastatic non-small-cell lung cancer. J Clin Oncol 2000; 18:122-130
- Sargent DJ, Wieand S, Benedetti J, Labianca R, Haller DG, Shepherd LE, Seitz JF, Francini G, De Gramont A, Goldberg RM. Disease-free survival (DFS) vs. overall survival (OS) as a primary endpoint for adjuvant colon cancer studies: Individual

patient data from 12,915 patients on 15 randomized trials. J Clin Oncol 2004; 22(14S): #3502

- Sartorelli AC, Booth BA. The synergistic anti-neoplastic activity of combinations of mitomycins with either 6-thioguanine or 5-fluourouracil. Cancer Res 1965; 25:1393-1400
- Sauer R, Becker H, Hohenberger W, Rodel C, Wittekind C, Fietkau R, Martus P, Tschmelitsch J, Hager E, Hess CF, Karstens JH, Liersch T, Schmidberger H, Raab R; German Rectal Cancer Study Group. Preoperative versus postoperative chemoradiotherapy for rectal cancer. N Engl J Med 2004; 351(17):1731-40
- Scheele J, Stang R, Altendorf-Hofmann A, Paul M. Resection of colorectal liver metastases. World J Surg 1995; 19:59-71
- Schilsky RL, Levin J, West WH, Wong A, Colwell B, Thirwell MP, Ansari RH, Bell WN, White RL, Yates BB, McGuirt PV, Pazdur R. Randomized, open-label, phase III study of a 28-day oral regimen of eniluracil plus fluorouracil versus intravenous fluorouracil plus leucovorin as first-line therapy in patients with metastatic/advanced colorectal cancer. J Clin Oncol 2002; 20(6):1519-26
- Selby P, Buick RN, Tannock I. A critical appraisal of the "human tumour stem-cell assay". N Engl J Med 1983; 308(3):129-134
- Sharma S, Neale MH, Di Nicolantonio F, Knight L, Whitehouse PA, Mercer SJ, Higgins BR, Lamont A, Osborne R, Hindley AC, Kurbacher CM, Cree IA. Outcome of ATP-based tumour chemosensitivity assay directed chemotherapy in heavily pre-treated recurrent ovarian carcinoma. BMC Cancer 2003; 3:19-29
- Shiff SJ, Rigas B. Nonsteroidal anti-inflammatory drugs and colorectal cancer: evolving concepts of their chemopreventive actions. Gastroenterology 1997; 113(6):1992-8
- Shirota Y, Stoehlmacher J, Brabender J, Xiong Y-P, Uetake H, Danenberg KD, Groshen S, Tsao-Wei DD, Danenberg PV, Lenz H-J. ERCC1 and thymidylate synthase mRNA levels predict survival for colorectal cancer patients receiving combination oxaliplatin and fluorouracil chemotherapy. J Clin Oncol 2001; 19:4298-304
- Silverstein FE, Faich G, Goldstein JL, Simon LS, Whelton A, Makuch R, Eisen G, Agrawal NM, Stenson WF, Burr AM, Zhao WW, Kent JD, Lefkowith JB, Verburg KM, Geis GS. Gastrointestinal toxicity with Celecoxib vs Nonsteroidal Anti-

inflammatory drugs for osteoarthritis and rheumatoid arthritis. The CLASS Study: A Randomized Controlled Trial. JAMA 2000; 284:1247-1255

