The Value of Regional Intra-Arterial Therapy

with

Novel Sustained-Release Ethiodol Based Preparations

for

Hepatic-Neoplastic Disease

Submitted for the Degree of Doctor of Medicine (M.D.)

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DEDICATION

"For Yasmin and Zachary, whose grace, charm, intellect and humanity continue to inspire me".

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

--	data not reported
5FU	Fluorouracil
CALGB	Cancer and leukaemia group B
CEA	Carcinoembryonic antigen
cGy	Centigray
cm	Centimetre
CT	Computed tomography
CVP	Central venous pressure
d	day
DFS	Disease free survival
dL	Decilitre
DNA	Deoxyribonucleic acid
ECOG	Eastern cooperative oncology group
EGFR	Epidermal growth factor receptor
EORTC	European organisation for research and cancer treatment
FDG	^{18}F-flourodeoxyglucose
GGT	Gamma glutamyl transferase
GI	Gastrointestinal
Gy	Gray
HAI	Hepatic arterial infusional
HLA	Human leukocyte antigen
HPLC	High-performance liquid chromatography
hrs	Hours

IV	Intravenous
IVC	Inferior vena cava
kHz	Kilohertz
LV	Leucovorin
min	Minutes
MRC	Medical research council
MSKCC	Memorial Sloan-Kettering cancer centre
mth	Month
NCCTG	North central cancer treatment group
NCI	National cancer institute
NCOG	Northern California oncology group
ng	nanogram
No.	Number
OS	Overall survival
PET	Positron emission tomography
pH	A measure of acidity or alkalinity
RNA	Ribonucleic acid
SGOT	Serum glutamic oxaloacetic transaminase or aspartate transaminase (AST)
SGPT	Serum glutamate pyruvate transaminase or alanine transaminase (ALT)
SYS	Systemic
SWOG	Southwest oncology group
UK	United Kingdom
VEGF	Vascular endothelial growth factor
wk	Week

1.0 INTRODUCTION AND BACKGROUND

1.1 Natural history of hepatic-metastatic disease

Liver cancer is the sixth most common cancer worldwide and the third most common cause of death from cancer (Parkin *et al*, 2005). The incidence of liver cancer however, is low in developed countries (Jemal *et al*, 2007; Parkin *et al*, 2005), and in the United States, the most common malignant tumours of the liver are metastatic in origin, and most common neoplasms will have spread to the liver by the time of death (Alexander, 2005). Hepatic metastases from colorectal carcinoma represent the majority of metastatic liver tumours with more than 50,000 patients affected each year in the United States (Blumgart & Fong, 1995; D'Angelica & Fong, 2004; Fong, 1999; McCarter & Fong, 2000).

Global cancer statistics for 2002 demonstrated that worldwide, colorectal cancer was the third most common cancer with almost one million new cases. Mortality was found to be about one half that of incidence with 529,000 estimated deaths, while prevalence was second overall, with an estimated 2.8 million persons alive within 5 years of diagnosis. Both incidence and mortality of colorectal cancer however, are higher in developed countries. There was at least a 25-fold variation in occurrence of colorectal cancer worldwide, with the highest incidence rates seen in North America, Australia/New Zealand, Western Europe, and especially in Japanese men (Parkin *et al*, 2005).

In the United Kingdom, current annual estimates show that colorectal carcinoma will be diagnosed in around 35,000 people and will cause more than 16,100 deaths. 2004

Cancer Research UK statistics[1] demonstrate that colorectal carcinoma is the second most common cause of cancer death in the United Kingdom. In the United States almost 153,000 new cases of, and 52,000 deaths from colorectal carcinoma, are estimated for 2007. 2004 *American Cancer Society* statistics demonstrate that similar to the United Kingdom, colorectal carcinoma is the second most common cause of cancer death in the United States (Jemal *et al*, 2007).

Conservative estimates indicate that at least 35% to 40% of patients diagnosed with colorectal cancer will develop hepatic metastases and about half will present with synchronous disease at the time of diagnosis (Alexander, 2005; Altendorf-Hofmann & Scheele, 2003; Khatri *et al*, 2005). For those patients initially without metastatic disease at the time of primary tumour resection, metachronous hepatic tumours usually develop within three years (Finlay & McArdle, 1986; Scheele *et al*, 1995).

Unlike metastases from other primary sites, almost one third of patients with metastases from colorectal cancer will develop liver metastases as the only, or major, site of metastatic disease (Gorog *et al*, 1997; Khatri *et al*, 2005; Weiss *et al*, 1986). Since drainage of the colon and rectum is via the portal vein and haematogenous spread usually occurs in a stepwise fashion, with spread initially to the liver and subsequently to the systemic circulation, it has been postulated that surgical resection of isolated hepatic metastases from colorectal cancer may be curative (Kemeny, 2006; Simmonds *et al*, 2006).

[1] Cancer Research UK Information Resource Centre; http://info.cancerresearchuk.org:8000/cancerstats/types/bowel/; last update November 2006; access date February 6th 2007.

The prognosis however, of these patients if left untreated is poor with median survival[1] of 4 to 13 months, and long term survivors are rare (Alexander, 2005; Altendorf-Hofmann & Scheele, 2003; Fong, 1999; Simmonds et al, 2006).

Studies have attempted to identify patients with limited hepatic-metastatic colorectal disease, to define the natural history of the disease in individuals who could be considered candidates for resection. Patients with limited but untreated disease had a one-year survival rate of 77%, a three-year survival rate of 14 to 23%, and a five-year survival rate of 2 to 8% (Wagner *et al*, 1984; Wood *et al*, 1976). Patients with isolated hepatic metastases have been seen to have better prognosis than those with more extensive metastatic disease, suggesting prognostic significance of this biological difference (Goslin *et al*, 1982; Lahr *et al*, 1983; Rougier *et al*, 1995; Stangl *et al*, 1994). However, even for those patients with limited liver-only metastases, few survive for five years (Goslin *et al*, 1982; Stangl *et al*, 1994). Therefore, although patients with solitary lesions or unilobar disease appear to have better prognoses than patients with diffuse hepatic disease, five-year

[1] Median survival refers to median overall survival. For overall survival, death from any cause is considered an event. For disease-free survival, an event is defined as the first documented evidence of local, regional, or distant recurrence, second primary cancer, or death without recurrence. Progression-free survival, in the context of a clinical trial, is defined as the time from enrolment or randomisation to disease progression or death, whichever comes first [Niimi M, Yamamoto S, Fukuda H, Ishizuka N, Akaza H (2002) The Influence of Handling Censored Data on Estimating Progression-free Survival in Cancer Clinical Trials (JCOG9913-A). *Jpn J Clin Oncol* **32:** 19-26]. Survival curves refer to plots generated by the method of Kaplan and Meier [Kaplan EL, Meier P Nonparametric estimation from incomplete observations, J Stat Assoc 53 (1958). *MathSciNet| Full Text via CrossRef*: 457–481]. Statistical significance for differences in survival is calculated using the logrank test [Bland JM, Altman DG (2004) The logrank test. *BMJ* **328:** 1073-]. A *P* value summarises the results of a statistical test of a hypothesis that two or more treatments or outcomes are equivalent. A very small *P* value provides evidence of treatment or survival differences, and many investigators regard the value of $P < 0.05$ as statistically significant (i.e. unlikely to have been caused by chance), however, this cutoff value is only a convention, and other information must be considered before deciding whether a difference is real and of clinical importance [Sterne JAC, Smith GD (2001) Sifting the evidence–what's wrong with significance testing. *BMJ* **322:** 226-331].

survival for any untreated patient is unusual (Fong, 1999; McCarter & Fong, 2000; Tanabe KK, 2006) (see **Table 1.1**). A study done from 1992 to 1996 recently reported five-year survival of 3.4% for patients who did not undergo hepatectomy (Kato *et al*, 2003).

Table 1.1

Natural history of liver metastasis from colorectal cancer

Reference	Survival					
	Number of Patients	Median (months)	Mean (months)	1 year (%)	3 year (%)	5 year (%)
(Abrams & Lerner, 1971)	58	6	-	24	-	2
(Baden & Andersen, 1975)	103	10	-	-	4	3
(Bengmark & Hafstrom, 1969)	39	-	6	-	0	0
(Bengtsson *et al*, 1981)	25	-	5	12	0	0
(Cady *et al*, 1970)	269	-	13	33	14	-
(Finan *et al*, 1985)	90	10	-	-	-	0
(Goslin *et al*, 1982)	125	13	-	-	-	0
(Lahr *et al*, 1983)	100	4	-	-	-	0
(Oxley & Ellis, 1969)	112	12	-	31	4	1
(Palmer *et al*, 1989)	30	12	16	-	-	-
(Wagner *et al*, 1984)	252	-	-	49	7	2
(Wood *et al*, 1976)	113	-	7	15	3	1
Data adapted from: Metastatic liver tumours (McCarter & Fong, 2000)						

1.2 Surgery for colorectal hepatic-metastatic disease

Early, small, single-institution series evaluating surgical treatment of hepatic metastases from colorectal cancer suggested potential survival benefit (Attiyeh et al, 1978; Wilson & Adson, 1976). An early multi-institutional study supported these results and demonstrated five-year survival of 20% (Foster, 1978).

Table 1.2

Results of hepatic resection for colorectal metastases

Study	Number of Patients	Operative Mortality (%)	Survival			
			1 year (%)	5 years (%)	10 years (%)	Median (months)
(Adson *et al*, 1984)	141	2	82	25	-	24
(Hughes *et al*, 1986)[1]	607	-	-	33	-	-
(Ringe *et al*, 1990)	157	-	23	-	-	35
(Schlag *et al*, 1990)	122	4	85	30	-	32
(Doci *et al*, 1991)	100	5	-	30	-	28
(van Ooijen *et al*, 1992)[1]	118	7.6	-	21	-	-
(Rosen *et al*, 1992)	280	4	-	25	-	-
(Savage & Malt, 1992)	104	-	-	18	-	-
(Gayowski *et al*, 1994)	204	0	91	32	-	33
(Gozzetti *et al*, 1994)	108	0.9	-	28	-	-
(Scheele *et al*, 1995)	469	4	83	33	20	40
(Doci *et al*, 1995a)	219	-	-	24	-	-
(Fuhrman *et al*, 1995)	107	2.8	-	44	-	-
(Nordlinger *et al*, 1996)[1]	1568	2	61	28	-	31
(Taylor *et al*, 1997)	123	0	-	34	-	-
(Fong *et al*, 1997)	577	4	85	35	-	40
(Jenkins *et al*, 1997)	131	4	81	25	-	33
(Rees *et al*, 1997)	150	1	94	37	-	-

[1] Multi-institution studies

Table 1.2 (continued) Results of hepatic resection for colorectal metastases						
(Jamison *et al*, 1997b)	280	4	84	27	20	33
(Ohlsson *et al*, 1998)	111	6	-	25	-	-
(Ambiru *et al*, 1999b)	168	3.5	-	26	-	-
(Bradley *et al*, 1999)	134	-	81	36	23	-
(Harmon *et al*, 1999)	110	4	-	46	27	-
(Yamamoto *et al*, 1999b)	96	-	94	51	-	-
(Fong *et al*, 1999a)	1001	3	89	37	22	42
(Iwatsuki *et al*, 1999)	305	-	-	32	-	-
(Minagawa *et al*, 2000)	235	0	-	35	26	37
(Kokudo *et al*, 2001)	174	-	-	43	-	-
(Lise *et al*, 2001)	135	-	-	29	-	29
(Scheele *et al*, 2001)	516	5.8	-	38	27	35
(Choti *et al*, 2002)[1]	226 (1984-99) 133 (1993-99)	- -	- -	40 58	26 -	46 -
(Yan *et al*, 2003)	146	0.6	89	19	-	28
(Kato *et al*, 2003)	585	0	-	33	-	-
(Laurent *et al*, 2004)	156	1.3	-	43	-	-
(Abdalla *et al*, 2004b)	190	-	-	58	-	-
(Fernandez *et al*, 2004)	100	-	-	58	-	-
(Wei *et al*, 2006)	423	1.6	93	47	28	-
(Figueras *et al*, 2007)	501	4	80	42	30	44
Data adapted from: Metastatic liver tumours (McCarter & Fong, 2000), Surgical therapy for hepatic colorectal metastases (Fong, 1999), and Critical review of the major indicators of prognosis after resection of hepatic metastases from colorectal carcinoma (Altendorf-Hofmann & Scheele, 2003)						

[1] Data from 226 consecutive patients undergoing potentially curative liver resection for colorectal metastases between 1984 and 1999 were analyzed. The median survival for the entire cohort was 46 months, with five and ten-year survival rates of 40% and 26% respectively. Ninety-three patients operated on between 1984 and 1992 were found to have an overall survival of 31% at 5 years, compared to 58% for the 133 patients operated on during the more recent period (1993-1999). Both OS and DFS were significantly better in the recent time period compared with the earlier period.

These early reports however, were viewed with scepticism (Silen, 1989) since it was assumed that haematogenous spread of tumour cells to the liver indicated widespread systemic metastasis (Fong, 1999). In addition, the historically higher morbidity and mortality associated with liver surgery were considered too great to justify resection of hepatic metastases (McCarter & Fong, 2000).

The acceptance of surgical resection as treatment for hepatic metastases from colorectal cancer has been based on increased safety of major liver resections (Fong *et al*, 1999a; Morris *et al*, 2006; Nordlinger *et al*, 1996), the knowledge that such metastases can be isolated to the liver (Hellman & Weichselbaum, 1995) and that resections can be potentially curative (Simmonds et al, 2006).

Liver resection has therefore become the standard treatment for metastatic lesions derived from colorectal primaries (Altendorf-Hofmann & Scheele, 2003; Biasco et al, 2006; Hao & Ji, 2006; Khatri et al, 2005; Morris et al, 2006). Several series have reported five-year survival of between 18 to 58%, with most recent series after 2004 reporting five-year survival of more than 40%. In addition, ten-year survival has been reported to be between 20 to 28%, with most recent series after 2000 reporting ten-year survival of more than 25%. Twenty-year survival of 18% has also been reported (Scheele et al, 1991), and median survival time post-resection ranges between 24 to 46 months, with postoperative mortality rates between 0 to 7.7% **(see Table 1.2)**.

The value of hepatic resection for colorectal liver metastases however, has never been demonstrated in a prospective randomised trial, and ethical considerations make it unlikely that such trials will be done since this could deny patients the potential for long-

term cure (Khatri *et al*, 2005; Simmonds *et al*, 2006). A recent systematic review[1] on published studies of hepatic resection reported that for patients undergoing R0[2] curative resection, median five-year survival was 30%, and 30-day mortality was generally low with median value of 2.8% (Simmonds *et al*, 2006).

Curative resection has been found to be possible in less than 25% of those patients with disease limited to the liver, which translates into 5% to 10% of the original group developing colorectal cancer (Adson, 1987; Khatri *et al*, 2005). Hence in the United States, it can be approximated that between 4000 to 6000 patients will ultimately become long-term survivors after undergoing hepatic resection for colorectal liver metastases (Altendorf-Hofmann & Scheele, 2003; Khatri *et al*, 2005). Efforts to increase the number of patients who could benefit from potentially curative hepatic resection include refinement of staging and prognostic factors to improve patient selection, plus advancements in surgical technique and novel approaches to permit curative hepatic resection (Khatri *et al*, 2005; Morris *et al*, 2006).

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[1] Systematic review was done to assess the published evidence for efficacy and safety regarding surgical resection of colorectal liver metastases, and to identify prognostic factors. Studies were identified by literature search, reference scan and investigator contact. Outcome measures included OS, DFS, postoperative morbidity and mortality, quality of life and cost effectiveness. The best available evidence came from prospective case series, but only two studies reported outcomes for all patients undergoing surgery. Only 30 of 529 independent studies met all the eligibility criteria for the review, and data on 30-day mortality and morbidity only were included from a further nine studies.

[2] R0 resection is defined as complete removal of all macroscopically detectable disease, with margins of resection being microscopically negative for tumour; this contrasts with R1 resection, in which margins are histologically involved and R2 resection in which gross tumour is left behind [Altendorf-Hofmann A, Scheele J (2003) A critical review of the major indicators of prognosis after resection of hepatic metastases from colorectal carcinoma. *Surgical oncology clinics of North America* **12:** 165-92, xi].

1.21 Improvements in patient selection

In an early multi-institutional study, factors considered to be contraindications to hepatic resection for colorectal metastases were the presence of positive porta-hepatis lymph nodes, extra-hepatic metastases, and four or more hepatic metastases. Patients with Duke's C[1] primary tumours, who presented either with multiple metastases or synchronous metastatic disease, were also noted to be poor candidates for resection (Hughes *et al*, 1986).

A retrospective study of 25-years of resection of colorectal liver metastases reported that independent determinants of long-term survival were low/moderate grade of liver tumour, absence of extra-hepatic tumour, few intra-operative blood transfusions, low preoperative serum CEA (carcinoembryonic antigen) level, and resection in the more recent time period[2]. No five-year survivors were seen when the resection margin was involved with tumour, extra-hepatic metastases or satellitosis were present, and with poorly differentiated liver metastases. The study concluded that although increased hepatic re-resection was partly responsible for the improved outcome after liver resection for colorectal metastases during recent years, patients with extra-hepatic metastases did not benefit from liver

[1] The Dukes classification system for colorectal cancer was developed in the 1930s to record the extent of spread of disease and focused on information obtained from pathologic examination of the surgical specimen. Tumours were classified as A, B, or C, with stage A indicating tumour limited to, but not through, the bowel wall; stage B indicating penetration through the bowel wall including direct continuity into adjoining structures; and stage C indicating spread to local and regional lymph nodes. Dukes subsequently modified his staging system, first dividing stage C into C1 (lymph nodes in the immediate vicinity of the tumour involved) and C2 (lymph nodes at the point of ligature involved) and later adding a fourth stage (subsequently known as stage D) for distant metastasis [Dukes CE (1978) The Surgical pathology of Rectal Cancer. *CA: A Cancer Journal for Clinicians* **28:** 249].

[2] The aim of this retrospective study was to analyze survival and prognostic factors in 111 consecutive patients undergoing curative resection of liver metastases from colorectal cancer. In addition, the time periods 1971–1984 and 1985–1995 were compared; criteria for first liver resection did not change with time, whereas the attitude toward re-resection was more aggressive during the latter period.

resection and that surgery should be performed with a clear resection margin and minimal blood loss (Ohlsson, 1998).

Studies have attempted to develop prognostic scoring systems for clinical and investigative use as methods for selecting optimal patients for hepatic resection (Fong *et al*, 1999a; Nordlinger *et al*, 1996). A clinical risk scoring system was derived from data collected on 1001 consecutive patients with hepatic-metastatic colorectal cancer subjected to liver resection (Fong *et al*, 1999a). Five clinical criteria that would be available preoperatively were chosen to develop the risk score and included: (1) lymph node positive primary tumour, (2) disease-free interval of less than 12 months between resection of primary and appearance of metastases, (3) tumour size greater than 5cm, (4) number of metastases greater than one and (5) CEA level greater than 200 ng/dl.

Each of these five adverse clinical criteria was assigned one point, and the cumulative 0 to 5 score proved to be highly predictive of long-term outcome. Patients with scores of 0 to 3 represented better candidates for liver resection with five-year survival between 20 to 60% and median survival of 33 to 74 months, while patients with scores of 4 or 5 had more guarded outlook with median survival of 20 to 22 months (Fong *et al*, 1999a; Morris *et al*, 2006) (see **Table 1.3**).

Table 1.3

Patient survival according to clinical risk score

Score	5-year survival (%)	Median survival (months)
0	60	74
1	44	51
2	40	47
3	20	33
4	25	20
5	14	22

Data adapted from: Memorial Sloan-Kettering Cancer Centre; Clinical risk score and survival (Fong *et al*, 1999a)

The clinical risk scoring system has been validated by data from a retrospective European series which found that the relative risk[1] (hazard rate) of tumour recurrence in patients with score 3 to 4 was 2.1, compared to that of patients with score 0 to 2. Five-year survival in the two groups was 12% and 40% respectively. Fourteen of 15 long-term survivors (with survival greater than five years) classified by the system had score of 2 or less (Mala *et al*, 2002). In a prospective study of 103 patients with potentially resectable liver metastases, the likelihood of occult metastatic disease identified at laparoscopy was 12% in patients with a risk score of 2 or less, compared to 42% for those with a score over 2 (Jarnagin *et al*, 2001b). Therefore the prognostic clinical risk score system appears useful however, the clinical role in patient selection remains to be defined in prospective studies (Khatri *et al*, 2005; Morris *et al*, 2006).

[1] A purpose of the study was to assess the predictive value of the clinical risk score system. The relationship between survival rates and explanatory variables was assessed with Cox regression analyses. The hazard rate expressed the risk of death from recurrence compared to the reference group [Cox DR (1972) Regression Models and Life-Tables. *Journal of the Royal Statistical Society Series B (Methodological)* **34:** 187-220].

Conventional indications for resection of colorectal hepatic-metastatic disease can be summarised as follows: (1) the presence of unilobar disease; (2) less than four metastases; (3) metastases size of less than 5cm; (4) absence of extra-hepatic disease; (5) resection margin greater than 1cm; (5) adequate hepatic parenchymal remnant; (6) resection of all macroscopic disease; (7) metachronous metastases; (8) absence of vena cava and hepatic vein confluence invasion, and (9) absence of hepatic pedicle lymph node metastases (Adson *et al*, 1984; Doci *et al*, 1991; Fong *et al*, 1997; Gayowski *et al*, 1994; Hughes *et al*, 1988; Jamison *et al*, 1997b; Jenkins *et al*, 1997; Kato *et al*, 2003; Scheele *et al*, 1995; Scheele *et al*, 1991; Schlag *et al*, 1990).

The modern aggressive approach to hepatic-resection however, challenges these traditional indications and differs as follows: (1) resection of bilobar and multiple-metastatic disease is acceptable, using neoadjuvant chemotherapy, staged resection and local ablative therapy; (2) large size of metastases is not a limitation; (3) extra-hepatic pulmonary metastases can be resected; (4) resection margin of less than 1cm can be managed with ablative treatment of narrow margin (using cryosurgery or radiofrequency); (5) preoperative portal vein embolization can be used to increase liver remnant volume; (6) metastases can be extirpated and R0 status can be achieved with combination of resection and local ablative therapy; (7) synchronous and metachronous metastases are both acceptable for resection; (8) vena cava and hepatic vein resection with reconstruction can be performed; and (9) in the absence of celiac axis metastases, hepatic pedicle lymph node metastases may be resected for improved 3-year survival (Adam et al, 2004b; Khatri et al, 2005; Petrelli et al, 2005).

A recent study reported on 259 patients with expanded indications for hepatic resection including 14 with liver metastases larger than 10cm, 194 with bilateral deposits, 140 with four or more metastases, and 73 with extra-hepatic disease. In this category, 28% had preoperative chemotherapy and 70% had postoperative chemotherapy. The overall survival rates at one, three, five, and ten-years were 88%, 67%, 45%, and 36% for patients with classic indications, and 84%, 53%, 34%, and 24% for patients with expanded indications (P = 0.0009). Although four or more liver metastases and extra-hepatic disease were independent predictors of poor outcome, the study suggested that liver resection combined with preoperative and postoperative chemotherapy offered the possibility of long-term survival to patients with multiple, bilateral, large metastases, even with resectable extra-hepatic disease, and recommended that surgery should be offered to these patients (Figueras et al, 2007).

Another recent study reported on 98 patients with hepatic resection for four or more colorectal hepatic metastases treated between 1998 and 2002. Fifty-five percent received preoperative chemotherapy, with a 35% response. Postoperative adjuvant chemotherapy was given to 92% and a further 52% received postoperative hepatic arterial infusion chemotherapy. There were no perioperative deaths and five-year survival was 33%. However, positive margins and extra-hepatic disease resection were independently associated with poor outcome, and the median disease-free survival was 12 months. The study suggested that long-term survival can be achieved after resection of multiple colorectal metastases; however, because most patients will experience recurrence of disease, effective adjuvant therapy and close follow-up are necessary (Kornprat *et al*, 2006).

A computer program (the OncoSurge model) has been developed to aid in selecting the best treatment option for individual patients with colorectal hepatic metastases. The model uses the RAND/University of California at Los Angeles (UCLA) appropriateness method (RAM)[1]. A multidisciplinary panel of sixteen experts in oncology, radiology, and liver surgery developed hypothetical patient profiles, and then rated the options of resection, chemotherapy, or local ablation using a health benefit-to-negative-consequence ratio on a scale of 1 to 9. Each expert independently rated specific interventions as appropriate (expected benefit outweighs possible consequences), inappropriate (no health benefits), or uncertain (benefits and risks nearly equal, or panel members disagreed), and the group rating was taken as the median value of all participants.

The following represent the general recommendations from this panel of experts that were then incorporated into a decision matrix: (1) resection was always preferred, if possible, over local ablation strategies (cryosurgery, radiofrequency ablation, laser techniques); (2) resection was absolutely contraindicated in the presence of unresectable extra-hepatic disease, extensive liver involvement (more than 70%, more than 6 segments, or involvement of all three hepatic veins), major liver insufficiency, or other conditions causing the patient to be unfit for surgery; (3) immediate resection was appropriate if adequate margins could be radiographically defined, there was no portal lymph node involvement, and there were four or fewer lesions; resection could be considered for more than four lesions if they were localized to a single lobe; (4) for patients with more than four metastases, or bilobar involvement, resection was considered appropriate only after tumour

[1] The RAM integrates a comprehensive literature review of clinical evidence with a subsequent consensus-based categorisation of therapeutic options for specified case scenarios by a panel of experts [Brook RH, Chassin MR, Fink A, Solomon DH, Kosecoff J, Park RE (1986) A method for the detailed assessment of the appropriateness of medical technologies. *Int J Technol Assess Health Care* **2:** 53-63].

shrinkage using neoadjuvant chemotherapy; 5FU/leucovorin was considered to be only rarely appropriate whereas 5FU in combination with either irinotecan or oxaliplatin was generally appropriate; (5) postoperative chemotherapy was considered appropriate for patients who had received preoperative chemotherapy, although for patients who had a complete resection, benefit was deemed uncertain (Poston *et al*, 2005).

It is apparent that as long as R0 resection can be achieved, while maintaining a functional residual liver, application of any combination of preoperative prognostic factors should not prohibit the use of surgery, since liver resection still remains the only option for potential cure (Khatri *et al*, 2005). However, further work is needed to more accurately define this group of patients and to determine whether the addition of adjuvant treatments results in improved survival (Simmonds *et al*, 2006).

1.22 Improvements in radiologic assessment

Whole-body positron emission tomography or PET scanning is an important innovation in imaging regarding surgical treatment of patients with metastatic colorectal cancer. The radioisotope used in PET scanning is ^{18}F-flourodeoxyglucose (FDG) which is a glucose analogue that cannot proceed down the glycolytic pathway, and as colorectal metastases are most often glucose-avid, FDG can be used to localize distribution of metastases. Whole body PET scans may therefore identify radiographically occult extra-hepatic disease, permitting the selection of appropriate candidates for hepatic resection (Fernandez *et al*, 2004; Flamen *et al*, 2001; Fong *et al*, 1999b; Ruers *et al*, 2002).

In a series of 51 patients with apparently isolated liver metastases, clinical management decisions based upon conventional diagnostic imaging were altered by PET

scan findings in ten (20%); six had unsuspected extra-hepatic disease. Eight patients were spared an unnecessary laparotomy, while two others were identified as candidates for resection. (Ruers *et al*, 2002). In another series of 40 patients considered for hepatic resection, but at high risk for unresectable disease by clinical criteria, PET scanning altered management in nine (23%). Six patients were spared laparotomy, and three others had PET-directed surgery that found extra-hepatic tumour and spared the patients unwarranted liver resection (Fong *et al*, 1999b).

A report examined the utility of PET scan in guiding surgical therapy for 43 patients referred for hepatic resection after conventional tumour staging with CT (computed tomography) scan. PET identified additional cancer not seen on CT in ten patients and surgery was contraindicated in six of these patients. Laparotomy was performed in 37 patients and 35 were resected (95%) with estimated three-year survival of 77%, which the authors noted was higher than three-year survival estimates found in previously published series. The report concluded that preoperative PET scan lessens the recurrence rate in patients undergoing hepatic resection for colorectal metastases to the liver by detection of disease not found on conventional imaging (Strasberg & Siegal, 2001).

Another report correlated PET findings with the clinical risk scoring system (see **Table 1.21**), in a subset of 63 patients presenting with a first occurrence of hepatic metastases from colorectal cancer. Among patients with a score of 0, no patient had extra-hepatic disease detected by PET and 57% had false positive readings, whereas among patients with a score of 1 or more, 14% were found to have additional disease that was detected only by PET, and there were no false positive readings ($P<0.001$, Fisher's exact

test[1]). The report concluded that patients with isolated hepatic colorectal metastases and a risk score of 0 should undergo conventional imaging alone prior to surgical exploration (Schussler-Fiorenza *et al*, 2004).

The real value of PET scans over other conventional diagnostic methods is difficult to ascertain in the absence of randomised trials. However, the superiority of PET scanning over CT was suggested in a systematic overview of available retrospective reports which utilized a scoring system to weight the individual studies according to the quality of the data and the clinical impact of the radiographic findings (Wiering *et al*, 2005). For the six articles that were judged to be of the highest quality (Fong *et al*, 1999b; Imdahl *et al*, 2000; Lai *et al*, 1996; Langenhoff *et al*, 2002; Ruers *et al*, 2002; Valk *et al*, 1999), the pooled sensitivity and specificity of PET scans were 79.9% and 92.3%, respectively, for hepatic metastases, and 91.2% and 98.4%, respectively, for extra-hepatic metastases. The corresponding values for CT were 82.7% and 84.1%, respectively, for hepatic metastases, and 60.9% and 91.1%, respectively, for extra-hepatic metastases. The percent change in clinical management from the performance of PET ranged from 20% to 32% (average 25%) (Wiering *et al*, 2005).

It has been reported that recent chemotherapy may alter the sensitivity of PET for the detection of colorectal metastases, and this is thought to be related to decreased tumour cellular metabolic activity. In a series of 42 patients undergoing surgery for hepatic colorectal metastases, 15 of 41 liver lesions (37%) were undetected by PET among the 13 who had received preoperative chemotherapy. Three of the 13 patients had lesions confirmed pathologically (one single, two multiple liver lesions) that were all undetectable

[1] [Fisher RA (1950) Fisher's exact test for 2 x 2 contingency table: In Statistical Methods for Research Workers, para 21.02 (Oliver and Boyd, ed.): Edinburgh].

by PET. In contrast, of the 29 patients who did not receive preoperative chemotherapy, 16 of 69 lesions (23%) were undetected by PET. Therefore the results of restaging PET scans (particularly if negative) should be interpreted in the context of recent therapy (Akhurst et al, 2005).

1.23 Advances in surgical approach

A. Repeat hepatic resection

The majority of patients who undergo hepatic resection for colorectal liver metastases will experience disease recurrence (Biasco *et al*, 2006; Hao & Ji, 2006; Simmonds *et al*, 2006).

Table 1.4

Results of studies on repeat hepatic resections

Reference	Survival						
	Number of Patients	Median [Mean] (months)	Operative Mortality (%)	2 year (%)	3 year (%)	4 year (%)	5 year (%)
(Fortner, 1988)	22	31	-	-	-	-	
(Stone *et al*, 1990)	10	25	0	-	-	-	-
(Bozzetti *et al*, 1992)	11	23	1	-	36	-	-
(Vaillant *et al*, 1993)	16	- [33]	6	67	57	-	30
(Elias *et al*, 1993)	28	-	2	-	21	-	-
(Que & Nagorney, 1994)	21	41	5	-	-	43	-
(Fong *et al*, 1994)	25	30	0	44	-	-	-
(Nordlinger *et al*, 1994)	116	-	0.9	57	33		
(Fernandez-Trigo *et al*, 1995)	170	34	0	-	45	-	32
(Bines *et al*, 1996)	13	24 [39]	8	-	-	-	23
(Riesener *et al*, 1996)	25	-	0		53	24	
(Neeleman & Andersson, 1996)	191	30	1.2	-	40	-	26
(Chu, 1997)	10	16	10	-	-	-	23
(Di Carlo *et al*, 1997)	12	-	0	-	71	-	42
(Tuttle *et al*, 1997)	23	40	0	-	-	-	32
(Adam *et al*, 1997b)	64	46	0	-	60	-	41
(Kin, 1998)	15	16	0	-	42	-	21
(Yamamoto *et al*, 1999a)	90	31 [47]	0	-	48	-	31
(Suzuki *et al*, 2001)	26	31	0	-	62	-	32
(Muratore *et al*, 2001)	29	-	3.5	-	35	-	-
(Yamada *et al*, 2001)	11	-	0	-	-	-	46
(Petrowsky *et al*, 2002)	126	37	1.6	-	51	-	34
(Nagakura, 2002)	28	21	-	-	-	-	44
(Takahashi *et al*, 2003)	22	0	23	-	49	-	-
(Tanaka *et al*, 2004)	26	-	0	-	48	-	48*
(Pessaux *et al*, 2006)	42	25	0	-	-	-	33
(Shaw *et al*, 2006)	66	56	0	-	68	-	44

Data adapted from: Repeat hepatectomy for recurrent colorectal liver metastases (Pessaux et al, 2006), Metastatic liver tumours (McCarter & Fong, 2000), and Repeat hepatectomy for colorectal metastases (Sugarbaker, 1999); note only results from reports with 10 or more patients included in table; * = DFS.

A report documented the site of first recurrence for 235 recurrences following 456 consecutive hepatic resections for colorectal metastases, with median follow-up of 37 months. Nearly half of these recurrences involved the liver, one-quarter involved the lungs and one-quarter involved other intra-abdominal and pelvic sites. The liver was the only identifiable site of disease in almost 40% of patients with recurrence (Fong *et al*, 1997), and similar findings have been reported by other investigators (Figueras *et al*, 2007; Kato *et al*, 2003; Kornprat *et al*, 2006).

Approximately one-third of patients with liver only recurrence are candidates for further hepatic resection (Bozzetti *et al*, 1992; Fortner, 1988; Khatri *et al*, 2005; McCarter & Fong, 2000). Several reports have documented satisfactory results for patients undergoing a second hepatic resection for treatment of recurrent colorectal liver metastases (see **Table 1.4**), and there are also reports of third and fourth hepatectomy for repeated liver recurrence (Pessaux *et al*, 2006).

The range for operative mortality for repeat hepatic-resection is between 1% to 10%, with more recent studies reporting operative mortality of less than 2%, and morbidity around 25%. The range for median survival for repeat hepatic-resection is between 16 to 56 months, with more recent studies reporting median survival greater than 20 months. Several series have reported five-year survival for repeat hepatic-resection between 21 to 44%, with the majority of later series reporting five-year survival of more than 30% (Pessaux *et al*, 2006; Petrowsky *et al*, 2002; Shaw *et al*, 2006; Tanaka *et al*, 2004).

A multi-institutional cooperative group report[1] on 170 patients from 20 different institutions around the world demonstrated five-year survival of 32% with median survival of 34 months, and concluded that repeat hepatectomy was a justifiable approach since surgery remains the only potentially curative treatment (Fernandez-Trigo *et al*, 1995; Sugarbaker, 1999). Similar results were confirmed in a report on the combined experience of repeat hepatectomy for recurrent colorectal liver metastases from American and European surgical oncology centres[2], which demonstrated five-year survival of 34% with median survival of 37 months (Petrowsky *et al*, 2002). It has been noted that the outcome for recurrent resectable hepatic metastases appears almost equivalent to that of initial hepatic resection (Khatri *et al*, 2005; Sugarbaker, 1999).

Some of the criteria for selecting patients for repeat hepatic resection include: (1) the presence of solitary recurrences, (2) metastases size of less than 5cm, (3) low operative risk, (4) the ability to achieve R0 resection, and (4) longer disease-free interval (Adam *et al*, 1997b; Petrowsky *et al*, 2002; Sugarbaker, 1999; Yamamoto *et al*, 1999a). After second hepatectomy however, the recurrence rate remains high and the liver remains the most common organ of subsequent recurrence (Khatri *et al*, 2005). Five-year survival for third and fourth hepatectomy has been reported between 21% to 36%, with median survival between 16 to 32 months (Adam *et al*, 1997b; Pessaux *et al*, 2006). Multivariate analysis in one report demonstrated that two factors were independently associated with

[1] A Registry of Repeat Resection of Hepatic Metastasis was started in 1991 with the cooperative effort of 20 different institutions around the world focused on liver surgery. The registry was able to accrue a total of 170 patients who had undergone repeat liver resections for colorectal liver metastasis. A retrospective study was carried out to determine the outcome of patients who had undergone repeated liver resections for colorectal metastatic disease and to identify those patients who were most likely to benefit from repeat liver resection [Sugarbaker PH (1999) Repeat hepatectomy for colorectal metastases. *Journal of Hepato-Biliary-Pancreatic Surgery* **6:** 30-38].

[2] Memorial Sloan-Kettering and the University of Frankfurt.

survival after second hepatic-resection: (1) a delay between the first and second hepatectomy over 1 year and (2) whether the second hepatectomy had been curative (Adam *et al*, 1997b).

B. Hepatic pedicle lymphadenectomy

The presence of portal or hilar[1] lymph node metastases have been traditionally considered extra-hepatic disease and a contraindication to hepatectomy for patients with colorectal liver metastases (Elias & Ouellet, 2003). This practice is based on past hepatectomy studies with limited[2] data on resected involved hilar lymph nodes which have reported five-year survival rate between 0 to 12% (Ambiru *et al*, 1999a; Elias *et al*, 2003; Hughes, 1988; Iwatsuki *et al*, 1999; Jamison *et al*, 1997a; Nordlinger *et al*, 1992).

A recent report on the outcome of complete hepatic pedicle lymphadenectomy[3] in patients with colorectal liver metastases however, has questioned whether complete lymphadenectomy may be of value. Patients with metastases limited to the portal triad had better prognosis at three-years than did those with lymphatic metastases along the common hepatic artery and celiac axis (38% versus 0%), and although five-year survival was rare, 19% of the patients survived three-years despite the presence of portal lymphatic metastases (Jaeck *et al*, 2002). This study has shown that the location of the hilar lymph node metastases was important, as involvement of certain nodal basins was associated with an improved prognosis. It has been suggested that prospective randomised trials are

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[1] Hilar lymph nodes refers to all lymph nodes located in the hepatoduodenal ligament.

[2] Refers to variations in surgical technique including *en bloc* dissection versus excision biopsy of lymph nodes [Elias DM, Ouellet JF (2003) Incidence, distribution, and significance of hilar lymph node metastases in hepatic colorectal metastases. *Surgical oncology clinics of North America* **12:** 221-9].

[3] Complete lymphadenectomy included the dissection of the hepatoduodenal ligament and retro-pancreatic and celiac lymphatics.

needed to further address this issue and determine whether improved survival can be achieved through selective complete lymphadenectomy, particularly in the era of neoadjuvant chemotherapy and preoperative downstaging (Roh, 2002).

C. IVC and hepatic vein resection

Metastatic involvement of the vena cava and hepatic vein (hepato-caval) confluence, or the inferior vena cava (IVC) and is often considered a contraindication to hepatic resection. However, aggressive surgical resection of these tumours with patch repair or graft replacement has been reported (Habib *et al*, 1996; Hemming *et al*, 2001). This approach has been associated with high surgical risks and poor long-term prognosis, and was precluded until the development of neoadjuvant chemotherapy, portal vein embolization, reinforced vascular prostheses, and technical advances in liver transplantation (Azoulay *et al*, 2006).