- Slatter JG, Schaaf LJ, Feenstra KL, Johnson MG, Bombardt PA, Cathcart KS, Verburg MT, Pearson LK, Compton LD, Miller LL, Baker DS, Pesheck CV, Lord RS 3rd. Pharmacokinetics, metabolism, and excretion of irinotecan (CPT-11) following I.V. infusion of [(14)C]CPT-11 in cancer patients. Drug Metab Dispos 2000; 28(4):423-33
- Smith E, Lumley CE, Stratford IJ, Adams GE. Chemosensitization in vitro: potentiation of melphalan toxicity by misonidazole, metronidazole and nitrofurazone. Int J Radiat Oncol Biol Phys 1982; 8:615-7
- South and West Cancer Intellegence Unit. Wessex Colorectal Cancer Audit: Final report, 5 year outcomes. Wessex: South and West Cancer Intelligence Unit, 2000
- Speciale A, Musumeci R, Blandino G, Milazzo I, Caccamo F, Nicoletti G. Minimal inhibitory concentrations and time-kill determination of moxifloxacin against aerobic and anaerobic isolates. Int J Antimicrobial Agents 2002; 19:111-118
- Stangl R, Altendorf-Hofmann A, Charnley RM, Scheele J. Factors influencing the natural history of colorectal liver metastases. Lancet 1994; 343:1405-10
- Steinbach G, Lynch PM, Phillips RKS, Wallace MH, Hawk E, Gordon GB, Wakabayashi N, Saunders B, Shen Y, Fujimura T, Su L-K, Levin B. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. N Engl J Med 2000; 342:1946-52
- Subbaramaiah K, Hart JC, Norton L, Dannenberg AJ. Microtubule-interfering agents stimulate the transcription of cyclooxygenase-2. Evidence for involvement of ERK1/2 and p38 mitogen-activated protein kinase pathways. J Biol Chem 2000; 275(20):14838-45
- Subbaramaiah K, Marmo TP, Dixon DA, Dannenberg AJ. Regulation of cyclooxygenase-2 mRNA stability by taxanes: evidence for involvement of p38, MAPKAPK-2, and HuR. J Biol Chem 2003; 278(39):37637-47
- Sugiyama Y, Kato T, Nakazato H, Ito K, Mizuno I, Kanemitsu T, Matsumoto K, Yamaguchi A, Nakai K, Inada K-I, Tatematsu M. Retrospective study on thymidylate synthase as a predictor of outcome and sensitivity to adjuvant chemotherapy in patients with curatively resected colorectal cancer. Anti-Cancer Drugs 2002; 13:931-938

- Sun Y, Tang XM, Half E, Kuo MT, Sinicrope FA. Cyclooxygenase-2 overexpression reduces apoptotic susceptibility by inhibiting the cytochrome c-dependent apoptotic pathway in human colon cancer cells. Cancer Res 2002; 62:6323-6328
- Surtees P, Ritchie JK, Phillips RKS. High versus low ligation of the inferior mesenteric artery in rectal cancer. Br J Surgery 1990; 77:618-621
- Swedish Rectal Cancer Trial. Improved survival with preoperative radiotherapy in resectable rectal cancer. N Engl J Med 1997; 336:980-7
- Tannock IF. In vivo interaction of anti-cancer drugs with misonidazole or metronidazole: methotrexate, 5-fluorouracil and adriamycin. Br J Cancer 1980; 42(6):861-70
- Taylor CG, Sargent JM, Elgie AW, Williamson CJ, Lewandowicz GM, Chappatte O, Hill JG. Chemosensitivity testing predicts survival in ovarian cancer. Eur J Gynaecol Oncol 2001; 22(4):278-82
- Tebbutt NC, Cattell E, Midgley R, Cunningham D, Kerr D. Systemic treatment of colorectal cancer. Eur J Cancer 2002; 38:1000-1015
- Thun MJ, Namboodiri MM, Heath CWJ. Aspirin use and reduced risk of fatal colon cancer. N Eng J Med 1991; 35:1593-1596
- Tournigand C, Andre T, Achille E, Lledo G, Flesh M, Mery-Mignard D, Quinaux E, Couteau C, Buyse M, Ganem G, Landi B, Colin P, Louvet C, de Gramont A. FOLFIRI followed by FOLFOX6 or the reverse sequence in advanced colorectal cancer: a randomized GERCOR study. J Clin Oncol 2004; 22(2):229-37
- Tramer MR, Moore RA, Reynolds DJ, McQuay HJ. Quantitative estimation of rare adverse events which follow a biological progression: a new model applied to chronic NSAID use. Pain 2000; 85:169-82
- Trujillo JM, Yang LY, Gercovich G, Su YZ, Lee J. Metronidazole enhances the cytotoxic synergism produced by the combination of 1-beta-arabinofuranosylcytosine and cis-diamminedichloroplatinum. Anticancer Res 1989; 9:1751-6
- Tsujii M, Kawano S, Tsuji S, Sawaoka H, Hori M, DuBois RN. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. Cell. 1998; 93(5):705-16
- Twelves C. Capecitabine as first-line treatment in colorectal cancer: pooled data from two large, phase III trials. Eur J Cancer 2002; 38:S15-S20