A report on 14 patients with colorectal hepatic metastases involving the IVC who had surgical resection with repair and reconstruction of the IVC demonstrated five-year survival of 22%, with median survival of 19 months, and morbidity and mortality rates of 25% and 6% (Miyazaki *et al*, 1999). Another report on 11 patients demonstrated five-year survival of 52% and morbidity and mortality rates of 58% and 6% (Nardo *et al*, 2005).

Ex-vivo liver surgery applies techniques and principles of hepatic transplantation to conventionally unresectable liver malignancies (Raab *et al*, 2000). The median survival in a report of six patients who had ex-vivo hepatic resection and vascular reconstruction for colorectal carcinoma metastatic disease was 21 months however, this extensive surgery was associated with a considerable mortality of 38% (Oldhafer *et al*, 2000). This modality

has not gained acceptance due to the substantial mortality associated with such an aggressive approach (Khatri *et al*, 2005).

D. Complex multi-visceral resection

The presence of local extension of colorectal hepatic metastases or associated focal extra-hepatic disease has traditionally been considered a contraindication for hepatic resection (Cady & McDermott, 1985; Ekberg *et al*, 1986; Hughes, 1988; Petrelli *et al*, 2005). A review of 747 hepatic resections, including 473 procedures for malignancy, revealed that a concomitant extra-hepatic procedure was the sole independent predictor of operative mortality in patients with no underlying liver disease (Belghiti *et al*, 2000).

However, several series of extended or complex hepatic resection with *en bloc* removal of adjacent intra-abdominal and intra-thoracic organs have been reported with encouraging results, and have failed to demonstrate a deleterious effect on postoperative mortality by performance of additional visceral resection. A report on a series of 105 patients including 39 who underwent complex hepatic resection for malignancy demonstrated sixty-day hospital mortality of 2.8% and morbidity of 33% (Sitzmann & Greene, 1994). Another report of 193 patients with malignancy who underwent complex hepatic resection demonstrated mortality of 3.1% and morbidity of 39.3% (Capussotti & Polastri, 1998). Mortality was 6% in a series of 226 consecutive patients who underwent extended hepatic resection and the combination of any two factors among preoperative cholangitis, elevated serum creatinine, elevated serum bilirubin, high operative blood loss, and vena cava resection, was associated with higher mortality (Melendez *et al*, 2001).

A recent prospective study of 75 patients who underwent R0 resection of extra-hepatic disease simultaneously with hepatectomy for colorectal liver metastases reported five-year survival of 28%, mortality of 2.7%, and morbidity of 25%. Multivariate analysis demonstrated that independent factors of poor prognosis for these patients included the presence of more than five liver metastases and multiple sites of extra-hepatic disease (Elias *et al*, 2004c). The study concluded that extra-hepatic disease in colorectal cancer patients with liver metastases should no longer be considered as a contraindication to hepatectomy however, it has been suggested that this concept needs to be validated in a prospective multi-institutional trial (Petrelli *et al*, 2005).

E. Improvements in surgical technique

The occurrence of major haemorrhage during hepatic resection has been reported to be between 1% to 3% (Doci *et al*, 1995b; Fong *et al*, 1997; Fortner *et al*, 1984; Scheele *et al*, 1995), and has been shown to be an independent risk factor for perioperative morbidity and mortality (Holm *et al*, 1989; Jarnagin *et al*, 2002). A variety of surgical occlusive techniques for hepatic inflow vascular control and combined inflow-outflow control have been described to reduce liver haemorrhage and facilitate complex resection (Abdalla *et al*, 2004a).

The most common approach to minimize blood loss is to perform hepatic pedicle clamping with the Pringle manoeuvre[1] (compression of the hepatoduodenal ligament),

[1] Pringle originally described this manoeuvre in 1908 following a case of hepatic haemorrhage due to trauma during which he "instructed his assistant to compress the portal vein and the hepatic artery between a finger and thumb and completely arrested all bleeding" [Pringle JH (1908) V. Notes on the Arrest of Hepatic Hemorrhage Due to Trauma. *Ann Surg* **48:** 541-549].

which interrupts most of the blood flow[1] to the liver but produces profound hepatic ischemia unless it is frequently released (Man *et al*, 1997). This is particularly true when there is underlying acute or chronic liver disease, making the liver very sensitive to hypoxia and reperfusion injury (Abdalla *et al*, 2004a; Huguet *et al*, 1994). However, studies have suggested that a short period of ischemia followed by brief reperfusion to the liver, or "ischemic preconditioning," might actually improve the tolerance to sustained ischemia (Clavien *et al*, 2000; Fung, 2001; Sindram *et al*, 2002).

A prospective randomized study compared continuous hepatic pedicle clamping to intermittent pedicle clamping[2] and demonstrated no difference in total operative blood loss, but better overall parenchymal tolerance to intermittent clamping was evident, particularly in patients with abnormal liver parenchyma, including steatosis and cirrhosis (Belghiti *et al*, 1999). Hemi-hepatic vascular occlusion is an alternative method which involves unilobar vascular inflow control, while preserving normal flow to the contralateral lobe, thereby decreasing ischemia time and minimizing reperfusion hepatic injury. Haemorrhage was found to be significantly lower in patients who had hemi-hepatic vascular occlusion in comparison with resections performed without vascular occlusion (Makuuchi *et al*, 1987b). Segmental clamping is a similar concept to minimise ischemic injury, in which the tumour portal territory is delineated in order to facilitate segment-oriented hepatic resection (Castaing *et al*, 1989; Shimamura *et al*, 1986).

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[1] Pringle's manoeuvre interrupts the arterial and portal venous inflow into the liver, but has no direct effect on backflow bleeding from branches of the hepatic veins [Abdalla EK, Noun R, Belghiti J (2004a) Hepatic vascular occlusion: Which technique? *The Surgical clinics of North America* **84:** 563-585].

[2] Continuous clamping was maintained until completion of hepatic transection; intermittent clamping consisted of alternating sessions of clamping (15 minutes) and unclamping (5 minutes), repeated until the end of the hepatectomy.

Hepatic vascular exclusion combines total inflow and outflow vascular occlusion of the liver and has been used for complex hepatic resections, particularly central hepatectomy or *en bloc* resection of the IVC. This technique allows prevention of profuse haemorrhage or air embolism but is not tolerated in 10% to 15% of patients (Abdalla *et al*, 2004a; Khatri *et al*, 2005). In a prospective randomised trial total vascular isolation was effective in reducing blood loss in major liver resections, but was also associated with unpredictable haemodynamic intolerance, increased postoperative complications with longer hospital stay, and the study concluded that this procedure should be restricted to lesions involving the hepato-caval intersection (Belghiti *et al*, 1996).

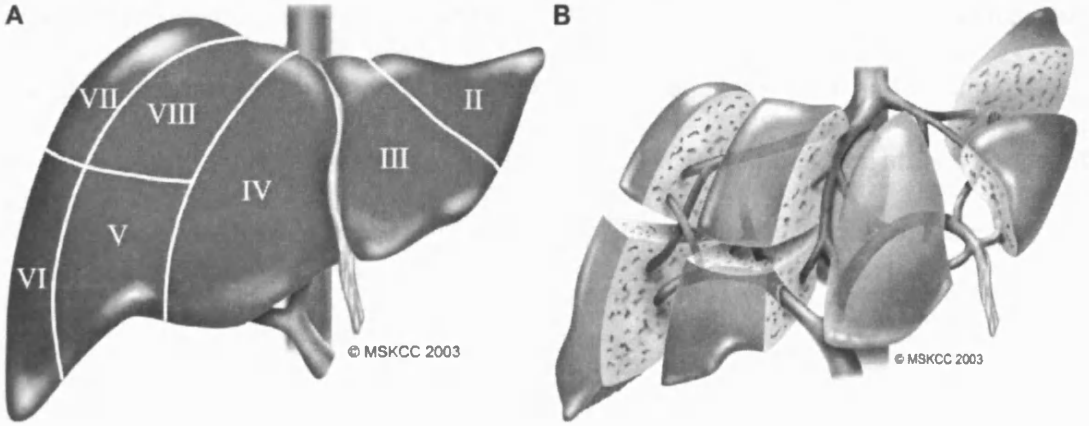
Haemorrhage can occur from injury to hepatic veins either during parenchymal transection or while achieving outflow control at their junction with the IVC. Reports have demonstrated that maintenance of low central venous pressure combined with extra-hepatic control of venous outflow, reduced the overall blood loss and mortality during major hepatic resections (Melendez *et al*, 1998). A report on the benefit of combined intermittent vascular inflow occlusion and low central venous pressure during hepatectomy demonstrated significantly less haemorrhage, morbidity and mortality, and improved patient outcomes (Chen *et al*, 2000).

Although haemorrhage during hepatic resection can result in the course of dissection of the vena cava and hepatic vein, it most commonly occurs during the parenchymal transection phase. Selective transection techniques using water-jet and ultrasonic dissection with the Cavitron ultrasonic aspirator or CUSA are devices that enhance haemostasis and reduce transfusion requirement (Fasulo *et al*, 1992; Izumi *et al*, 1993). Other useful surgical devices that enhance haemostasis include the argon beam coagulator

and radiofrequency electrocautery for parenchymal transection, and slim linear endovascular stapling devices for vascular transection (Gananadha & Morris, 2004; Khatri *et al*, 2005).

Parenchymal sparing segmental[1] hepatic resection offers the same benefits as classic lobar resections with less risk than is associated with removal of a large volume of functional liver tissue (Billingsley *et al*, 1998; Scheele & Stangl, 1994). Advantages of segment-oriented resection include preservation of normal liver parenchyma, tailoring the extent of resection to the extent of the pathology and minimizing postoperative liver failure, and segmental resections have been demonstrated to be superior to non-anatomic wedge resections with respect to blood loss and tumour clearance (DeMatteo *et al*, 2000). This approach allows liver resection while minimizing the blood loss and morbidity that may be associated with a more extensive resection. A report on experience from 1803 consecutive hepatic resections with increasing use of segmental resection reported a significant decline

[1] The eight anatomic segments of the liver (A) are defined by the distribution of the hepatic and portal venous systems (B). Each segment has independent biliary drainage and vascular inflow and outflow and it is therefore possible to remove an individual segment without disrupting the blood flow or biliary drainage of the remaining segments [Couinaud C (1957) *Le foie: études anatomiques et chirurgicales*edn.: Masson].



Segment I lies between the IVC and segment IV (not shown) [from Liau KH, Blumgart LH, Dematteo RP (2004) Segment-oriented approach to liver resection. *The Surgical clinics of North America* **84:** 543-561].

in blood loss, the use of blood products, hospital stay and operative mortality (Jarnagin *et al*, 2002).

F. Intra-operative ultrasound

Intra-operative ultrasound has become an essential surgical tool in oncologic hepatobiliary surgery (Bismuth *et al*, 1987; Makuuchi *et al*, 1981; Makuuchi *et al*, 1985; Makuuchi *et al*, 1987a). Tumour characteristics and resectability can be evaluated according to sonographic appearance and relationship to intra-hepatic vasculature (Patel & Roh, 2004). Findings of unsuspected proximity or invasion of vessels can alter the operative approach (Parker *et al*, 1989). Lesions that may not be detected on preoperative imaging or bimanual palpation can be detected by intra-operative ultrasound, and several reports have shown that in comparison with preoperative imaging, ultrasonography discovers additional lesions in between 10% to 35% of patients.

Detection of these occult lesions by routine use of intra-operative ultrasound may improve the therapeutic value of hepatic resection for colorectal cancer by improving patient selection (Bismuth *et al*, 1987; Boutkan *et al*, 1992; Cerwenka *et al*, 2003; Jarnagin *et al*, 2001a; Machi *et al*, 1991; Ozsunar & Skjoldbye, 2000; Solomon *et al*, 1994; Stadler *et al*, 1991; Staren *et al*, 1997; Zacherl *et al*, 2002). Intra-operative ultrasound has also facilitated performance of parenchymal-sparing segmental resection and minimally-invasive laparoscopic techniques, guidance of ablative modalities, and allows continual verification of distance between the dissection plane and tumour, ensuring adequate margins during parenchymal dissection (Patel & Roh, 2004).

1.24 Innovative approaches for curative resection

A Portal vein embolization

Postoperative hepatic failure is a rare but devastating complication after major hepatic resection and is a common cause of perioperative mortality. In order to estimate the risk of postoperative hepatic failure, preoperative assessment includes evaluation of hepatic functional reserve and regenerative capacity (Schneider, 2004). Tumours are usually considered unresectable when it would be impossible to remove the malignant hepatic disease and maintain a sufficient residual amount of functional liver parenchyma (which is estimated to be at least 30% of initial preoperative liver volume). In patients treated with prolonged preoperative chemotherapy, an additional volume allowance has been recommended due to the possibility of hepatic damage, and the functional liver remnant volume considered at high risk for failure is higher at approximately 40% (Adam *et al*, 2004b).

It has been demonstrated that fatal hepatic failure did not occur after major liver resection when the portal vein of the resected lobe was obstructed and this knowledge led to development of preoperative portal vein embolization as a method to initiate compensatory hypertrophy of the future hepatic remnant, enabling more confident resection of tumours with clear margins (Adam *et al*, 2004b; Azoulay *et al*, 1995; Kawasaki *et al*, 1994; Makuuchi *et al*, 1990).

A study reported on 30 patients with colorectal hepatic metastases which were considered unresectable because of inadequate liver remnant, who were treated with

chemotherapy followed by preoperative portal vein embolization[1]. Embolization led to a median 42% gain in liver remnant volume and facilitated resection in 63% of patients, with 19% undergoing removal of four or more segments. The complication rate was 3% and there was no evidence of stimulating contralateral tumour growth. Postoperative liver failure did not occur and the five-year survival for the resected patients was 40% (Azoulay *et al*, 2000). Other reports have confirmed the applicability of this novel approach to increase the rate of hepatic resection for colorectal liver metastases (Akasu *et al*, ; Jaeck *et al*, 2003; Kianmanesh *et al*, 2003).

A review of preoperative portal vein embolization reported that complications typically occurred in less than 5% of patients, no specific embolic substance (cyanoacrylate, thrombin, coils or absolute alcohol) emerged as superior, the increase in remnant liver volume averaged 12% of the total liver volume, the morbidity rate of resection after treatment was less than 15%, and the mortality rate was 6 to 7% with cirrhosis and 0 to 6·5% without cirrhosis. Embolization was used for patients with a normal liver when the anticipated liver remnant volume was 25% or less of the total liver volume, and for patients with compromised liver function when the liver remnant volume was 40% or less (Abdalla *et al*, 2001).

A prospective clinical trial assessed the impact of liver hypertrophy of the future liver remnant volume induced by preoperative portal vein embolization on the immediate postoperative complications after a standardized major liver resection[2]. The hypertrophy

[1] Preoperative portal vein embolization involves a percutaneous, interventional radiologic approach for depriving portal blood flow to the liver tissues in the planned resection. Over a period of 3–4 weeks, atrophy of the embolized liver occurs with contralateral remnant liver hypertrophy.

[2] Right hepatectomy was defined as removal of Couinaud segments 5, 6, 7, and 8.

of remnant induced by embolization had no beneficial effect on the postoperative course in patients with normal liver. In contrast, in patients with chronic liver disease, the hypertrophy of the remnant induced by embolization decreased significantly the rate of postoperative complications (Farges *et al*, 2003).

Therefore improved understanding of the benefits of liver regeneration has allowed preoperative portal vein embolization to increase the pool of patients who can safely undergo potentially curative hepatic resection (Adam *et al*, 2004b; Khatri *et al*, 2005). Preoperative portal vein embolization is currently used mainly in patients who have cirrhosis, severe fibrosis or steatosis, and in patients with normal non-cancerous parenchyma who will be subjected to extended resections. It is estimated that use of this technique will likely expand as more patients present with liver parenchymal damage from multiple preoperative chemotherapeutic regimens (Morris *et al*, 2006).

B. Staged resection

Despite chemotherapy and preoperative portal vein embolization, curative one-stage resection may not be possible in some patients with bilobar hepatic metastases. Two-stage hepatectomy consists of a sequential strategy to extend the benefits of curative resection (Adam *et al*, 2004b). A study reported on thirteen patients where the initial resection removed the highest number of metastases possible, followed by chemotherapy to limit residual tumour growth while the remnant liver hypertrophied. When adequate parenchymal hypertrophy had occurred and after documenting absence of disease progression, patients underwent a second hepatectomy. Three-year survival was 35%, and median survival was 31 months from the second hepatectomy and 44 months from the time of initial diagnosis of metastases. The perioperative death rate however, was 15% and this was thought to be a consequence of high tumour burden, the detrimental effects of

prolonged chemotherapy, and the need for performing more technically demanding surgical procedures (Adam *et al*, 2000).

Another report demonstrated an alternative staged-approach, where the first stage involved clearing the left liver of metastases by local resection and concomitantly performing a right portal vein ligation. The second stage involved a right hepatectomy after hypertrophy of the cleared left liver through a different abdominal approach, thereby avoiding the need for extensive lysis of adhesions from the earlier procedure (Kianmanesh *et al*, 2003).

C. Ablative therapies as surgical adjuncts

Local hepatic ablative therapies aim to preserve residual normal functional tissue by ablating only the metastatic lesions and a surrounding margin of normal tissue. Cryotherapy and radiofrequency are effective ablative therapies which have been used either in conjunction with surgical resection or as an alternative to resection (Khatri & McGahan, 2004).

Reports on small numbers of patients with unresectable colorectal liver metastases treated by cryosurgery[1] combined with hepatic resection suggest that benefits are similar to

[1] Cryosurgery destroys tumours through the formation of ice crystals. A vacuum-insulated cryoprobe is inserted either percutaneously or intra-operatively using ultrasound guidance. One or more probes are placed centrally within the lesion based on size. The probe is secured in place by rapidly lowering the temperature to -100°C. Freezing occurs by circulating liquid nitrogen via the probe at -196°C. One to three cycles of freezing for 15 minutes are applied between periods of spontaneous thaw. Lethal temperatures of -20°C to -30°C are reached, and during freeze-thaw cycles, intracellular and extracellular ice forms in an area termed the ice ball, leading to cell necrosis and tumour destruction. Freezing is continued until the ice ball extends at least 1 cm beyond the tumour to achieve adequate tissue margins [Khatri VP, McGahan J (2004) Non-resection approaches for colorectal liver metastases. *The Surgical clinics of North America* **84:** 587-606].

that of resection alone, with a five-year survival rates of almost 30% (Adam *et al*, 1997a; Adam *et al*, 2004b). Radiofrequency[1] has also being used increasingly as an adjunct to surgical resection of colorectal liver metastases, (Oshowo *et al*, 2003b; Scudamore *et al*, 1999; Tepel *et al*, 2004), and a report has suggested equivalence of outcome when compared with surgical resection for solitary metastases (Oshowo *et al*, 2003a). Since the growth rate of metastases can be more rapid than that of liver parenchyma during hepatic regeneration, radiofrequency can also be used in conjunction with preoperative portal vein embolization to ablate metastatic nodules located in non-embolised liver segments and prevent tumour progression, (Elias *et al*, 1999; Kokudo, 2001).

The presence of a 1cm tumour-free surgical resection margin has been identified as a significant prognostic factor after resection of colorectal liver metastases, and it has been reported that there is a doubling of recurrence rates in patients with positive margins, and a significantly higher rate of recurrence in patients with margins less than 1cm (Cady & Stone, 1991). A report demonstrated that cryotherapy of the resection edge, after resection of colorectal liver metastases with involved or inadequate resection margins, improved local disease control and allowed a greater proportion of patients to undergo potentially curative treatment (Seifert & Morris, 1998). Early reports also indicate that following a

[1] Radiofrequency thermal ablation involves the conversion of radiofrequency waves into heat. A rapidly alternating current in the range of radiofrequency waves (460 kHz) is applied from a generator and passed from an uninsulated electrode tip into the surrounding tissue. The current causes ionic vibration as the ions attempt to follow the changes in the direction of the rapidly alternating current. Heat is generated when the ionic agitation leads to localized frictional heating of the tissue surrounding the electrode. Heating tissue to temperatures above 50°C results in extracellular and intracellular desiccation, and thermal coagulative necrosis. In addition to the direct effect of thermal energy, local hyperthermia has also been shown to cause the secondary effects of recruiting peripheral blood effector cells, inducing cytotoxic cytokines, expressing heat-shock proteins, and inducing apoptosis [Ibid.].

narrow margin hepatic resection, radiofrequency can be used to treat the inadequate resection edge and create a zone of ablated tissue (Di Carlo *et al*, 2003; Ogata *et al*, 2005).