- Twelves C, Wong A, Nowacki MP, Abt M, Burris H 3rd, Carrato A, Cassidy J, Cervantes A, Fagerberg J, Georgoulias V, Husseini F, Jodrell D, Koralewski P, Kroning H, Maroun J, Marschner N, McKendrick J, Pawlicki M, Rosso R, Schuller J, Seitz JF, Stabuc B, Tujakowski J, Van Hazel G, Zaluski J, Scheithauer W. Capecitabine as adjuvant treatment for stage III colon cancer. N Engl J Med 2005; 352(26):2696-704
- Umpleby HC, Williamson RC. Anastomotic recurrence in large bowel cancer. Br J Surgery 1987; 74:873-8
- Van Cutsem E, Twelves C, Cassidy J, Allman D, Bajetta E, Boyer M, Bugat R, Findlay M, Frings S, Jahn M, McKendrick J, Osterwalder B, Perez-Manga G, Rosso R, Rougier P, Schmiegel WH, Seitz J-F, Thompson P, Vieitez JM, Weitzel C, Harper P. Oral Capecitabine compared with intravenous Fluorouracil plus Leucovorin in patients with metastatic colorectal cancer: Results of a large phase III study. J Clin Oncol 2001; 19(21):4097-4106
- Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002; 3(7): research 0034.1-0034.11
- van Moorsel CJ, Veerman G, Bergman AM, Guechev A, Vermorken JB, Postmus PE, Peters GJ. Combination chemotherapy studies with gemcitabine. Semin Oncol 1997; 24(2 Suppl 7):17-23
- van Moorsel CJ, Pinedo HM, Smid K, Comijn EM, Voorn DA, Veerman G, Lakerveld B, Van der Vijgh WJ, Postmus PE, Peters GJ. Schedule-dependent pharmacodynamic effects of gemcitabine and cisplatin in mice bearing Lewis lung murine non-small cell lung tumours. Eur J Cancer 2000; 83(8):2420-9
- Vasen HF, Watson P, Mecklin JP, Lynch HT. New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative group on HNPCC. Gastroenterology 1999; 116(6):1453-6
- Veronese ML, Sun W, Giantonio B, Berlin J, Shults J, Davis L, Haller DG, O'Dwyer PJ. A phase II trial of gefitinib with 5-fluorouracil, leucovorin, and irinotecan in patients with colorectal cancer. Br J Cancer 2005; 92(10):1846-9

- Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert AC, Nakamura Y, White R, Smits MM, Bos JL. Genetic alterations during colorectal-tumour development. N Eng J Med 1988; 319:525-532
- Vogelstein B, Lane D and Levine AJ. Surfing the p53 network. Nature 2000; 408:307-310
- Von Hoff DD. He's not going to talk about in vitro predicitive assays again, is he? J Natl Cancer Inst 1990; 82(2):96-101
- Wakaki S, Marumo H, Tomioka K, Shimizu M, Kato E, Kamada H, Kudo S, Fujimoto Y. Purification and isolation study on gancidins. J Antibiot (Tokyo) 1958; 11(4):150-5
- Weisenthal LM, Marsden JA, Dill PL, Macaluso CK. A novel dye exclusion method for testing *in vitro* chemosensitivity of human tumours. Cancer Res 1983; 43:749-57
- Weisenthal LM, Kern DH. Prediction of drug resistance in cancer chemotherapy: the Kern and DiSC assays. Oncology (Huntingt) 1991; 5(9):93-103
- Whitehouse PA, Knight LA, Di Nicolantonio F, Mercer SJ, Sharma S, Cree IA, on behalf of the Portsmouth Colorectal Cancer Multidisciplinary Team. Heterogeneity of chemosensitivity of colorectal adenocarcinoma determined by a modified *ex vivo* ATP-tumour chemosensitivity assay (ATP-TCA). Anti-Cancer Drugs 2003; 14:369-375
- Wolmark N, Rockette H, Mamounas E, Jones J, Wieand S, Wickerham DL, Bear HD, Atkins JN, Dimitrov NV, Glass AG, Fisher ER, Fisher B. Clinical trial to assess the relative efficacy of fluorouracil and leucovorin, fluorouracil and levamisole, and fluorouracil, leucovorin and levamisole in patients with Dukes' B and C carcinoma of the colon: results from the National Surgical Adjuvant Breast and Bowel Project C-04. J Clin Oncol 1999; 17:3533-9
- Wolmark N, Wieand HS, Hyams DM, Colangelo L, Dimitrov NV, Romond EH, Wexler M, Prager D, Cruz AB, Gordon PH, Petrelli NJ, Deutsch M, Mamounas E, Wickerham DH, Fisher ER, Rockette H, Fisher B. Randomized trial of postoperative adjuvant chemotherapy with or without radiotherapy for carcinoma of the rectum: National Surgical Adjuvant Breast and Bowel Project Protocol R-02. J Natl Cancer Inst 2000; 92:388-96

- Wu MH, Yan B, Humerickhouse R, Dolan ME. Irinotecan activation by human carboxylesterases in colorectal adenocarcinoma cells. Clin Cancer Res 2002; 8:2696-2700
- Xu JM, Song ST, Tang ZM, Jiang ZF, Liu XQ, Zhou L, Liu XW. Predictive chemotherapy of advanced breast cancer directed by MTT assay *in vitro*. Breast Cancer Res Treat 1999; 53(1):77-85
- Yamaguchi K, Kazior A, Dannenberg AJ, Subbaramaiah K. Sn-38, a topoisomerase I inhibitor, blocks the activation of cyclooxygenase-2 transcription. Proceed Am Ass Cancer Res 2003; 44(2nd ed): Abstract #1599
- Yamaue H, Tanimura H, Nakamori M, Noguchi K, Iwahashi M, Tani M, Hotta T, Murakami K, Ishimoto K. Clinical evaluation of chemosensitivity testing for patients with colorectal cancer using MTT assay. Dis Colon Rectum 1996; 39(4):416-22
- Yoshinare K, Kubota T, Watanabe M, Wada N, Nishibori H, Hasegawa H, Kitajima M, Takechi T, Fukushima M. Gene expression in colorectal cancer and in vitro chemosensitivity to 5-fluorouracil: A study of 88 surgical specimens. Cancer Sci 2003; 94(7):633-638
- Zamboni WC, Stewart CF, Cheshire PJ, Richmond LB, Hanna SK, Luo X, Poquette C, McGovren JP, Houghton JA, Houghton PJ. Studies of the efficacy and pharmacology of irinotecan against human colon tumour xenograft models. Clin Cancer Res 1998; 4(3):743-53
- Zalcberg J, Kerr D, Seymour L, Palmer M. Haematological and Non-haematological toxicity after 5FU/FA in patients with advanced CRC and age. Eur J Cancer 1998; 34:1871-1875
- Zeghari-Squalli N, Raymond E, Cvitkovic E, Goldwasser F. Cellular pharmacology of the combination of the DNA topoisomerase I inhibitor SN-38 and the diaminocyclohexane platinum derivative oxaliplatin. Clin Cancer Res 1999; 5:1189-1196
- Zhengdong L, Duhong B. Experimental study on the mechanism of cisplatin resistance and its reversion in human ovarian cancer. Chinese Medical Journal 1996; 109(5):353-5

APPENDICES

APPENDIX A – Antibiotic Validation Raw Data

Table A1. AUC, IC50, IC90 and $Index_{SUM}$ for individual experiments on SK-MEL-28 cell line performed using 2.5μg/ml Amphotericin B added to the culture media