It should be noted that comparison of intra-operative radiofrequency ablation and cryoablation for hepatic malignancies has demonstrated that complications occurred much less frequently following radiofrequency, and early local tumour recurrence was less frequent following radiofrequency (Decadt & Siriwardena, 2004; Pearson *et al*, 1999).

In addition, a recent study evaluated treatment of 159 patients with four or more colorectal liver metastases. Almost 90% of patients received neoadjuvant chemotherapy and 29.0% had resection only, 7.5% had radiofrequency ablation only, and 63.5% had resection plus radiofrequency ablation. The overall median survival was 62 months and five-year survival was 51%. Patients who underwent radiofrequency ablation as part of their surgical procedure and those with a positive surgical resection margin were found to be more likely to have a shorter time to recurrence. The study concluded that patients with four or more metastases should be considered for aggressive surgical treatment, including liver resection with or without radiofrequency ablation, in order to improve the chance of long-term survival (Pawlik et al, 2006). These results regarding the role and value of radiofrequency ablation are encouraging, but require validation in further prospective clinical trials (Petrelli *et al*, 2005).

D. Preoperative chemotherapy and downstaging

Although hepatic resection for colorectal liver metastases offers the best chance for cure and long-term survival, it has been estimated that only 10% to 25% (or about 100,000 of such patients per year worldwide) will be candidates for resection (Leonard *et al*, 2005).

For the majority of patients (or more than 300,000 per year worldwide) with non-resectable colorectal liver metastases, the standard of care has been systemic therapy. However, despite recent advances in chemotherapy, five-year survivors are rare and systemic therapy has been considered palliative. (Kemeny, 2006; Leonard *et al*, 2005).

In addition, a recent report has confirmed that in most patients receiving chemotherapy for colorectal liver metastases, even a complete objective response[1] on CT scan does not mean cure, since persistent disease and recurrence has been observed in 83% of these patients (Benoist *et al*, 2006; Leichman, 2006). Although no randomised studies of hepatic resection versus systemic therapy have been performed (Kemeny, 2006), in contrast to systemic therapy, patients with colorectal liver metastases who undergo hepatic resection for cure in modern times can expect five-year survival of at least 30% (Khatri *et al*, 2005).

Preoperative or neoadjuvant chemotherapy is an approach for which the rational is as an attempt to make more patients amenable to surgical resection and cure. Because of the improved response rates associated with modern chemotherapy, a neoadjuvant approach has the potential ability to render formerly unresectable patients resectable.

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[1] Objective response reported for a cohort, is the sum of the percent complete and partial response observations for the entire cohort. "Objective response" refers to the WHO definition of reporting of response to therapy, for measurable and unmeasurable cancer disease. Complete response is defined as the disappearance of all known measurable disease, determined by two observations, not less than four weeks apart; or complete disappearance of all known unmeasurable disease for at least four weeks. Partial response for measurable disease, is defined as 50% or more decrease in total tumour size of the lesians which have been measured to determine the effect of therapy, by two observations not less than four weeks apart, and there can be no appearance of new lesions or progression of any lesion; or for unmeasurable disease, estimated decrease in tumour size of 50% or more for at least for weeks. [WHO (1979) WHO Handbook for Reporting Results of Cancer Treatment Vol. Publication number 48. WHO Offset Publications, Geneva].

Historically, palliative treatment of metastatic colorectal cancer with systemic 5FU/LV regimens produced response rates between to 10% to 20%, with a median overall survival time of between 6 to 12 months (Meta-analysis-Group-In-Cancer, 1998a). Objective response rates for first-line combination oxaliplatin/5FU/LV systemic chemotherapeutic regimens range between 31 to 54% (de Gramont *et al*, 2000; Giacchetti *et al*, 1999; Giacchetti *et al*, 2000; Goldberg *et al*, 2004; Hospers & Schaapveld, 2004; Tournigand, 2003); response rates for first-line combination irinotecan/5FU/LV systemic regimens range between 31 to 56% (Douillard *et al*, 2000; Goldberg *et al*, 2004; Hurwitz *et al*, 2004; Pozzo, 2004; Saltz *et al*, 2000b; Tournigand, 2003); and response rates for first-line combination irinotecan/oxaliplatin systemic regimens range between 35 to 53% (Goldberg *et al*, 2004; Heinemann *et al*, 2004; Schalhorn *et al*, 2005; Souglakos *et al*, 2004).

Although the definition of unresectable disease is not definitively established or uniform (Bilchik *et al*, 2005), for resectable or near-resectable patients, neoadjuvant chemotherapy may also increase the prospect of complete resection rate and facilitate limited hepatic resection, sparing normal liver parenchyma and improving postoperative recovery. Neoadjuvant chemotherapy also treats micro-metastatic disease and can be used as a test of chemo-responsiveness from which an optimal postoperative chemotherapy regimen can be determined. A trial of neoadjuvant chemotherapy may also allow the identification of patients with particularly aggressive disease in whom surgery would be inappropriate (Chong & Cunningham, 2005; Kemeny, 2006; Leonard *et al*, 2005; Morris *et al*, 2006).

(i) Systemic chemotherapy

There have been several reports regarding the influence of systemic chemotherapy on conversion of previously non-resectable colorectal hepatic-metastases to resectable disease, but study comparisons are made difficult by variations in study design and patient selection criteria (**see Table 1.5 for selective reports using regimens containing oxaliplatin and irinotecan**).

Early reports on small numbers of patients rendered resectable by use of systemic chemotherapy (Fowler *et al*, 1992; Shankar *et al*, 2001) prompted further investigation, and the first large retrospective study demonstrated that of 330 unresectable patients treated with oxaliplatin/FU/LV, 16% were able to proceed with hepatic resection with no operative mortality, and with five-year survival of 40% (Bismuth *et al*, 1996).

Table 1.5

Hepatic-resection rates post systemic chemotherapy

Reference	Regimen	Number of patients	Resection rate (%)	*P* value
(Bismuth *et al*, 1996)	Oxaliplatin/5FU/LV[1]	330	14	-
(Giacchetti *et al*, 1999)	Oxaliplatin/5FU/LV	151	38	-
(Wein *et al*, 2001)	5FU/LV	53	11	-
(Gaspar, 2003)	FOLFOX	37	27	-
(Tournigand, 2003)	FOLFIRI/FOLFOX6[2] FOLFOX6/FOLFIRI	109 111	9 22	0.02
(Adam *et al*, 2004a)	Oxaliplatin/5FU/LV	1104	12.5	-
(Quenet *et al*, 2004)	Oxaliplatin/5FU/LV/ Irinotecan	34	37.5	-
(Falcone *et al*, 2004) (Masi *et al*, 2006)	Oxaliplatin/5FU/LV/ Irinotecan	74	26	-
(Pozzo, 2004) (Pozzo *et al*, 2006)	FOLFIRI	40	32.5	-
(De La Camara *et al*, 2004)	Oxaliplatin/5FU/LV/ Irinotecan	22	40.9	-
(De Gramont *et al*, 2004)	FOLFOX4[3] FOLFOX7[4]	262 264	10.7 9.9	-
(Rougier *et al*, 2004)	Cetuximab/FOLFIRI	42	16.7	-
(Alberts *et al*, 2005)	FOLFOX4	42	40	-

[1] FOLFOX: combination Oxaliplatin/5FU/LV chemotherapy regimen.

[2] FOLFOX6: Oxaliplatin 100 mg/m^2 over 2 hrs; LV 400 mg/m^2 concurrently, followed by 5FU bolus 400 mg/m^2, then 5FU 2400-3000 mg/m^2 infusion over 46-48 hrs; cycle repeated every 14 days;
FOLFIRI: Irinotecan 180 mg/m^2 over 2 hrs; LV 400 mg/m^2 concurrently, followed by 5FU bolus 400 mg/m^2, then 5FU 2400-3000 mg/m^2 infusion over 46-48 hrs; cycle repeated every 14 days [Tournigand C (2003) FOLFIRI Followed by FOLFOX 6 or the Reverse Sequence in Advanced Colorectal Cancer: A Randomized GERCOR Study. *Journal of Clinical Oncology* **22:** 229-237].

[3] FOLFOX4: Oxaliplatin 85 mg/m^2 over 2 hrs; LV 200 mg/m^2 concurrently, followed by 5FU bolus 400 mg/m^2, then 5FU 600 mg/m^2 infusion over 22 hrs; Oxaliplatin given day 1 only, all other agents given days 1 and 2; cycle repeated every 14 days [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 (2000) Leucovorin and Fluorouracil With or Without Oxaliplatin as First-Line Treatment in Advanced Colorectal Cancer. *J Clin Oncol* **18:** 2938-2947].

[4] FOLFOX7: Oxaliplatin 130 mg/m^2 over 2 hrs; LV 400 mg/m^2 concurrently, followed by 5FU bolus 400 mg/m^2, then 5FU 2400 mg/m^2 infusion over 46 hrs; cycle repeated every 14 days [Maindrault-Goebel F, de Gramont A, Louvet C, Andre T, Carola E, Mabro M, Artru P, Gilles V, Lotz JP, Izrael V (2001) High-dose intensity oxaliplatin added to the simplified bimonthly leucovorin and 5-fluorouracil regimen as second-line therapy for metastatic colorectal cancer (FOLFOX 7). *European Journal of Cancer* **37:** 1000-1005].

Table 1.5 (continued) Hepatic-resection rates post systemic chemotherapy				
(Delaunoit *et al*, 2005)	IFL[1] FOLFOX4 IROX[2]	264 267 265	0.8 4.1 4.2	0.02[3]
(Köhne *et al*, 2005)	5FU/LV Irinotecan/5FU/LV	216 214	6.5 2.8	-
(Diaz Rubio *et al*, 2005)	Cetuximab/FOLFOX4	43	18.6	-
(Martoni *et al*, 2005)	FOLFOX Capecitabine/oxaliplatin	48 52	4.1 9.6	-
(Falcone *et al*, 2006)	FOLFIRI FOLFOXIRI[4]	122 122	6 14	0.02[5]
Selective reports using regimens containing oxaliplatin and irinotecan; data adapted from: Hepatic colorectal metastases: methods of improving resectability (Adam *et al*, 2004b), Management of liver metastases from colorectal cancer (Kemeny, 2006), Neoadjuvant chemotherapy before liver resection for patients with unresectable liver metastases from colorectal carcinoma (Leonard *et al*, 2005)				

[1] IFL: Irinotecan 125 mg/m^2 over 90 mins, followed by LV 20 mg/m^2 by brief infusion, then bolus 5FU 500 mg/m^2; all treatments repeated weekly for 4 wks, and repeated every 6 wks [Saltz LB, Cox JV, Blanke C, Rosen LS, Fehrenbacher L, Moore MJ, Maroun JA, Ackland SP, Locker PK, Pirotta N (2000a) Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. Irinotecan Study Group. *N Engl J Med* **343:** 905-14].

[2] IROX: bolus oxaliplatin 85 mg/m^2 and irinotecan 200 mg/m^2 every 3 weeks [Wasserman E, Sutherland W, Cvitkovic E (2001) Irinotecan plus oxaliplatin: a promising combination for advanced colorectal cancer. *Clin Colorectal Cancer* **1:** 149-53].

[3] The rate of curative intent resection was significantly higher for patients treated with oxaliplatin-containing regimens.

[4] FOLFOXIRI: Irinotecan 165 mg/m^2 day 1, oxaliplatin 85 mg/m^2 day 1, LV 200 mg/m^2 day 1, 5FU 3200 mg/m^2 48 hr infusion. starting on day 1; treatments were repeated every 2 weeks [Falcone A, Masi G, Brunetti I, Benedetti G, Bertetto O, Picone V, Chiara S, Merlano M, Vitello S, Ricci S (2006) The triplet combination of irinotecan, oxaliplatin and 5FU/LV (FOLFOXIRI) vs the doublet of irinotecan and 5FU/LV (FOLFIRI) as first-line treatment of metastatic colorectal cancer (MCRC): Results of a randomized phase III trial by the Gruppo Oncologico Nord Ovest (G.O.N.O.). *J Clin Oncol (Meeting Abstracts)* **24:** 3513-].

[5] Increased response allowed a radical secondary resection of hepatic metastases in a greater percentage of patients in the FOLFOXIRI arm (6% versus 14%, $P = 0.05$, among all 244 patients; and 12% versus 36%, $P = 0.02$, among 81 patients with liver metastases only) [Ibid.].

These retrospective results were updated for 1104 initially non-resectable patients and 12.5% of responders underwent hepatic resection with 0.7% operative mortality and 28% postoperative morbidity, with five-year survival of 33% and ten-year survival of 23%. However, there was a high rate of recurrence (111 of 138 patients or 80%), 40 of which were hepatic (29%), 12 extra-hepatic (9%), and 59 both hepatic and extra-hepatic (43%). Recurrence was treated in 52 patients by repeat hepatectomy and in 42 patients by extra-hepatic resection. When compared with survival of patients primarily resected within the same period, survival for these initially non-resectable patients was decreased (48% versus 30%, $P = 0.01$) (Adam *et al*, 2004a).

Another retrospective study of 151 patients used a similar oxaliplatin/FU/LV regimen and reported that 38% had curative resection with median survival was 48 months for the patients who underwent resection and 15.5 months in the patients who did not undergo resection. However, the fact that some patients with stable disease had surgery implies that some patients may have been resectable before neoadjuvant chemotherapy. (Giacchetti *et al*, 1999).

A randomized prospective study, designed primarily to evaluate efficacy of FOLRIRI and FOLFOX6 regimens, and to determine the best sequence to treat patients with metastatic colorectal cancer, demonstrated response rates of 56% for FOLRIRI and 54% for FOLFOX, implying no significant difference in these regimens if used for the purposes of neoadjuvant therapy. As a first-line therapy, secondary surgery to remove metastases was performed in 10 patients (9%) in the FOLRIRI arm versus 24 patients (22%) in the FOLFOX arm B ($P = 0.02$). Secondary surgery to remove metastases after

second-line therapy could only be performed in two patients in the FOLRIRI arm and 1 patient in the FOLFOX arm (Tournigand, 2003).

A study attempted to identify and describe patients treated on Intergroup study N9741 with initially inoperable metastatic colorectal cancer, who obtained sufficient chemotherapeutic benefit to allow removal of their metastatic disease. A total of 795 patients had been randomised to treatment with IFL, FOLFOX4 or IROX regimens, and the rate of curative intent resection was significantly higher for patients treated with oxaliplatin-containing regimens (P=0.02). The response rate for FOLFOX4 was also significantly better than for IFL (P=0.02) or for IROX (P=0.01), and response rates for IFL and IROX did not differ. The median overall survival time in the resected group was 42.4 months and the median time to relapse was 18.4 months (Delaunoit *et al*, 2005).

In a recent study from the Mayo Clinic, patients with liver-only metastases from colorectal cancer deemed unresectable by a surgeon experienced in liver surgery were considered eligible. The determination that a patient was unresectable was based on the distribution of multiple lesions or the proximity of large lesions adjacent to major vasculature structures that would preclude resection with tumour-free margins. This included the following: (1) metastatic disease adjacent to or apparently involving all three major hepatic veins, the portal vein bifurcation, or the retro-hepatic vena cava; (2) metastatic disease adjacent to or involving the main right or main left portal vein and the main hepatic vein of the opposite lobe; and (3) metastatic disease that would require more than a right or left trisegmentectomy. The presence of six or more metastatic lesions distributed diffusely in both lobes of the liver was also considered a contraindication to surgery. Patients requiring a resection that would jeopardize postoperative liver function

were also considered candidates for the study. Forty-two patients were treated with FOLFOX4 and there was a 60% response rate, with 40% undergoing hepatic resection after six months of treatment. Median survival time was 26 months, however a high recurrence rate after surgery was observed, which involved the liver in 73% of patients. The study concluded that FOLFOX4 had a high response rate in patients with liver-only metastases from colorectal cancer, allowing for successful resection of disease in a portion of patients initially not judged to be optimally resectable (Alberts *et al*, 2005).

Although high response rates have been seen with first-line therapy, response rates for second-line systemic chemotherapy therapy are generally low. In a study of previously untreated metastatic colorectal cancer patients randomised to either FOLFOX followed by FOLFIRI or the reverse sequence regimen, response rates of only 15% and 4% were demonstrated in patients who received second-line FOLFOX and FOLFIRI (Tournigand, 2003). Patients whose tumours have progressed on first-line therapy have a low likelihood of being rendered resectable with second-line systemic chemotherapy however, new biologic agents offer promise for further response (Leonard *et al*, 2005).

Preoperative chemotherapy has also been associated with pathologic changes of liver, including vascular changes and steatohepatitis (Bilchik *et al*, 2005; Nordlinger & Benoist, 2006). Several reports have demonstrated that oxaliplatin-based chemotherapy has been associated with an increased risk of vascular lesions of the liver (Aloia *et al*, 2006; Kooby *et al*, 2003; Rubbia-Brandt, 2004). Other reports have demonstrated that irinotecan-based chemotherapy has been associated with an increased risk of steatosis and steatohepatitis, and the latter is also observed more frequently after chemotherapy in

patients with a higher body mass index (Fernandez *et al*, 2005; Kooby *et al*, 2003; Parikh *et al*, 2003; Vauthey *et al*, 2006).

In a review of the effect of chemotherapy and liver injury on perioperative outcome in 406 patients who underwent hepatic-resection, oxaliplatin was associated with sinusoidal dilation compared with no chemotherapy (18.9% versus 1.9%; $P < 0.001$), and irinotecan was associated with steatohepatitis compared with no chemotherapy (20.2% versus 4.4%; $P < 0.001$). Importantly, patients with steatohepatitis had an increased 90-day mortality compared with patients who did not have steatohepatitis (14.7% versus 1.6%; $P = 0.001$) (Vauthey *et al*, 2006).

A report on preoperative oxaliplatin-based chemotherapy demonstrated that perioperative mortality and morbidity rates were not increased, but patients who had received more than 12 cycles of preoperative chemotherapy had a higher risk of reoperation and longer hospital stay (Aloia et al, 2006). In addition early toxicity data from the European Organisation for Research and Treatment of Cancer (EORTC) study 40983 which included 364 patients, and compared surgery alone with perioperative chemotherapy with FOLFOX, six cycles before surgery followed by six cycles after, showed that morbidity and mortality rates were thus far similar for both treatment arms (Nordlinger *et al*, 2005).

It has been therefore been recommended that patients with initially unresectable liver metastases can receive chemotherapy to render their metastases resectable, provided treatment is carefully monitored, and surgery can proceed as soon as the metastases become resectable without waiting for best radiographic response to chemotherapy.

Protracted chemotherapy with subsequent surgical delay may result in increased toxicity and liver damage, which may negatively impact resection (Nordlinger & Benoist, 2006).

(ii) Biologic therapy

The integration of novel targeted biologic agents, including use as neoadjuvant therapy, is transforming the treatment of patients with advanced colorectal cancer, and it certainly is possible that this could increase the rate of resection (Bilchik *et al*, 2005; Ellis *et al*, 2005; Leonard *et al*, 2005; Nordlinger & Benoist, 2006).