		AUC	IC50	IC90	$Index_{SUM}$
Cisplatin					
1	Control	10002	243	82	364
	2.5μg/ml	11364	215	67	349
2	Control	5558	297	173	479
	2.5μg/ml	6883	247	149	449
3	Control	8283	240	108	456
	2.5μg/ml	6515	227	150	427
4	Control	11728	222	48	326
	2.5μg/ml	10866	220	70	398
5-FU					
1	Control	14244	194	36	259
	2.5μg/ml	15606	137	14	210
2	Control	15736	119	11	197
	2.5μg/ml	16218	99	12	186
Gemcitabine					
1	Control	13619	275	4	170
	2.5μg/ml	14063	269	4	155
2	Control	11264	283	5	238
	2.5μg/ml	10810	374	5	248
3	Control	10960	184	120	277
	2.5μg/ml	11746	305	5	226
4	Control	14942	231	4	134
	2.5μg/ml	13269	260	4	177
MMC					
1	Control	15641	88	36	260
	2.5μg/ml	16316	79	30	238
2	Control	15819	98	25	216
	2.5μg/ml	16964	71	22	187
Oxaliplatin					
1	Control	4784	432	240	529
	2.5μg/ml	6699	363	202	472
2	Control	6882	333	169	476
	2.5μg/ml	7639	328	112	449
Topotecan					
1	Control	14823	159	18	225
	2.5μg/ml	14300	223	11	215
2	Control	16588	182	6	138
	2.5μg/ml	15417	206	6	171

Table A1 continued.

		AUC	IC50	IC90	$Index_{SUM}$
Treosulfan					
1	Control	10700	253	60	257
	2.5μg/ml	12143	248	27	328
2	Control	8630	247	137	387
	2.5μg/ml	8741	296	129	389
Vinorelbine					
1	Control	17768	75	4	93
	2.5μg/ml	17610	79	4	99
2	Control	16412	85	25	202
	2.5μg/ml	16449	82	34	200

Table A2. AUC, IC50, IC90 and $Index_{SUM}$ for individual experiments using tumour cells performed using 2.5μg/ml Amphotericin B with 1μg/ml or 2μg/ml Metronidazole added to culture media

		AUC	IC50	IC90	$Index_{SUM}$
5-FU					
02-0096	Control	13264	199	25	269
	1μg/ml	14081	185	32	261
	2μg/ml	14992	162	27	218
02-0097	Control	8080	478	49	321
	1μg/ml	13088	202	33	318
	2μg/ml	11781	203	56	389
02-0098	Control	10158	237	79	380
	1μg/ml	9941	237	81	405
02-0116	Control	12003	202	58	381
	1μg/ml	11749	199	70	345
	2μg/ml	13182	173	58	317
02-0249	Control	13906	147	41	357
	1μg/ml	13581	156	43	378
02-0357	Control	14088	124	43	326
	1μg/ml	16468	93	14	215
Gemcitabine					
02-0357	Control	12796	268	9	236
	1μg/ml	13919	243	22	295
Irinotecan					
02-0096	Control	17205	67	18	199
	1μg/ml	16318	72	33	240
	2μg/ml	16551	72	27	237
02-0097	Control	14041	161	39	290
	1μg/ml	16410	70	28	246
	2μg/ml	9923	165	85	489

Table A2 continued

		AUC	IC50	IC90	$Index_{SUM}$
Irinotecan					
02-0098	Control	14304	126	42	323
	1μg/ml	14333	124	41	325
02-0249	Control	13267	188	43	362
	1μg/ml	13250	151	69	339
Mitomycin C					
02-0249	Control	14870	203	22	256
	1μ/ml	15284	168	22	252
02-0357	Control	10260	201	50	410
	1μg/ml	10012	225	82	484
Oxaliplatin					
02-0096	Control	7255	1232	685	362
	1μg/ml	5623	869	483	434
	2μg/ml	1010	3160	1756	611
02-0097	Control	1504	684	380	620
	1μg/ml	1591	473	263	577
	2μg/ml	873	567	315	575
02-0098	Control	2130	1214	674	548
	1μg/ml	2435	845	470	561
02-0116	Control	3504	916	509	485
	1μg/ml	4087	720	400	493
	2μg/ml	3911	1139	633	592
02-0357	Control	-15907	-322	-179	1294
	1μg/ml	-12639	-309	-172	1189
	2.5μg/ml	17722	68	4	79

APPENDIX B - Colorectal Adenocarcinoma - Patient details

No.	TCA Number	Tumour Site	Age	Sex	Sample Type	Evaluability
1	TORC02-0008	colon	39	F	solid	Yes
2	TORC02-0053	rectum	61	F	solid	Yes
3	TORC02-0061	rectum	69	M	solid	Infected
4	TORC02-0071	colon	61	M	solid	Yes
5	TORC02-0080	colon	69	F	solid	Yes
6	TORC02-0096	rectum	73	M	solid	Yes
7	TORC02-0097	rectum	53	M	solid	No
8	TORC02-0098	colon	58	M	solid	Yes
9	TORC02-0108	colon	78	M	solid	Yes
10	TORC02-0116	colon	67	M	solid	Yes
11	TORC02-0121(A)	colon	87	F	solid	Yes
12	TORC02-0121(B)	colon	87	F	solid	Yes
13	TORC02-0121(N)	lymph node	87	F	solid	Yes
14	TORC02-0124	rectum	59	M	solid	Yes
15	TORC02-0129	rectum	65	M	solid	Yes
16	TORC02-0132	colon	69	M	solid	Yes
17	TORC02-0133	rectum	58	M	solid	Yes
18	TORC02-0139	colon	63	F	solid	Yes
19	TORC02-0143	colon	77	F	solid	Yes
20	TORC02-0146	colon	78	F	solid	Yes
21	TORC02-0149	colon	48	F	solid	Yes
22	TORC02-0157	colon	76	F	solid	Yes
23	TORC02-0161	colon	39	F	ascites	Yes
24	TORC02-0172	colon	75	M	solid	Yes
25	TORC02-0174	colon	73	F	solid	Yes
26	TORC02-0176	colon	57	F	Solid	Yes
27	TORC02-0181	rectum	73	M	solid	Yes
28	TORC02-0189	rectum	76	M	solid	Yes
29	TORC02-0194	colon	72	M	solid	Yes
30	TORC02-0195	rectum	74	M	solid	Yes
31	TORC02-0205	colon	74	M	solid	Yes
32	TORC02-0206	colon	80	F	solid	Yes
33	TORC02-0209	rectum	68	M	solid	Yes
34	TORC02-0214	colon	72	F	solid	Yes
35	TORC02-0215	colon	63	M	solid	Yes
36	TORC02-0221	colon	68	F	solid	Yes
37	TORC02-0229	colon	75	M	solid	No cells
38	TORC02-0248	colon	58	M	Pleural effusion	Yes
39	TORC02-0249	colon	73	M	solid	Yes
40	TORC02-0251	rectum	71	F	solid	Yes
41	TORC02-0258	colon	76	M	solid	No cells
42	TORC02-0259	rectum	58	M	solid	Yes
43	TORC02-0269	colon	73	M	solid	No cells
44	TORC02-0273	colon	76	M	solid	Yes
45	TORC02-0276	colon	64	M	solid	Yes

Appendix B continued.

No.	TCA Number	Tumour Site	Age	Sex	Sample Type	Evaluability
46	TORC02-0286	colon	69	M	solid	Yes
47	TORC02-0291	rectum	72	M	solid	Yes
48	TORC02-0292	colon	56	M	solid	Yes
49	TORC02-0303	colon	84	F	solid	Yes
50	TORC02-0306	colon	78	M	solid	Infected
51	TORC02-0307	colon	72	M	solid	Yes
52	TORC02-0309	colon	61	M	solid	Yes
53	TORC02-0312	colon	79	M	solid	No
54	TORC02-0325	colon	64	M	solid	Infected
55	TORC02-0328	colon	81	F	solid	Yes
56	TORC02-0334	colon	53	M	solid	Yes
57	TORC02-0340	rectum	52	M	solid	Yes
58	TORC02-0356	colon	75	F	solid	Yes
59	TORC02-0368	rectum	64	M	solid	Yes
60	TORC02-0370	colon	88	F	solid	Yes
61	TORC02-0385	rectal	74	F	solid	Infected
62	TORC02-0386	colon	61	F	solid	Infected
63	TORC02-0393	rectal	71	M	solid	Infected
64	TORC02-0405	colon	67	F	solid	Yes
65	TORC02-0407	rectum	76	M	solid	Yes
66	TORC03-0001	colon	70	M	solid	Yes
67	TORC03-0028	colon	75	F	solid	Yes
68	TORC03-0043	colon	74	F	solid	Infected
69	TORC03-0045	colon	71	F	solid	Infected
70	TORC03-0046	colon	71	M	solid	Yes
71	TORC03-0056	rectum	69	M	solid	Yes