As first-line therapy for metastatic colorectal cancer, 813 patients were randomised to combination IFL and the anti-angiogenesis agent bevacizumab[1] or IFL plus placebo. There was progression-free survival advantage (10.6 versus 6.2 months; $P < 0.001$), median overall survival advantage (20.3 versus 15.6 months; $P < 0.001$) and response rate of (44.8% versus 34.8%; $P = 0.004$) for the bevacizumab/IFL regimen versus IFL/placebo (Hurwitz *et al*, 2004). A phase 2 trial compared bevacizumab/5FU/LV to 5FU/LV as first-line therapy in 209 patients considered non-optimal candidates for first-line irinotecan. Addition of bevacizumab was found to provide clinically significant patient benefit, including improvement in progression-free survival (Kabbinavar *et al*, 2005b).

Eastern Cooperative Oncology Group (ECOG) study E3200 randomised 829 patients previously treated (progression after IFL therapy) for advanced colorectal cancer, to high-dose bevacizumab (10mg/kg) plus FOLFOX4 or FLOFOX4 alone, and found improved

[1] Bevacizumab is a humanized monoclonal antibody that binds to vascular endothelial growth factor [Presta LG, Chen H, O'Connor SJ, Chisholm V, Meng YG, Krummen L, Winkler M, Ferrara N (1997) Humanization of an Anti-Vascular Endothelial Growth Factor Monoclonal Antibody for the Therapy of Solid Tumors and Other Disorders. *Cancer Res* **57:** 4593-4599].

progression-free ($P < 0.0001$) and overall survival ($P = 0.0018$) for the bevacizumab arm (Giantonio *et al*, 2005). Furthermore, the addition of bevacizumab to three different oxaliplatin-based regimens as first-line treatment for metastatic colorectal cancer in the TREE-2 study, demonstrated improved response rates and time to progression with acceptable tolerability, and no unexpected toxicity (Hochster *et al*, 2006).

Bevacizumab has been considered responsible for increased risk of organ perforation (Giantonio *et al*, 2005; Hurwitz *et al*, 2004; Kabbinavar *et al*, 2005b), bleeding, and decreased wound healing (Scappaticci *et al*, 2005). Due to the critical role of vascular endothelial growth factor (VEGF) in liver regeneration, it is also possible that hepatic regeneration could be impaired in patients who undergo surgery after having received anti-VEGF therapy. It has been recommended that a six to eight week interval (two half-lives of bevacizumab) should be allowed between the last administration of bevacizumab and surgery (Ellis *et al*, 2005; Nordlinger & Benoist, 2006).

Encouraging results have similarly been seen for the epidermal growth factor receptor (EGFR) inhibitor cetuximab1. In the second-line setting, cetuximab has also shown significant activity. Response rates seen in trials using single-agent cetuximab or cetuximab combined with irinotecan were 9% and 25%, in patients who had previously experienced disease progression on irinotecan (Chung *et al*, 2005; Saltz *et al*, 2004).

[1] Cetuximab is a chimeric human/murine monoclonal antibody that binds to the ligand binding domain of epidermal growth factor receptor [Sato JD, Kawamoto T, Le AD, Mendelsohn J, Polikoff J, Sato GH (1983) Biological effects in vitro of monoclonal antibodies to human epidermal growth factor receptors. *Mol Biol Med* **1**: 511-29].

A prospective study randomised 329 patients whose disease had progressed during or within three months after treatment with an irinotecan-based regimen, to receive either cetuximab plus irinotecan or cetuximab monotherapy. Cetuximab had clinically significant activity when given alone or in combination with irinotecan, with higher response rates in the combination therapy group (22.9% versus 10.8%; $P = 0.007$) and improved time to progression ($P < 0.001$) (Cunningham *et al*, 2004).

A multi-centre study evaluated the antitumour activity of cetuximab in 346 patients with metastatic colorectal cancer, whose tumours demonstrated EGFR immunostaining and were refractory to irinotecan, oxaliplatin, and fluoropyrimidines. Cetuximab was found to be active and well tolerated (Lenz *et al*, 2006). In a phase 2 trial of 20 patients assessable for response, the partial response rate using combination cetuximab and FOLFOX was 70%, and two patients underwent subsequent curative liver resection (Tabernero *et al*, 2004).

Cetuximab however, does not appear to increase the complication rate of surgery and there is no current evidence that EGFR inhibitors impair wound healing or liver regeneration (Ellis et al, 2005). The encouraging response rates seen with biologic agents in combination with chemotherapy suggest that they will play an important role in the development of effective neoadjuvant chemotherapy regimens. However, data specifically addressing the role of biologic agents as neoadjuvant therapy is currently lacking (Leonard et al, 2005).

(iii) Regional chemotherapy

Regional drug delivery is an approach designed to improve the selectivity of chemotherapy. When compared with systemic drug administration, regional delivery can potentially increase drug concentrations at tumour sites and/or lower systemic drug exposure (Collins, 1984).

Hepatic arterial infusion (HAI) therapy maximizes drug exposure to the liver by delivering the drug via the hepatic artery, and agents such as floxuridine (FUDR) in particular have a high first pass hepatic extraction (almost 95%) (Ensminger *et al*, 1978), and HAI therapy therefore appears to be an ideal choice for neoadjuvant therapy. HAI therapy has demonstrated high response rates, both as first and second-line treatments (Kemeny *et al*, 2006; Meta-Analysis-Group-In-Cancer, 1996), and although results from neoadjuvant HAI therapy trials are encouraging (**see Table 1.6**), it is difficult to compare these trials to neoadjuvant systemic chemotherapy trials because of heterogeneity in study design and patient populations. Current data support the use of either neoadjuvant HAI or systemic chemotherapy, and randomised trials are needed to further determine the optimal approach (Leonard *et al*, 2005).

Some reports have evaluated FUDR-based HAI therapy. A study evaluated 168 patients treated with four HAI regimens, with two arms containing FUDR only and two other arms containing 5FU-based chemotherapy. The overall resection rate was 5%, but despite similar response rates in all arms, all of the resected patients came from the 5FU-regimen arms. The median survival for patients in the FUDR groups was 20.8 months, compared with 19.8 months for 5FU/LV alone and 27.4 months for the FU/LV/ mitoxantrone/mitomycin regimen.

A possible explanation for the poor results seen with HAI FUDR may be related to differences in patient baseline characteristics, and sclerosing cholangitis/cirrhosis (seen in up to 25% of the FUDR treated patients compared with 0% in 5FU treated patients) which may have prevented liver resection in the FUDR group (Link *et al*, 1999a; Link *et al*, 1999b). Another study also used HAI FUDR or 5FU-based therapy to render 4.2% of 383 patients resectable, and the authors suggested that prior treatment failure with disease progression was an explanation for the observed low response and resection rates (Meric *et al*, 2000).

Several trials have also evaluated 5FU-based HAI therapy. A report showed that 5.8% of 239 patients were rendered resectable when treated with 5FU-based HAI, and at mean follow-up of 36 months post HAI, five of nine patients treated for colorectal liver metastases were disease free (Elias *et al*, 1995). Another study treated 36 patients with 5FU-based HAI in combination with systemic therapy, and 11% became resectable. Survival of patients who underwent resection ranged from 24 to more than 39 months and was superior to the survival of 13 months seen for all 36 patients (Milandri *et al*, 2003).

Table 1.6

Hepatic-resection rates post regional chemotherapy

Reference	Regimen	Number of patients	Resection rate (%)	p value
(Elias *et al*, 1995)	HAI: 5FU/mitomycin Cisplatin/ pirarubicin	239	5.8	-
(Link *et al*, 1999b) (Link *et al*, 1999a)	HAI: 5FU/LV/ Mitomycin/mitoxantrone	74	12.2	-
(Meric *et al*, 2000)	HAI: FUDR or FU/LV/mitomycin	383	4.2	-
(Clavien *et al*, 2002)	HAI: FUDR	23	26	-
(Milandri *et al*, 2003)	HAI: 5FU/mitomycin + SYS: 5FU	31	9.7	-
(Zelek, 2003)	HAI: pirarubicin + SYS: 5FU/LV/irinotecan	31	35	-
(Leonard *et al*, 2004)	HAI: FUDR + SYS: FOLFOX/IROX	44	20	-
(Noda *et al*, 2004)	HAI: 5FU + SYS: UFT	51	47	-
(Kemeny *et al*, 2005b)	HAI: FUDR + SYS: irinotecan/oxaliplatin	21	33.3	-
(Garassino *et al*, 2005)	HAI: 5FU + SYS: Oxaliplatin/LV	34	15	-
(Ducreux *et al*, 2005)	HAI: Oxaliplatin + SYS: FU/LV	28	17.8	-
(Bouchahda *et al*, 2006)	HAI: Oxaliplatin/ Irinotecan/FU	25	3	-

Selective reports using HAI regimens in neoadjuvant setting containing 5FU or FUDR ± systemic therapy (SYS); data adapted from: Management of liver metastases from colorectal cancer (Kemeny, 2006), Neoadjuvant chemotherapy before liver resection for patients with unresectable liver metastases from colorectal carcinoma (Leonard *et al*, 2005).

Fifty-one patients were treated with 5FU HAI plus oral uracil and tegafur, and achieved a response rate of 78%, rendering 31 patients resectable. Of interest, eight patients chose to continue HAI therapy without surgery and had a five-year survival rate of 0%, whereas the 24 resected patients had a 5-year survival rate of 42% (Noda *et al*, 2004).

In a phase 1 study of systemic oxaliplatin/irinotecan plus HAI chemotherapy in 44 unresectable patients, with 70% of patients receiving this treatment as second or third-line therapy, 34% became resectable. The median survival of the entire group was 36 months,

and median survival for the resected group has not yet been reached (Kemeny *et al*, 2005b). A similar phase 2 study of concomitant 5FU HAI plus systemic oxaliplatin/5FU in 34 patients (51% previously treated) showed response rates of 53% and 29% for chemonaive and pretreated patients, and 15% underwent radical surgery on the liver. The median time to progression was 9 months (range, 2 to 43) and the median overall survival was 22 months (range, 2 to 51+), and better results were obtained in chemonaive patients and in those without extra-hepatic disease (Garassino *et al*, 2005).

Complications of neoadjuvant HAI chemotherapy are mainly chemotherapy or catheter related toxicities. HAI therapy most commonly requires positioning of a catheter into the gastroduodenal artery. Prior studies have demonstrated that only approximately 60% of patients have normal anatomy, and arterial ablation is sometimes necessary to facilitate pump placement (Skitzki & Chang, 2002).

Surgical complications include hepatic artery occlusion, and catheter thrombosis or displacement, and are increased with inexperience. Complications usually occur in less than 10% of patients and are more frequent with external than with implanted infusion pumps. Mortality from HAI therapy has been less than 1% (Barnett & Malafa, 2001; Campbell *et al*, 1993; Heinrich *et al*, 2003).

The main HAI chemotherapy toxicities have been gastrointestinal symptoms (22%), chemical hepatitis (19%), and bone marrow toxicity (8%), and sclerosing cholangitis and chemical hepatitis are associated with the use of FUDR, while the use of 5FU is associated with bone marrow toxicity (Barnett & Malafa, 2001).

1.3 Non-surgical treatment for colorectal hepatic-metastatic disease

Prospective studies have demonstrated that chemotherapy as treatment for metastatic colorectal cancer prolongs survival and improves quality of life in comparison to palliative care alone (Allen-Mersh *et al*, 1994; Cunningham *et al*, 1998; Nordic-Gastrointestinal-Tumour-Adjuvant-Therapy-Group, 1992; Scheithauer *et al*, 1993). A meta-analysis of 13 randomised controlled trials representing a total of 1365 patients demonstrated that palliative chemotherapy was associated with a 35% reduction in the risk of death, which translated into an absolute improvement in survival of 16% at both six and twelve months and an improvement in median survival of 3.7 months (Cochrane, 2000; Simmonds, 2000). For several years the fluoropyrimidines represented the only active agents for treatment of metastatic colorectal cancer, however this changed markedly with the recent development of other effective new drugs including irinotecan, oxaliplatin, and two humanized monoclonal antibodies that target vascular endothelial growth factor (bevacizumab) and the epidermal growth factor receptor (cetuximab) (Kelly & Goldberg, 2005; Meyerhardt & Mayer, 2005).

The value of early chemotherapy for treatment of asymptomatic patients with metastatic colorectal cancer has not been adequately resolved. A lack of clear benefit for early treatment was suggested in a meta-analysis of 168 asymptomatic patients enrolled in two trials randomly testing early versus delayed 5FU-based chemotherapy. This demonstrated similar median overall and progression-free survival, and overall quality of life (Ackland *et al*, 2005). However, another trial in which 182 asymptomatic patients were randomly assigned to initial or deferred chemotherapy with methotrexate/5FU/LV, earlier treatment was associated with improvement in median survival (14 versus 9 months; $P < 0.02$), symptom-free interval and time to progression (8 versus 4 months; $P <$

0.001) (Nordic-Gastrointestinal-Tumour-Adjuvant-Therapy-Group, 1992). Neither of these reports involved treatment with more modern regimens including irinotecan or oxaliplatin.

The optimal duration of therapy, particularly the benefit of a chemotherapy-free interval in patients with responding or non-progressive disease remains controversial. A study randomised 354 patients who responded or had stable disease after receiving 12 weeks of first-line regimens, to either intermittent (a break in chemotherapy, re-starting on the same drug on progression), or continuous chemotherapy until progression. Results demonstrated no clear evidence of a benefit in continuing therapy indefinitely until disease progression, and showed that it was safe to stop chemotherapy after 12 weeks and re-start the same treatment on progression in patients with chemo-sensitive disease (Maughan *et al*, 2003).

Another study involved 55 patients with 5FU-refractory disease who received irinotecan monotherapy for 6 months and did not progress. Patients were then randomised to either discontinue irinotecan, or continue until disease progression occurred. No difference was seen in failure-free survival, overall survival and quality-of-life. The study concluded that there was little benefit from continuing irinotecan in these patients, though the drug was well tolerated without any deterioration in quality of life (Lal *et al*, 2004). However, early results form the OPTIMOX2 study which randomised 177 non-progressing patients following FOLFOX7 treatment to either maintenance chemotherapy with 5-FU/LV or chemotherapy-free interval, demonstrated a benefit for maintenance chemotherapy with improved progression-free survival ($P = 0.01$) (Maindrault-Goebel *et al*, 2006).

1.31 Systemic therapy

A. Fluoropyrimidines

Synthesized in 1957on the premise that uracil metabolism might represent a potential target for cancer chemotherapy (Heidelberger *et al*, 1957), 5FU is a fluorinated pyrimidine[1] which impairs DNA synthesis, and acts primarily by inhibiting thymidylate synthase (the rate-limiting enzyme in pyrimidine nucleotide synthesis) and incorporating its metabolites into RNA and DNA (Longley *et al*, 2003; Sobrero *et al*, 2000). The deoxyribonucleoside derivative 5-fluoro-2'-deoxyuridine (Floxuridine or FUDR) is limited in its clinical use given its rapid degradation in normal and tumour tissues, hence it is not administered systemically, but rather its use has been strictly limited to hepatic arterial infusions (Ensminger *et al*, 1978). Capecitabine (N^4-pentoxycarbonyl-5'-deoxy-5-fluorocytidine,) is a novel oral fluoropyrimidine carbamate that was designed as a 5FU pro-drug to facilitate intact passage through intestinal mucosa and selective 5FU activation in tumour tissue (Shimma *et al*, 2000).

5FU is inactive and requires intracellular activation to exert cytotoxic effects. 5FU readily enters cells via the facilitated uracil transport mechanism, whereas FUDR is a substrate for the facilitated nucleoside transport system. Anabolism of these compounds to cytotoxic forms occurs via several biochemical pathways. 5FU is converted to FUDR by thymidine phosphorylase. Subsequent phosphorylation of FUDR by thymidine kinase results in formation of the active metabolite 5-fluoro-2'-deoxyuridine monophosphate (or FdUMP). In the presence of the reduced folate cofactor 5,10-methylenetetrahydrofolate, FdUMP forms a stable covalent complex with thymidylate synthase. Thymidylate synthase catalyzes the sole intracellular de novo formation of thymidine-5'-monophosphate

[1] A fluorine atom is substituted in place of hydrogen at the C5 position of the pyrimidine ring.

from deoxyuridine monophosphate (dUMP). Inhibition of thymidylate synthase leads to depletion of deoxythymidine triphosphate (dTTP), thus interfering with DNA biosynthesis and repair. 5FU is also metabolized to fluorouridine monophosphate through the sequential action of uridine phosphorylase and uridine kinase. In the presence of 5'-phosphoribosyl-1-pyrophosphate, orotic acid phosphoribosyltransferase directly converts 5FU to fluorouridine monophosphate. This metabolite is further metabolized to fluorouridine diphosphate and then to the triphosphate form (FUTP), which is subsequently incorporated into RNA (Ismail & Grem, 2001; Longley *et al*, 2003)

Thymidylate synthase inhibition by FdUMP is considered one of the principal mechanisms of 5FU action. The thymidylate synthase/FdUMP/folate ternary complex is slowly dissociable, and the intracellular level of 5,10-methylenetetrahydrofolate is critical for ternary complex formation as well as for maintaining enzyme inhibition. Depletion of intracellular reduced folate pools prevents ternary complex formation. Pharmacologic concentrations of 5-formyltetrahydrofolate (Leucovorin or LV) modulate or enhance the cytotoxicity of 5FU by expanding the intracellular pools of 5,10-methylenetetrahydrofolate and thereby increasing the extent and duration of thymidylate synthase inhibition (Longley *et al*, 2003; Mini *et al*, 1990; Park *et al*, 1988; Sotos *et al*, 1994).

The metabolite FUTP is incorporated into both nuclear and cytoplasmic RNA species. This interferes with normal RNA processing and function, and the extent of RNA incorporation correlates with cytotoxicity. Inhibition of thymidylate synthase leads not only to depletion of dTTP but to accumulation of dUMP. Both FdUMP and dUMP may be subsequently metabolized to their respective triphosphate forms. Incorporation of deoxyuridine triphosphate (dUTP) and fluorodeoxyuridine triphosphate (FdUTP) into cellular DNA, with resultant inhibition of DNA synthesis and function, may represent

another mechanism of cytotoxicity. The enzyme dUTP nucleotidehydrolase degrades triphosphate nucleotides and limits the intracellular accumulation of dUTP/ FdUTP. The nucleotide excision repair enzyme uracil-DNA glycosylase attempts to repair DNA that contains uracil and 5FU however, this is unsuccessful if the intracellular nucleotide ratio favours dUTP/ FdUTP over dTTP. The combined effects of dTTP depletion and dUTP/ FdUTP DNA incorporation result in inhibition of nascent DNA chain elongation, altered DNA stability, production of DNA single-strand breaks, and interference with DNA repair. The genotoxic stress resulting from thymidylate synthase inhibition may also activate programmed cell death pathways in susceptible cells, which leads to induction of parental DNA fragmentation, and factors operating downstream from thymidylate synthase may influence the cellular response (Chu & Allegra, 1996; Fisher, 1993; Longley *et al*, 2003; Lowe *et al*, 1993).

5FU is not administered by the oral route because its bioavailability is erratic due to high levels of the enzyme dihydropyrimidine dehydrogenase present in the gut mucosa. 5FU is normally given intravenously, and it has a short metabolic half-life on the order of 15 minutes (Hahn *et al*, 1975; Meropol, 1998). The rate-limiting enzyme in 5FU catabolism is dihydropyrimidine dehydrogenase, which converts 5FU to dihydrofluorouracil. More than 80% of administered 5FU is normally catabolised primarily in the liver, where dihydropyrimidine dehydrogenase is abundantly expressed and displays a circadian pattern of activity (Diasio & Harris, 1989; Diasio & Lu, 1994). Inter-patient variation in the activity of dihydropyrimidine dehydrogenase may account for differences in toxicity, and individuals who lack dihydropyrimidine dehydrogenase develop severe, potentially fatal side effects (van Kuilenburg *et al*, 2001).

(i) Fluorouracil

From the late 1950s until recently, the antimetabolite fluorouracil or 5FU was the foundation of systemic therapy for metastatic colorectal cancer (Kelly & Goldberg, 2005; Meyerhardt & Mayer, 2005). Bolus 5FU as a single agent has limited activity, as almost 90% of patients do not achieve an objective response. Higher response rates can be achieved when 5FU is administered by continuous infusion, and with the addition of LV to bolus 5FU (O'Dwyer *et al*, 2001). In a meta-analysis of randomised trials comparing continuous infusion versus bolus administration of 5FU monotherapy, continuous infusion therapy was associated with modestly higher response rate (22% versus 14%; $P = 0.0002$)) and marginally longer median survival (12 versus 11 months; $P = 0.04$). Gastrointestinal toxicity was similar, while moderately severe haematologic toxicity (particularly neutropenia) was more common with bolus 5FU (31% versus 4%). Hand-foot syndrome[1] was more common with continuous infusion therapy (34% versus 13%) (Meta-analysis-Group-In-Cancer, 1998a; Meta-Analysis-Group-In-Cancer, 1998b)

Randomised clinical trials in advanced colorectal cancer and meta-analyses of almost 3,300 patients indicate that compared with bolus 5FU alone, combined bolus 5FU/LV improved response rates (11% versus 21%; $P < 0.0001$) and median survival (11.7 versus 10.5 months; $P = 0.004$), which were primarily seen in the first year (Buyse *et al*, 2000; Piedbois *et al*, 1992; Thirion *et al*, 2004).

In order to optimize therapy, several different schedules and doses of bolus LV modulated 5FU have been studied for treatment of metastatic colorectal cancer.

[1] Palmar–plantar erythrodysaesthesia.

Developed in the United States, the most common are the monthly Mayo Clinic[1] and weekly Roswell Park[2] regimens, and the comparative efficacy of these regimens has been explored in several studies. A study randomised 372 patients to either the low-dose Mayo regimen or the Roswell Park regimen. Both regimens produced similar response rates (35% versus 31%) and median survival (9.3 versus 10.7 months), but the toxicity profiles differed. The monthly Mayo regimen was associated with more leucopenia and stomatitis, but less diarrhoea and need for hospitalization compared to the weekly Roswell Park regimen (Buroker *et al*, 1994).

A second study randomised 96 previously untreated patients to either weekly IV bolus lower dose 5FU 400 mg/m^2 plus lower dose LV 20 mg/m^2 or IV bolus 5FU 425 mg/m^2 plus LV 20 mg/m^2 for five consecutive days every four to five weeks. Similar response (14% versus 11%) and median survival (16 versus 18 months) was reported for both regimens. Monthly treatment however, was more toxic, with more moderately severe diarrhoea (15% versus 2%) and stomatitis (9% versus 0%). Haematologic toxicity was minimal in both arms (Wang *et al*, 2000).

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[1] The Mayo regimen consists of bolus 5FU 425 mg/m^2 plus low-dose LV 20 mg/m^2, both given on days 1 to 5 every four to five weeks, or a modification consisting of 5FU 370 mg/m^2 plus high-dose LV 200 mg/m^2 each given on days 1 to 5, every four to five weeks [Poon MA, O'Connell MJ, Moertel CG, Wieand HS, Cullinan SA, Everson LK, Krook JE, Mailliard JA, Laurie JA, Tschetter LK (1989) Biochemical modulation of fluorouracil: evidence of significant improvement of survival and quality of life in patients with advanced colorectal carcinoma. *J Clin Oncol* **7:** 1407-18].

[2] The Roswell Park regimen consists of weekly 5FU 500 mg/m^2 plus LV 500 mg/m^2 for six of every eight weeks [Petrelli N, Herrera L, Rustum Y, Burke P, Creaven P, Stulc J, Emrich LJ, Mittelman A (1987) A prospective randomized trial of 5-fluorouracil versus 5-fluorouracil and high-dose leucovorin versus 5-fluorouracil and methotrexate in previously untreated patients with advanced colorectal carcinoma. Ibid. **5:** 1559-65].

Lower versus higher weekly LV doses were directly compared in a third study in which 291 patients were randomised to weekly 5FU 500 mg/m^2 plus either low dose 20 mg/m^2 or high dose 500 mg/m^2 LV. Both regimens had similar response rates (22% versus 18%), response duration (25 versus 23 weeks), and progression-free intervals (29 versus 30 weeks). Severe side effects were infrequent and did not differ between the two groups, although the overall toxicity profile favoured the lower dose of weekly LV (Jager *et al*, 1996).

Overall, the monthly schedule of leucovorin-modulated 5FU is associated with higher rates of neutropenia and stomatitis, while the weekly schedule causes more diarrhoea. The five-day bolus regimen also appears to be more toxic in women than in men. The lower rates of neutropenia and stomatitis and the ability to stop or modify therapy if toxicity occurs, favours the weekly Roswell Park regimen. Although the equivalence of low-dose (20 mg/m^2 per dose) versus high-dose (500 mg/m^2 per dose) leucovorin for the weekly schedule has not been firmly established for treatment of metastatic disease, use of the lower dose is common (Saltz, 2003).

Infusion schedules have been advocated as methods to gain additional therapeutic advantage for 5FU/LV regimens (Sobrero *et al*, 1997). Reports of short-term infusion schedules have demonstrated further improved response rates for combined 5FU/LV regimens and support this concept. This was demonstrated in a trial of 448 patients who were randomised to either a monthly regimen of LV 20 mg/m^2 plus bolus 5FU 425 mg/m^2 on days 1 to 5 every four weeks, or a bimonthly regimen (the de Gramont regimen) of LV 200 mg/m^2 over two hours followed by bolus 5FU 400 mg/m^2 and a 22 hour infusion of 5FU 600 mg/m^2, with both drugs given daily for two consecutive days every two weeks.

Improved response rates (33% versus 14%; $P = 0.0004$), median progression-free survival (28 versus 22 weeks; $P = 0.0012$), and a trend toward longer median survival (62 versus 57 weeks; $P = 0.067$) were seen with the infusional regimen, which also caused less haematologic and gastrointestinal toxicity (de Gramont *et al*, 1997). Another study randomised 497 patients to either a monthly Mayo type 5FU/LV regimen or 5FU 2600 mg/m^2 as a 24 hour infusion alone or in combination with 500 mg/m^2 LV, all given weekly for six weeks, followed by a two week rest period. Similar to the prior report, the 5FU/LV 24 hour infusion in this report was found to have the best response rate (17%) with improved progression-free survival ($P = 0.029$) (Kohne *et al*, 2003).

Circadian differences in thymidylate synthase activity in normal tissues and dihydropyrimidine dehydrogenase levels are the basis for the existence of diurnal variation of toxicity profiles for 5FU based therapy (Ii *et al*, 2000; Wood *et al*, 2006). Chronomodulation refers to a method of drug administration that is variable over a 24 hour period and takes into account circadian rhythm differences and drug interactions. For patients receiving 5FU-based regimens, chronomodulated administration schedules have generally increased response rates and have been associated with less toxicity than non-chronomodulated schedules however, survival benefits have not been clearly established (Cure *et al*, 2002; Levi *et al*, 1997; Levi *et al*, 1994).

(ii) Orally active fluoropyrimidines

In contrast to 5FU, capecitabine is rapidly and extensively absorbed by intestinal mucosa, with nearly 80% oral bioavailability. It is inactive in original form and must undergo enzymatic conversion via three successive steps. First it is hydrolyzed in the liver

by a hepatic carboxylesterase to an intermediate, 5'-deoxy-5-fluorocytidine, which is then converted to 5'-deoxy-5-fluorouridine by the enzyme cytidine deaminase. The third step occurs in tumour tissue and involves conversion of 5'-deoxy-5-fluorouridine to 5FU by the enzyme thymidine phosphorylase. Thymidine phosphorylase is present at higher levels in colorectal tumour cells compared to normal tissue, thereby providing the basis for enhanced selectivity for tumour cells, and may also be a predictive marker for clinical response.

After ingestion of capecitabine, peak plasma levels are attained in 1.5 hours, with peak 5FU blood levels occurring at 2 hours. Capecitabine and capecitabine metabolites are primarily excreted by the kidneys, and caution must be taken in the presence of renal dysfunction, with formal recommendations for dosage reduction. Dose-limiting toxicities include nausea, vomiting, diarrhoea, and hand-foot syndrome. Uncommon side effects include myelosuppression and hyperbilirubinaemia (Di Costanzo *et al*, 2000; Meropol *et al*, 2006; Pentheroudakis & Twelves, 2002; Schüller *et al*, 2000).

Two large randomised trials have shown similar efficacy for capecitabine monotherapy with 1250 mg/m ^{2} twice daily, for 14 of every 21 days, compared to the 5FU/LV with the Mayo regimen, as first-line treatment of metastatic colorectal cancer. In one study 605 patients were randomised and capecitabine was associated with a modestly higher response rate than 5FU/LV (25% versus 16%; $P = 0.005$), but similar median time to tumour progression (4.3 versus 4.7 months), and overall survival (12.5 versus 13.3 months). The incidence of moderately severe diarrhoea, stomatitis, nausea, and neutropenic sepsis were less in the capecitabine group ($P < 0.0001$), and only hyperbilirubinaemia and hand-foot syndrome were more common compared to 5FU/LV (*P*

< 0.0001). Similar results were noted a second randomised trial of 602 patients, except that response rates were similar (19% versus 15%), as was time to progression (5.2 versus 4.7 months), and median survival (13.2 versus 12.1 months) (Hoff *et al*, 2001; Van Cutsem *et al*, 2004; Van Cutsem *et al*, 2001).

In contrast to these results of first-line therapy, objective response rates with second-line capecitabine monotherapy are quite low in patients with 5FU/LV refractory metastatic colorectal cancer (Hoff *et al*, 2004; Lee *et al*, 2004).

UFT is a combination of uracil and tegafur (a prodrug of 5FU) in a fixed molar ratio of 4:1, which competitively inhibits dihydropyrimidine dehydrogenase to improve the absorption and bioavailability of tegafur, resulting in sustained 5FU concentrations in both plasma and tumour (Hoff *et al*, 1999; Sulkes *et al*, 1998). This orally active prodrug has equivalent activity to the Mayo 5FU/LV regimen. In one report, response rates were approximately 25% when UFT was administered as a single agent, and 40% in combination with oral LV 150mg daily. The dose-limiting toxicity is diarrhoea, and myelosuppression and hand-foot syndrome are infrequent. In two randomised studies however, UFT therapy resulted in response rates and median overall survival similar to those obtained with parenteral 5FU/LV (Carmichael *et al*, 2002; Douillard *et al*, 2002; Mayer, 2001).

B. Irinotecan

Camptothecin is a naturally occurring alkaloid and cytotoxic extract from the bark and wood of the Chinese plant, *Camptotheca acuminata*. Due to poor aqueous solubility

and formulation for initial clinical testing as the less active carboxylate sodium salt, early studies demonstrated objective tumour responses, but were associated with severe and unpredictable toxicities, such as hemorrhagic cystitis (Takimoto & Arbuck, 2001).

The semi synthetic camptothecin derivative irinotecan or CPT11, exerts a cytotoxic effect through its interaction with the enzyme topoisomerase I, which is involved in the uncoiling of DNA for replication and transcription, and causes single-stranded DNA breaks. Such breaks are normally transient and repaired; however, camptothecin stabilizes these breaks, leading to DNA fragmentation and cell death through interaction with the replication fork[1] (Hsiang & Liu, 1988; Iyer, 1998; Liu *et al*, 2000).

Hepatic carboxylesterases hydrolyze the inactive prodrug irinotecan to its active metabolite SN-38, which is detoxified to an inactive, glucuronidated form by uridine diphosphate glucuronosyltransferase isoform 1A1 (UGT1A1) and excreted in the urine and bile, and additional inactive metabolites are also formed through oxidative metabolism by cytochrome P-450 enzymes (Klein *et al*, 2002; Mathijssen *et al*, 2001).

Irinotecan as monotherapy has demonstrated clinical benefit after 5FU failure in patients with metastatic colorectal cancer. A study randomised 267 patients who had failed to respond to first-line 5FU or whose disease had progressed after treatment with first-line 5FU. Patients treated with irinotecan lived for significantly longer than patients on 5FU ($P = 0.035$). Survival at 1 year was increased from 32% in the 5FU group to 45% in the

[1] When DNA is ready to replicate itself, helicase breaks the hydrogen bonds holding the two DNA strands together and the resulting structure which has two branches or "prongs", each one made up of a single strand of DNA, is known as a replication fork [DePamphilis ML, Wassarman PM (1980) Replication of Eukaryotic Chromosomes: A Close-up of the Replication Fork. *Annual Review of Biochemistry* **49:** 627-666].

irinotecan group. Median survival was 10.8 months in the irinotecan group and 8.5 months in the 5FU group. Median progression-free survival was longer with irinotecan (4.2 versus 2.9 months; $P = 0.030$). Both treatments were equally well tolerated and quality of life was similar in both groups (Rougier *et al*, 1998b).

A trial of 189 patients with 5FU-refractory disease randomised either to best supportive care with or without irinotecan. The irinotecan group had superior one-year survival (36% versus 14%; $P = 0.0001$) and quality of life (Cunningham *et al*, 1998). The toxic effects of irinotecan include diarrhoea, bone marrow suppression, nausea, vomiting, and alopecia (Fuchs *et al*, 2003) and polymorphisms of UGT1A1 appear to correlate with the severity of the gastrointestinal effects and bone marrow suppression (Ando *et al*, 1998; Innocenti *et al*, 2004; Iyer *et al*, 2002).

Large phase 3 randomised trials have demonstrated survival benefits for combined irinotecan plus 5FU/LV compared to 5FU/LV alone. In a study 387 previously untreated patients were randomised to 5FU/LV with or without irinotecan. Irinotecan therapy could be administered either weekly with irinotecan 80 mg/m^2, 5FU 2300 mg/m^2 over 24 hours, LV 500 mg/m^2 or every other week with irinotecan 180 mg/m^2 on day 1, 5FU 400 mg/m^2 bolus followed by 600 mg/m^2 over 22 hours, both on days one and two, and LV 200 mg/m^2 on days one and two (the Douillard FOLFIRI regimen). The control arm allowed weekly 5FU 2600 mg/m^2 over 24 hours plus LV 500 mg/m^2 or every other week regimens 5FU 400 mg/m^2 bolus followed by 600 mg/m^2 over 22 hours, both on days 1 and 2, and LV 200 mg/m^2 on days one and two. Irinotecan therapy was associated with a higher response rate (49% versus 31%; $P < 0.001$), as well as longer time to progression (6.7 versus 4.4 months; $P < 0.001$) and median survival (17.4 versus 14.1 months; $P = 0.031$). Although some

toxicities were more common with irinotecan (moderately severe diarrhoea and neutropenia), they were predictable, reversible, non-cumulative, and manageable (Douillard *et al*, 2000).

Another trial comparing weekly infusional 5FU/LV with and without irinotecan as first-line therapy in 430 patients with metastatic colorectal cancer demonstrated similar results. As seen with the prior report, the addition irinotecan was associated with higher response rates (62% versus 34%; $P < 0.0001$), progression-free survival (8.5 versus 6.4 months; $P < 0.0001$), and median survival (20.1 versus 16.9 months; $P = 0.2779$) (Köhne *et al*, 2005).

An American study included irinotecan/5FU/LV combination regimen that included bolus 5FU/LV (the Saltz regimen or IFL). Treatment with irinotecan/5FU/LV resulted in longer progression-free survival (median, 7.0 vs. 4.3 months; P =0.004), a higher rate of confirmed response (39% versus 21%; $P < 0.001$), and longer median survival (14.8 versus 12.6 months; $P = 0.04$) (Saltz *et al*, 2000b). However, there was greater toxicity than expected compared to triple combination regimens that include short-term infusional 5FU/LV. A study further investigated the sequence effect of irinotecan and 48-hour infusion of modulated 5FU/LV on the plasma pharmacokinetics of irinotecan and its metabolites, the toxicity profile of this combination, and irinotecan's maximum-tolerated dose. It was confirmed that the sequence of treatment with irinotecan and infusional 5FU affected the tolerability of this combination. This was explained in part by a reduced SN-38 *AUC* (area under the concentration versus time curve)[1] when irinotecan preceded

[1] The area under the concentration versus time curve is frequently used as a kinetic parameter to assess systemic drug exposure in formal pharmacodynamic studies of anticancer agents. Numerous pharmacodynamic studies have validated use of this parameter as a predictor of anticancer drug toxicity and

infusional 5FU (Falcone *et al*, 2001). Hence, irinotecan-based combinations that include bolus 5FU/LV are no longer considered an appropriate choice for irinotecan/5FU/LV therapy (Cutsem *et al*, 2001; Delaunoit *et al*, 2004; Ledermann *et al*, 2001; Rothenberg *et al*, 2001).

Irinotecan plus capecitabine combinations (XELIRI) have been investigated as first-line therapy in several early studies and evaluation is ongoing, however gastrointestinal toxicity/diarrhoea has been a major concern and these regimens have required some alteration in dose and administration schedule (Bajetta *et al*, 2004; Borner *et al*, 2005; Patt *et al*, 2004; Tewes, 2003). In addition, preliminary results from the BICC-C study indicate that efficacy may be inferior and toxicity rates greater for irinotecan/capecitabine regimens compared with more conventional irinotecan/5FU/LV regimens such as FOLFIRI (Fuchs *et al*, 2006).

Since oxaliplatin-based regimens are increasingly used as first-line therapy for metastatic colorectal cancer, the efficacy of irinotecan as second-line or salvage therapy is emerging as an important issue. The randomised GERCOR study demonstrated that second-line FOLFIRI after FOLFOX6 achieved a 4% response rate and 2.5 months median progression free survival (Tournigand *et al*, 2004). A phase 2 study of 35 patients pretreated with FOLFOX and treated with bifractionated bimonthly schedule of infusion irinotecan/5FU/LV demonstrated a response rate of 20% and median progression free

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efficacy in preclinical and clinical models. *AUC* is related to clearance by drug dose and it is also useful for estimating other pharmacokinetic parameters such as clearance and volume of distribution at steady-state in a noncompartmental pharmacokinetic analyses. The *AUC* can be directly estimated from concentration versus time data using relatively simple mathematical integration methods such as the linear trapezoidal rule or the log-linear trapezoidal rule with extrapolation to infinity [Ratain MJ, Schilsky RL, Conley BA, Egorin MJ (1990) Pharmacodynamics in cancer therapy. *J Clin Oncol* **8:** 1739-1753].

survival of 7.1 months (Recchia *et al*, 2004). The efficacy of irinotecan monotherapy in the third-line setting was also evaluated in Intergroup study N9841. In a preliminary report, patients receiving single agent irinotecan after failing FOLFOX4 (and having failed 5FU before FOLFOX4), had an objective response rate of only 4 percent; with median time to progression from crossover of 2.7 months, and median survival of 8.7 months (Rowland *et al*, 2005). Modern randomised trials of first-line chemotherapy are listed in Table 1.7.

Table 1.7

Modern randomised trials of first-line chemotherapy for metastatic colorectal cancer

Reference	Number	Regimen	Response (%)	Survival (months)	*P* value
(Saltz *et al*, 2000b)	226 231 226	5FU/LV (Mayo) IFL Irinotecan	28 50 29	12.6 14.8 12	0.04[1]
(de Gramont *et al*, 2000)	210 210	5FU/LV FOLFOX4	22.3 50.7	14.7 16.2	0.12
(Douillard *et al*, 2000)	188 199	5FU/LV (infusion) FOLFIRI	31 49	14.1 17.4	0·031
(Giacchetti *et al*, 2000)	100 100	5FU/LV (chronotherapy) 5FU/LV/oxaliplatin	16 53	19.9 19.4	> 0.05
(Grothey *et al*, 2002)	129 123	5FU/LV (Mayo) 5FU/LV/oxaliplatin	23 49	16.1 19.7	> 0.05
(Goldberg *et al*, 2004)	264 267 264	IFL FOLFOX4 IROX	31 45 35	15 19.5 17.4	0.0001[2] 0.04
(Tournigand *et al*, 2004)	109 111	FOLFIRI FOLFOX6	56 54	21.5 20.6	0.99
(Hurwitz *et al*, 2004)	402 411	IFL/bevacizumab IFL	44.8 34.8	20.3 15.6	< 0.001
(Souglakos *et al*, 2004)	101 102	5FU/LV/oxaliplatin/irinotecan[3] FOLFIRI	45 31	21.1 16.5	0.09
(Hurwitz *et al*, 2005)	110 100	5FU/LV/bevacizumab IFL	40 37	18.3 15.1	0.25
(Köhne *et al*, 2005)	214 216	5FU/LV/irinotecan 5FU/LV	62.2 34.3	20.1 16.9	0.2779
(Colucci *et al*, 2005)	164 172	FOLFIRI FOLFOX4	31 34	14 15	> 0.05
(Schalhorn *et al*, 2005)	157 142	FOLFIRI IROX	46 50	21.1 18.6	0.23

[1] *P* value for IFL versus 5FU/LV control arm; median survival of patients assigned to receive irinotecan alone was similar to 5FU/LV control arm ($P > 0.05$).