PUBLICATIONS

- Whitehouse PA, Knight LA, Di Nicolantonio F, Mercer SJ, Sharma S, Cree IA. Heterogeneity of chemosensitivity of colorectal adenocarcinoma determined by a modified ex vivo ATP-tumour chemosensitivity assay (ATP-TCA). Anti-Cancer Drugs 2003; 14:369-375

- Whitehouse PA, Mercer SJ, Knight LA, Di Nicolantonio F, O'Callaghan A, Cree IA. Combination chemotherapy in advanced gastrointestinal cancers: *ex vivo* sensitivity to Gemcitabine and Mitomycin C. B J Cancer 2003; 89(12):2299-304

- Mercer SJ, Somers SS, Knight LA, Whitehouse PA, Sharma S, Di Nicolantonio F, Glaysher S, Toh S, Cree IA. Heterogeneity of Chemosensitivity of esophageal and gastric carcinoma. Anti-Cancer Drugs 2003; 14:397-403
- Sharma S, Neale MH, Di Nicolantonio F, Knight LA, Whitehouse PA, Mercer SJ, Higgins BR, Lamont A, Osborne R, Hindley AC, Kurbacher CM, Cree IA. Outcome of ATP-based tumour chemosensitivity assay directed chemotherapy in heavily pre-treated recurrent ovarian carcinoma. BMC Cancer 2003; 3:19-29
- Di Nicolantonio F, Knight LA, Di Palma S, Sharma S, Whitehouse PA, Mercer SJ, Charlton PA, Norris D, Cree IA. *Ex vivo* characterization of XR11576 (MLN576) against ovarian cancer and other solid tumors. Anti-Cancer Drugs 2004; 15(9):849-60
- Di Nicolantonio F, Knight LA, Glaysher S, Whitehouse PA, Mercer SJ, Sharma S, Mills L, Prin A, Johnson P, Charlton PA, Norris D, Cree IA. *Ex vivo* reversal of chemoresistance by tariquidar (XR9576). Anti-Cancer Drugs 2004; 15(9):861-9
- Di Nicolantonio F, Knight LA, Whitehouse PA, Mercer SJ, Sharma S, Charlton PA, Norris D, Cree IA. The ex vivo characterization of XR5944 (MLN944) against a panel of human clinical tumor samples. Mol Cancer Ther. 2004: 3(12):1631-7
- Knight LA, Di Nicolantonio F, Whitehouse P, Mercer S, Sharma S, Glaysher S, Johnson P, Cree IA. The in vitro effect of gefitinib ('Iressa') alone and in combination with cytotoxic chemotherapy on human solid tumours. BMC Cancer. 2004; 4(1):83
- Mercer SJ, Di Nicolantonio F, Knight LA, Gabriel FG, Whitehouse PA, Sharma S, Fernando A, Bhandari P, Somers SS, Toh SK, Cree IA; for the NHS Collaborative Research Programme on Predictive Oncology. Rapid up-regulation of cyclooxygenase-2 by 5-fluorouracil in human solid tumors. Anticancer Drugs. 2005; 16(5):495-500
- Di Nicolantonio F, Mercer SJ, Knight LA, Gabriel FG, Whitehouse PA, Sharma S, Fernando A, Glaysher S, Di Palma S, Johnson P, Somers SS, Toh S, Higgins B, Lamont A, Gulliford T, Hurren J, Yiangou C, Cree IA. Cancer cell adaptation to chemotherapy. BMC Cancer. 2005; 5(1):78