[2] *P* values are for FOLFOX4 AND IROX versus the IFL control arm.

[3] Arm-A: Irinotecan 150 mg/m^2 30–90 min infusion given on day 1, oxaliplatin 65 mg/m^2 2 hr infusion on day 2 simultaneously but in different lines with LV 200 mg/m^2 on days 2 and 3 followed by 5FU 400 mg/m^2 bolus and 600 mg/m^2 22 hr infusion on days 2 and 3. Arm-B: Irinotecan 180 mg/m^2 30–90 min infusion given on day 1, LV 200 mg/m^2 on days 1 and 2 followed by 5FU 400 mg/m^2 bolus and 600 mg/m^2 22 hr infusion on days 1 and 2. Cycles were repeated every 2 weeks [Souglakos J, Ziras N, Polyzos A, Athanasiadis A, Kakolyris S, Giannakakis T, Tselepatiotis E, Kalbakis K, Vardakis N, Georgoulias V (2004) Oxaliplatin (L-OHP) combined with irinotecan (CPT-11), leucovorin (LV) and fluorouracil (5-FU) compared with irinotecan, leucovorin and fluorouracil as first-line treatment for metastatic colorectal cancer (MCC): Preliminary results of a multicenter randomized phase III trial. *J Clin Oncol (Meeting Abstracts)* **22:** 3532-].

(Fuchs *et al*, 2006)	430 (Total)	FOLFIRI IFL Irinotecan/capecitabine	47 42 38	23.1 17.6 18.8	> 0.05
(Hospers *et al*, 2006)	151 151	5FU/LV (Mayo) FOLFOX4	18.5 33.8	13.3 13.8	0.619
(Giacchetti *et al*, 2006)	282 282	chronoFLO4[1] FOLFOX2	42 44.3	19.6 18.7	0.55
(Goldberg *et al*, 2006)	151 154	reduced IFL FOLFOX4	32 48	16.3 19	0.026
(Falcone *et al*, 2006)	122 122	FOLFIRI FOLFOXIRI	41 66	16.7 22.6	0.032
Selective reports of large (>100 patients/arm), modern (year 2000 and after), phase 3 randomised trials as first-line therapy; data adapted from: Systemic therapy for colorectal cancer (Meyerhardt & Mayer, 2005); Management of liver metastases from colorectal cancer (Kemeny, 2006); Neoadjuvant chemotherapy before liver resection for patients with unresectable liver metastases from colorectal carcinoma (Leonard *et al*, 2005).					

In a recent review, irinotecan-based therapy as second-line treatment after disease progression on oxaliplatin-based first-line therapy demonstrated modest response rates between 4% to 22% and median survival between 6 to 10.7 months (Leonard *et al*, 2005).

A novel approach to selection of appropriate therapy involves the application of gene expression signatures to identify which patients will respond to, and benefit from FOLFIRI. Tumour samples from 21 patients with advanced colorectal cancer were analyzed with gene expression profiling using Human Genome GeneChip arrays U133.

[1] chronoFLO4: Four-day course of chronomodulated infusions of FU/LV from 2215-0945 hrs with a peak at 0400 hrs, and oxaliplatin from 1015-2145 hrs with a peak at 1600 hrs. FOLFOX2: Oxaliplatin and LV as 2 hr infusion on day 1 and LV only on day 2, starting between 0900 and 1600 hrs. FU infusion delivered at a constant rate for 22 hours on days 1 and 2. Courses were repeated every 14 days. All patients received the same doses of FU (3,000 mg/m^2), LV (1,200 mg/m^2), and oxaliplatin (100 mg/m^2) on the first course. An escalation of FU by 400 mg/m^2/course on the second and by 200 mg/m^2 on the third course was planned if no grade moderately severe toxicity had occurred [Giacchetti S, Bjarnason G, Garufi C, Genet D, Iacobelli S, Tampellini M, Smaaland R, Focan C, Coudert B, Humblet Y, Canon JL, Adenis A, Re GL, Carvalho C, Schueller J, Anciaux N, Lentz M-A, Baron B, Gorlia T, Levi F (2006) Phase III Trial Comparing 4-Day Chronomodulated Therapy Versus 2-Day Conventional Delivery of Fluorouracil, Leucovorin, and Oxaliplatin As First-Line Chemotherapy of Metastatic Colorectal Cancer: The European Organisation for Research and Treatment of Cancer Chronotherapy Group. *J Clin Oncol* **24:** 3562-3569].

This report determined a fourteen gene expression signature that predicted chemotherapy response to FOLFIRI, and further clinical evaluation is ongoing (Del Rio *et al*, 2007).

C. Oxaliplatin

The third-generation platinum derivative oxaliplatin, is a diaminocyclohexane containing platinum derivative, and has a spectrum of activity, and mechanisms of action and resistance, that appear to be different from those of other platinum-containing compounds (Raymond *et al*, 2002b). Oxaliplatin forms bulky DNA adducts and induces cellular apoptosis, and preclinical data suggested that oxaliplatin would be effective in the treatment of colorectal cancer (Raymond *et al*, 1998; Rixe *et al*, 1996). In addition, oxaliplatin and 5FU were shown to be highly synergistic, in both preclinical models and in subsequent clinical evaluation (deBraud *et al*, 1998; Raymond *et al*, 1997; Rothenberg *et al*, 2003a). A potential mechanism for this synergy is the down-regulation of thymidylate synthase by oxaliplatin, which thereby potentiates the efficacy of 5FU (Raymond *et al*, 2002a).

Oxaliplatin has a different toxicity profile compared to cisplatin and carboplatin, and renal dysfunction, alopecia, and ototoxic effects are uncommon, but neuropathy is more frequent. A majority of patients experience transient dysaesthesias, manifested as numbness or tingling of the hands and feet and the oral or perioral regions, which are exacerbated by exposure to low temperatures. Furthermore, after months of therapy, patients may have a cumulative, dose-dependent sensory neuropathy in which peripheral dysaesthesias and paraesthesias persist between cycles of therapy; these effects usually diminish after the cessation of treatment (de Gramont *et al*, 2000).

Oxaliplatin as a single-agent therapy has demonstrated limited efficacy when administered as first-line or second-line treatment for patients with metastatic colorectal cancer, and most clinicians consider single agent oxaliplatin to be an inappropriate choice for first-line therapy (Armand *et al*, 2000; Becouarn *et al*, 1998; Diaz-Rubio *et al*, 1998; Levi *et al*, 1993; Machover *et al*, 1996; Meyerhardt & Mayer, 2005; Rothenberg *et al*, 2003a).

As first-line combination therapy, the benefit of adding oxaliplatin to 5FU/LV was demonstrated in a prospective trial in which 420 patients with previously untreated metastatic colorectal cancer were randomised to either the de Gramont 5FU/LV regimen or FOLFOX4, and patients treated with FOLFOX4 had higher objective response rate (51% versus 22%) and longer progression-free survival (9 versus 6.2 months), but similar median survival (16.2 versus 14.7 months). Moderately severe neutropenia (42% versus 5%) and diarrhoea (12% versus 5%) were both more common with oxaliplatin (de Gramont *et al*, 2000).

A second prospective trial randomised 252 patients to either the low dose Mayo regimen, or combination oxaliplatin 50 mg/m^2 over two hours plus 5FU 2000 mg/m^2 over 24 hours and LV 500 mg/m^2 on days 1, 8, 15, and 22, every 36 days. Combination oxaliplatin treatment was associated with more neurotoxicity, improved objective response rate (48% versus 23%) and progression-free survival (7.8 versus 5.3 months), however median survival was similar (19.7 versus 16.1 months) (Grothey *et al*, 2002).

A third prospective trial randomised 200 patients to a five-day course of chronomodulated 5FU/LV 700/300 mg/m^2, with or without oxaliplatin 125 mg/m^2 as a six-

hour infusion on the first day of each course, and each course was repeated every 21 days. Combination oxaliplatin treatment was again associated with more neurotoxicity (13%), improved objective response rate (53% versus 16%) and progression-free survival (8.7 versus 6.1 months), but median survival was similar (19.9 versus 19.4 months) (Giacchetti *et al*, 2000).

In the United States, the North Central Cancer Treatment Group or Intergroup 9741 trial was a prospective, phase 3, randomised study which began as a six-arm trial. Bolus 5FU/LV was the control arm, with two irinotecan plus 5FU/LV schedules, two oxaliplatin plus 5FU/LV schedules, and a non-fluoropyrimidine irinotecan plus oxaliplatin arm. After the study was initiated, emerging data showed improved survival for irinotecan/5FU/LV compared with 5FU/LV. Therefore, a new standard treatment for patients with metastatic colorectal cancer was established, and the 5FU/LV control arm was deleted. In addition, the arms containing a daily times five bolus schedule of 5FU/LV with either oxaliplatin or irinotecan, which were regimens that had not undergone extensive previous clinical testing, proved too toxic and were also discontinued. Hence, the 9741[1] trial was reduced to three arms consisting of (1) weekly irinotecan/5FU/LV or IFL, (2) biweekly oxaliplatin/bolus plus infusional 5FU/LV or FOLFOX4, and (3) three-weekly irinotecan plus oxaliplatin or IROX. Approximately one year after the three-arm design was implemented, real-time

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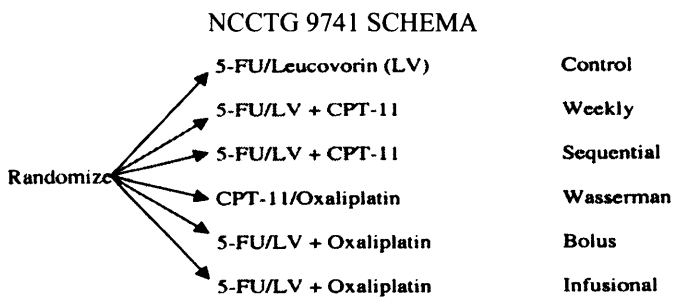
[1]



[Goldberg RM, Sargent DJ, Morton RF, Mahoney MR, Krook JE, O'Connell MJ (2002) Early Detection of Toxicity and Adjustment of Ongoing Clinical Trials: The History and Performance of the North Central Cancer Treatment Group's Real-Time Toxicity Monitoring Program. Ibid. **20:** 4591-4596].

toxicity monitoring identified an excessive frequency of toxic deaths on the IFL arm (4.6% 60-day mortality rate), and the study was once again amended, with a reduction of the starting doses of irinotecan and 5FU on the IFL arm, a regimen designated as rIFL (Goldberg *et al*, 2002; Meropol, 2006; Rothenberg *et al*, 2001; Sargent *et al*, 2001).

The Intergroup 9741 study ultimately randomised 795 patients and clearly demonstrated the superiority of FOLFOX4 over IFL and IROX. The primary end point, time to progression, was prolonged from 6.9 to 8.7 months by FOLFOX4 over IFL (P = 0.0014). Treatment with FOLFOX4 reduced the risk of dying by 34% and prolonged median survival from 15 to 19.5 months in comparison to IFL (P = 0.0001). The IROX combination performed slightly better than IFL, but was less effective than FOLFOX4 in all outcome measures. Toxicity of both IROX and IFL was greater than with FOLFOX4, with the exception of peripheral neuropathy (Goldberg *et al*, 2004).

A subsequent report provided outcomes for the 305 patients randomised on the rIFL versus FOLFOX4 subset study. A total of 151 patients had FOLFOX4 and treatment of 151 patients on the rIFL arm consisted of irinotecan 100 mg/m^2 and bolus 5FU 400 mg/m^2 plus LV 20 mg/m^2 on days 1, 8, 15, and 22 every 6 weeks. The results were superior for FOLFOX4 compared with rIFL for time to progression (9.7 versus 5.5 months; $P <$ 0.0001), response rates (48% versus 32%; P = 0.006), and median overall survival (19.0 versus 16.3 months; P = 0.026). Toxicity profiles were not significantly different between regimens for nausea, vomiting, diarrhoea, febrile neutropenia, dehydration, or 60-day all-cause mortality. Sensory neuropathy (14.4% versus 0.7%; $P < 0.0001$) and neutropenia (58.9% versus 26.7%; $P < 0.0001$) were more common with FOLFOX4. Approximately 75% of patients in both arms received second-line therapy; 58% of rIFL patients received

oxaliplatin-based second-line therapy, and 55% of FOLFOX4 patients received irinotecan-based regimens as second-line therapy. The study concluded that FOLFOX4 led to superior response, time to progression, and overall survival compared with rIFL, and the survival benefit for FOLFOX4 observed in the earlier stage of the study was preserved with equal use of either irinotecan or oxaliplatin as second-line therapy (Goldberg *et al*, 2006). These findings are also similar to those previously reported when comparing FOLFOX4 with standard-dose IFL before the Intergroup 9741 protocol modification (Meropol, 2006).

In contrast to the results from Intergroup 9741 trial, two European trials suggest similar efficacy for combinations of irinotecan or oxaliplatin with short-term infusional LV-modulated 5FU. The GERCOR trial compared FOLFOX6 to FOLFIRI, and patients were allowed to crossover to the alternative regimen at progression. This trial accrued 226 patients and was powered to detect a 20% difference in progression-free survival at 15 months with 80% certainty. No difference in progression-free survival after first-line therapy, first-line response rate, or overall median survival between strategies emerged. After progression, FOLFOX6 was significantly more effective than FOLFIRI with longer progression-free survival (4.2 and 2.5 months; $P = 0.003$) and better objective response rate (15% *v* 4%; $P = 0.05$). The degree of toxicity to therapy was generally similar, though initial therapy with FOLFOX6 led to more moderately several toxicities than did FOLFIRI (74% and 53%; $P = 0.001$). FOLFOX6 was associated with more neuropathy and neutropenia, while FOLFIRI caused more diarrhoea, febrile neutropenia, nausea and vomiting, and fatigue. Sixty-day all-cause mortality was similar, 4% with FOLFIRI and 3% with FOLFOX6. This study has been criticized as being underpowered however, it is has been suggested that it is highly unlikely that with a *P* value of .99 for the comparison of

median overall survival, 21.5 months for FOLFIRI and 20.6 months for FOLFOX, that a truly meaningful difference in overall survival would be uncovered with a larger sample size (Kelly & Goldberg, 2005; Tournigand *et al*, 2004).

A lack of superiority for FOLFOX4 was also noted in a second trial, which randomised 360 patients to either FOLFIRI or FOLFOX4. Overall response rates were similar (31% and 34%, respectively), as were progression-free survival, median survival (14 versus 15 months), and toxicity profiles. Differences in trial design may provide at least part of the explanation for these disparate results between trials from the United States and Europe however, it has been recommended that when irinotecan is selected for initial therapy of metastatic colorectal cancer, it should be combined with infusional (as per the FOLFIRI regimen) rather than bolus 5FU (as per the IFL or rIFL regimens). The available data support the view that efficacy of first-line FOLFOX is similar to that of FOLFIRI (Meropol, 2006).

Furthermore, in two of the above randomised trials of oxaliplatin/FU/LV versus irinotecan/FU/LV regimens, more patients who received FOLFOX as their first-line therapy were able to go on to have hepatic resection of metastatic disease than patients receiving irinotecan-based therapy. In Intergroup N9741, a total of 24 patients underwent subsequent resection, consisting of 11 on the FOLFOX arm, 11 on the IROX arm, and two on the IFL arm. Median survival of the resected patients was 42 months, and seven (29%) remained relapse free (Delaunoit *et al*, 2005). Of those receiving FOLFOX on the GERCOR trial, 22% were able to undergo hepatic resection compared to 9% treated with FOLFIRI ($P = 0.02$), and the median overall survival of the resected patients was greater than 46 months (Tournigand *et al*, 2004).

As a first-line combination oxaliplatin/irinotecan regimen, the IROX regimen was not found to be superior to first-line IFL or FOLFOX4 in United States Intergroup 9741 trial. Other studies of first-line combination oxaliplatin/irinotecan regimen provide variable results. A preliminary report of the FIRE trial, which compared FOLFIRI and IROX as first-line treatment of metastatic colorectal cancer, suggested that FOLFIRI and IROX regimens had similar efficacy (objective response rate 46% versus 50%, and median survival 21.1 versus 18.6 months respectively) (Schalhorn *et al*, 2005).

Two other prospective randomised phase 3 trials comparing FOLFOXIRI and FOLFIRI have come to different conclusions. A randomised trial of 283 patients from the Hellenic Oncology group reported similar outcomes with both regimens (Souglakos *et al*, 2004). In contrast, a preliminary report of a randomised trial of 244 patients suggested better outcomes with FOLFOXIRI, including higher response rate (66% versus 41%; P = 0.0002), a greater number of patients eligible for secondary surgical cytoreduction (14% versus 6%; P = 0.05), and longer median progression free survival (9.8 versus 6.9 months; P = 0.0006) and overall survival (22.6 versus 16.7 months; P = 0.032) (Falcone *et al*, 2006).

The efficacy and tolerability of capecitabine plus oxaliplatin combination regimens (XELOX or CAPOX) has been evaluated in the first-line setting. In three separate phase 2 studies using oxaliplatin 130 mg/m^2 day 1, followed by capecitabine 1000 mg/m^2 twice daily for 14 of every 21 days, objective response rates were between 36% and 55%, and median time to progression was between 5.8 to 10.5 months (Cassidy *et al*, 2004; Feliu *et al*, 2006; Scheithauer *et al*, 2003). Lower initial doses of oxaliplatin 85 mg/m^2 every three weeks, were combined with capecitabine 1000 mg/m^2 orally twice daily for 14 of every 21

days, in a trial that focused on first-line treatment in patients 70 years of age or older. If therapy was tolerated, the oxaliplatin dose was increased to 110 mg/m^2 and then to 130 mg/m^2 for the second and third cycles. The objective response rate was 41% and the median overall survival was 14.4 months. Treatment was well tolerated with only 5% of patients developing moderately severe haematologic toxicity, 8%peripheral neuropathy, and 13%severe hand-foot syndrome (Comella *et al*, 2005).

The TREE-1 trial prospectively randomised 147 patients with previously untreated metastatic colorectal cancer to modified FOLFOX6, CAPOX (capecitabine 1000 mg/m^2 twice daily for 14 of every 21 days plus oxaliplatin 130 mg/m^2 day 1), or bFOL (bolus 5FU 500 mg/m^2, and LV 20mg/m^2 weekly for three of every four weeks plus oxaliplatin 85 mg/m^2 days 1 and 15). Results were similar for both FOLFOX6 and CAPOX arms in terms of response rate (45% versus 35%), time to progression (5.9 versus 8.7 months) and median survival (19.2 versus 17.2 months). However, the CAPOX group had the highest incidence of moderately severe nausea, vomiting, diarrhoea (19%, 21%, and 27%, respectively), and neuropathy (17%) in the first 12 weeks, and therapy was more often discontinued because of toxicity. Patients receiving FOLFOX6 had the highest rate of moderately severe neutropenia (35% versus 12% with CAPOX) but no episodes of febrile neutropenia (Hochster *et al*, 2006). However, similar efficacy as well as safety results were reported with the same capecitabine/oxaliplatin regimen as compared to oxaliplatin (85 mg/m^2 every other week) plus infusional 5FU (2250 mg/m^2 over 48 hours once weekly) in a preliminary report of a prospectively randomised Spanish trial involving 348 previously untreated patients (Sastre *et al*, 2005).

A third phase 3 trial randomised 476 patients with previously untreated metastatic colorectal cancer to FUFOX (5FU 2000 mg/m^2 over 24 hours, LV 500 mg/m^2, and oxaliplatin 50 mg/m^2 on days 1,8,15, and 22 of every 5 week cycle) or XELOX using a lower oxaliplatin dose (capecitabine 1000 mg/m^2 twice daily for 14 of every 21 days plus oxaliplatin 70 mg/m^2 day 1). In a preliminary report, the objective response rates were similar for both FUFOX and XELOX arms (45% and 42%, respectively), as was progression-free survival (35 versus 30 weeks). Toxicity profiles were also comparable with the exception of more hand-foot syndrome in the XELOX arm (Arkenau *et al*, 2005). The available data from these reports suggest that when compared to infusional 5FU/oxaliplatin combinations, XELOX or CAPOX has approximately similar antitumour efficacy, but may be more toxic, especially in terms of diarrhoea and hand-foot syndrome.

In a recent review, oxaliplatin-based therapy as second-line treatment after disease progression on irinotecan-based first-line therapy demonstrated modest response rates between 11% to 40% and median survival between 8.7 to 12 months (Leonard *et al*, 2005).

A prospective study randomised 463 patients with metastatic colorectal cancer who progressed after IFL therapy to oxaliplatin 85 mg/m^2 monotherapy, the de Gramont 5FU/LV regimen or FOLFOX 4. FOLFOX4 proved superior to 5FU/LV in all measures of clinical efficacy. Objective response rates were 9.9% for FOLFOX4 versus 0% for 5FU/LV ($P < 0.0001$). Median time to progression was 4.6 months for FOLFOX4 versus 2.7 months for 5FU/LV ($P < .0001$). Relief of tumour related symptoms occurred in 33% of patients treated with FOLFOX4 versus 12% of patients treated with 5FU/LV ($P < 0.001$). Single-agent oxaliplatin was not superior to 5FU/LV in any measure of efficacy. Patients treated with FOLFOX4 experienced a higher incidence of clinically significant

toxicities than patients treated with 5FU/LV, but these toxicities were predictable and did not result in a higher rate of treatment discontinuation or 60-day mortality rate (Rothenberg *et al*, 2003a; Rothenberg *et al*, 2003b).

Another prospective study randomised 214 patients who progressed after sequential 5FU and irinotecan monotherapy to the de Gramont regimen of 5FU/LV or FOLFOX4. Objective response rates for 5FU/LV versus FOLFOX4 were 2% versus 13% ($P = 0.0027$). Median time to disease progression was 2.4 versus 4.8 months ($P < 0.0001$), and median survival was 11.4 versus 9.9 months ($P = 0.20$) for 5FU/LV and FOLFOX4. Among the 72 patients who crossed over from 5FU/LV to FOLFOX4, 6% responded. Symptomatic improvement was significantly better for patients in the FOLFOX4 arm (32% versus 18% for 5FU/LV; $P = 0.05$). Moderately severe toxicities for 5FU/LV and FOLFOX4 were neutropenia (13% versus 42%), diarrhoea (6% versus 16%), and overall neuropathy (0% versus 6%) (Kemeny *et al*, 2004).

In addition, a phase 2 study evaluated XELOX (capecitabine 1000 mg/m^2 orally twice daily on days 1–14 and oxaliplatin 130 mg/m^2 as a 30 min infusion on day 1) in 70 patients with advanced colorectal cancer resistant to irinotecan. The response rate was 17%, median time to progression was 5.4 months and median survival 9.5 months (Pfeiffer *et al*, 2006).

As a second-line combination oxaliplatin/irinotecan regimen, IROX was studied in a phase 2 trial that randomised 62 patients with 5FU-refractory disease to IROX or a triple regimen of 5FU/LV plus alternating irinotecan and oxaliplatin. The IROX regimen was associated with a higher response rate (23% versus 6%), slightly longer median survival

(12.3 versus 9.8 months), and a more favourable toxicity profile (Becouarn *et al*, 2001). A phase 2 cohort study reported a 39% response rate with oxaliplatin plus IFL in patients failing first-line IFL (Stathopoulos *et al*, 2005). The clinical significance of these findings is limited in that most patients receive either an oxaliplatin or irinotecan-containing regimen as first-line therapy.

Chronomodulated administration schedules of oxaliplatin plus 5FU appear to be less toxic than constant rate infusions. At least three prospective randomised trials have explored the benefit of this approach however none have demonstrated a clear survival benefit for chronotherapy. Despite the suggestion of better tolerability and at least similar efficacy, the data are insufficient to conclude that chronomodulated administration of 5FU and oxaliplatin is a more valuable strategy (Caussanel *et al*, 1990; Giacchetti *et al*, 2006; Giacchetti *et al*, 2000; Levi *et al*, 1997).

D. Biologic therapy

(i) Bevacizumab

As previously discussed, it has been demonstrated in prospective randomised phase 3 trials that bevacizumab, when combined with IFL as first-line treatment of metastatic colorectal cancer, and with FOLFOX as second-line treatment, leads to increased median survival, progression-free survival, and response rates, compared with cytotoxic chemotherapy regimens alone (Giantonio *et al*, 2005; Hurwitz *et al*, 2004).

Furthermore, a combined analysis of three randomised phase 2 studies has demonstrated that bevacizumab also increased the activity of FU/LV in the first-line

setting, and increased median survival (17.9 versus 14.6 months; $P = 0.0081$)., progression-free survival (8.7 versus 5.5 months; $P = 0.0001$), and response rates (34.1% versus 24.5%; $P = 0.019$), compared with cytotoxic chemotherapy alone (Hurwitz *et al*, 2005; Kabbinavar *et al*, 2003; Kabbinavar *et al*, 2005a; Kabbinavar *et al*, 2005b).

The TREE-2 trial prospectively randomised 213 patients with previously untreated metastatic colorectal cancer to modified FOLFOX6, CAPOX, or bFOL regimens with bevacizumab, and these arms were compared to the same chemotherapy regimens without bevacizumab (or TREE-1 arms). In a preliminary report, the addition of bevacizumab in the TREE-2 study improved objective response rates, median time to progression, and probability of survival at 18 months. At the time of the report, 122 TREE-2 pts (57%) were alive compared to 56 TREE-1 pts (38%). The addition of bevacizumab in TREE-2 also caused more moderately severe hypertension, impaired wound healing, and bowel perforation in each arm. The study concluded that the addition of bevacizumab to oxaliplatin/fluoropyrimidine-based regimens improved response rate and time to progression with acceptable tolerability, and no unexpected toxicity (Hochster *et al*, 2006).

(ii) EGFR targeted monoclonal antibodies

As previously discussed, cetuximab is a human/mouse chimeric monoclonal antibody that binds to EGFR, competitively inhibiting ligand binding, and inducing receptor dimerisation and downregulation. It is however unclear that this represents the only mechanism of antitumour action. Immunohistochemical studies have demonstrated that there is a lack of correlation between the presence or intensity of EGFR positivity and clinical response to cetuximab, and reports have documented partial response in patients with EGFR-negative tumours (Chung *et al*, 2005; Lenz *et al*, 2004). Hence, it is difficult

to select or exclude patients for cetuximab therapy based upon EGFR immunostaining and other molecular determinants of cetuximab efficacy are currently being investigated (Moroni *et al*, 2005; Vallbohmer *et al*, 2005).

Cetuximab has been found to be safe and effective when combined with FOLFOX4 in first-line treatment of patients with metastatic colorectal cancer (Tabernero *et al*, 2004). A prospective phase 3 trial randomised 238 patients with untreated metastatic colorectal cancer to FOLFOX or FOLFIRI with or without cetuximab, independent of EGFR status. Preliminary results suggested that FOLFIRI and FOLFOX were similar in efficacy, and that adding cetuximab to either in first-line treatment appeared to increase objective response rates (42% versus 34% for FOLFIRI; 55% versus 32% for FOLFOX), however progression-free survival and duration of response did not appear different at this point of analysis (Venook *et al*, 2006).

Furthermore, cetuximab has been found to demonstrate activity as second-line monotherapy in EGFR expressing metastatic colorectal cancer refractory to irinotecan (Lenz *et al*, 2006; Saltz *et al*, 2004), oxaliplatin, and fluoropyrimidine (Lenz *et al*, 2006), and also as combination cetuximab/irinotecan second-line therapy in EGFR expressing metastatic colorectal cancer refractory to irinotecan (Cunningham *et al*, 2004).

Panitumumab is a human monoclonal antibody that targets EGFR (Yang et al, 2001). Clinical activity has been demonstrated, most notably in patients with EGFR positive metastatic colorectal cancer who have failed prior therapy. Early phase 3 trial results with panitumumab monotherapy indicate a 46% reduction in the rate of tumour progression in treated patients compared with those who received best supportive care alone.

Panitumumab is well tolerated, with acneiform rash the most common dose-dependent adverse effect. Studies thus far indicate a low rate of moderately severe infusion-related reactions (1%) (Gibson *et al*, 2006; Saadeh & Lee, 2007).

E. Consensus statement

In late 2006, an international panel of experts produced a consensus statement regarding guidelines for systemic chemotherapy for unresectable colorectal hepatic metastatic disease. The current guidelines are as follows (Bartlett *et al*, 2006):

- Standard of care is either infusional 5FU/LV plus irinotecan (most commonly FOLFIRI) in combination with bevacizumab, or infusional 5FU/LV plus oxaliplatin (most commonly FOLFOX) with bevacizumab.
- If FOLFOX plus bevacizumab are used as first-line therapy, irinotecan or FOLFIRI should be the second-line regimen. Upon progression, cetuximab should be added. While it may be reasonable to add cetuximab immediately in the second-line regimen, rather than waiting for progression on irinotecan, current United States, Food and Drug Administration (FDA) approval would suggest the first plan.
- If FOLFIRI plus bevacizumab is administered first, then second-line therapy can be FOLFOX or irinotecan plus cetuximab.
- Testing of the tumour for EGFR expression has been suggested prior to cetuximab use, but this is of no predictive value.
- Enrolment in clinical trials, particularly the cooperative group trials, remains crucial to determining the optimal use of available agents.

1.32 Regional therapy

As previously discussed, for patients with metastatic colorectal cancer, liver involvement is a major source of morbidity, and eventually leads to death in the vast majority of such individuals. Considerable interest has focused on the potential advantage and use of hepatic arterial infusion or HAI therapy, given that hepatic metastases derive most of their blood supply from the hepatic artery, whereas the normal liver parenchyma derives blood supply from the portal vein circulation, and that certain drugs are largely extracted by the liver during the first pass, allowing for minimal systemic toxicity (Kemeny *et al*, 2006).

A. Hepatic intra-arterial approaches to regional therapy

(i) Tumour Blood Supply

Hepatic metastases are secondary to haematogenous spread, and for colorectal carcinoma and other gastrointestinal malignancies, this occurs via the portal vein (Weiss *et al*, 1986). As tumours grow in the liver, unlike normal hepatocytes which derive most of their blood supply from the portal circulation, they develop an almost exclusive arterial blood supply (Ackerman, 1974; Breedis & Young, 1954; Elias *et al*, 2004b). There is evidence that tumours as small as 0.5 mm are predominantly supplied by the hepatic artery (Archer & Gray, 1989).

Angiogenesis is a critical step for establishment of metastatic disease in colorectal cancer (Meyers & Watson, 2003). During angiogenesis, the existing vessels become leaky in response to growth factors released by normal or tumour cells, the basement membrane and the interstitial matrix dissolve, pericytes (cells that provide support for the endothelial cells) dissociate from the vessel, endothelial cells migrate and proliferate to form an array

or sprout, the process of canalization occurs in which a lumen is formed in the sprout, branches and loops are formed by confluence and anastomoses of sprouts to permit blood flow, and finally, these immature vessels are invested in basement membrane and pericytes. During normal physiologic angiogenesis, these vessels differentiate into mature arterioles, capillaries, and venules, whereas in tumours they may remain immature (Jain, 2005a).

The resulting tumour vasculature is structurally and functionally abnormal. Blood vessels are leaky, tortuous, dilated, and saccular and have a haphazard pattern of interconnection. The endothelial cells lining these vessels have aberrant morphology, pericytes are loosely attached or absent, and the basement membrane is often abnormal, at times being unusually thick or entirely absent. These structural abnormalities contribute to spatial and temporal heterogeneity in tumour blood flow. In addition, solid pressure generated by proliferating cancer cells compresses blood and lymphatic vessels within the tumour, which further impairs not only the blood flow but also the lymphatic flow (Carmeliet & Jain, 2000; Padera *et al*, 2004).

High tumoural interstitial fluid pressure is caused in part by tumour vessel hyperpermeability. In normal tissues, the vessel is able to maintain a gradient of fluid pressure from inside the vessel to the outside. In tumours, this gradient disappears and the pressure outside the blood vessels or interstitial fluid pressure, tends to become equal to that inside, or microvascular pressure. Similarly, in normal tissues, the colloid osmotic pressure (osmotic pressure exerted by large proteins) inside blood vessels is much higher compared to that outside. In tumours, these two become approximately equal due to vessel leakiness. The loss of these pressure gradients between the vessels and the tumour

impedes the delivery of large molecular weight therapeutics to the tumour. Uneven tumour perfusion impedes the delivery of all blood-borne molecules, including oxygen and nutrients as well as chemotherapeutics (Jain, 2005a).

Table 1.8

Interstitial fluid pressure in normal and neoplastic human tissues

Type of Tissue	Number of Patients	Mean Pressure (mm Hg)
Normal breast	8	0.0
Normal skin	5	0.4
Renal cell carcinoma	1	38.0
Cervical carcinoma	26	23.0
Colorectal liver metastases	8	21.0
Head & neck carcinoma	27	19.0
Breast carcinoma	13	29.0
Metastatic melanoma	14	21.0
Lung carcinoma	26	10.0
Data adapted from: Transport in solid tumours (Jain, 1999).		

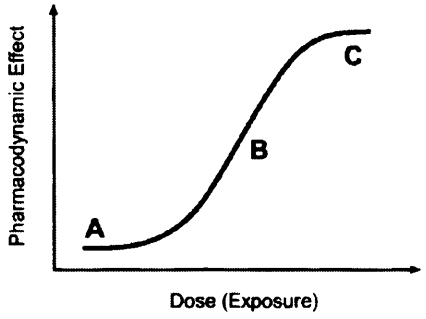
Collectively these vascular abnormalities lead to an abnormal tumour microenvironment characterized by interstitial hypertension (elevated hydrostatic pressure outside the blood vessels) (See **Table 1.8**), hypoxia, and acidosis. Impaired blood supply and interstitial hypertension interfere with the delivery of therapeutics to solid tumours. Hypoxia renders tumour cells resistant to both radiation and several cytotoxic drugs. Independent of these effects, hypoxia also induces genetic instability and selects for more malignant cells with increased metastatic potential. Hypoxia and low pH may also compromise the cytotoxic functions of immune cells that infiltrate a tumour, however, cancer cells are able to survive in this abnormal microenvironment. Therefore, the abnormal vasculature of tumours and the resulting abnormal microenvironment together pose a formidable barrier to the delivery and efficacy of cancer therapy (Jain, 2005b).

(ii) Tumour Pharmacokinetics

Regional drug delivery is an approach designed to improve the selectivity of chemotherapy. When compared with systemic drug administration, regional delivery can potentially increase drug concentrations at tumour sites and/or lower systemic drug exposure. Drugs with a steep dose-response curve[1] are more useful when given by HAI, because provided that maximal biologic effect has not been achieved (i.e. the upper plateau of the curve), small increases in the concentration of drug usually result in a large improvement in response. The use of drugs that are largely extracted by the liver during the first pass results in high local concentrations of drug with minimal systemic toxicity. Drugs with a high total body clearance are also more useful for hepatic infusion. The area under the concentration-time curve is a function not only of drug clearance but also of hepatic arterial flow. Because hepatic arterial blood flow has a high regional exchange rate (100 to 1500 mL/min), drugs with a high clearance rate are needed. If a drug is not rapidly cleared, recirculation through the systemic circulation mitigates the advantage of HAI over systemic therapy (Collins, 1984; Collins, 1986).

Pharmacokinetics (the study of drug concentrations in the body) can predict the relative advantage of regional drug delivery and some drugs and sites of delivery are more

[1] Conventional sigmoid dose-response curve relating pharmacodynamic (biologic) effect to drug exposure



[Ensminger WD (2002) Intrahepatic arterial infusion of chemotherapy: pharmacologic principles. *Semin Oncol* **29:** 119-25].

favourable than others. The ratio of total body clearance of a drug to its regional exchange rate is the principal determinant of therapeutic advantage. Therefore, the most favourable circumstances for regional delivery are the use of drugs with high total body clearances and/or sites of delivery with low exchange rates. The formula defining the advantages of HAI over an IV infusion in terms of exposure within the hepatic arterial tree is given below (see **Figure 1.1**). Regional drug delivery is seen as a method of circumventing the restriction imposed by the maximum tolerated dose (Collins, 1984; Ensminger, 2002).

$$\text{Regional advantage} = 1 + \frac{\text{Total body clearance of drug}}{\text{Hepatic artery flow rate (1 — fraction of drug extracted across liver)}}$$

Figure 1.1 Formula for regional advantage.

Therefore essential properties for drugs used for HAI therapy are therefore (1) efficacy for the particular tumour type being treated, with near linear dose-response curve and systemic administration producing exposure at the low end of the dose-response curve, (2) pharmacokinetic properties which generate higher exposure with HIA, including high total body clearance and high hepatic extraction, (3) physical properties compatible with infusion pump technology, including the ability to infuse drug in small volumes and drug stability at 37°C for protracted periods, and (4) well tolerated and non-sclerotic to arteries and subcutaneous tissue (Ensminger, 2002).

It has been demonstrated that with HAI 94% to 99% of FUDR and 19% to 51% of 5FU is extracted by the liver in one pass. Hepatic venous levels, which are one measure of intra-hepatic drug concentration in the hepatic and tumour capillary bed, were found to be 4-fold higher for FUDR infusion, and 1.5-fold higher for 5FU infusion, when drug was

given by the hepatic arterial route. Systemic FUDR levels with HAI were only about 25% of corresponding systemic levels with peripheral venous infusion. Systemic 5FU levels with hepatic arterial infusion were also lower and were about 60% of corresponding systemic levels with peripheral venous infusion. Of the drugs studied, FUDR demonstrated near linear pharmocokinetics and superior properties for HAI therapy. Assuming a total body clearance of 15,000 mL/min, a hepatic artery blood flow of 250 mL/min, and 95% extraction across the liver, the calculated increased arterial exposure should be 1,200-fold greater with HAI versus IV administration. The increased exposure achieved with HAI FUDR is likely to place drug exposure levels well into the maximum pharmacodynamic effect range or the upper plateau of the dose-response curve. 5FU however, does have significant saturable, non-linear pharmacokinetics, and the regional advantage of 5FU HAI can fall with increasing dose rate. In addition, 5FU HAI requires larger daily dose volume compared with FUDR (which means FUDR can be give via smaller implantable pumps), and 5FU solutions are alkaline and sclerotic to arteries, making thrombosis likely over extended time periods (Ensminger, 2002; Ensminger *et al*, 1978).

(iii) Modification of tumour blood flow

Improvement in regional advantage is obtained by decreasing blood flow to the target organ or reduction in hepatic artery flow rate. This is the basis for treatment of hepatic metastatic colorectal cancer with arterial ligation or embolization, alone or combined with regional cytotoxic therapy and vasoactive agents. This theoretically accomplishes increased contact time between drugs and tumour cells in the liver (Kokudo & Makuuchi, 2004). Surgical de-arterialisation of the liver may be performed as simple ligation of the hepatic artery, or as a more extensive devascularising procedure, where

potential collateral vessels are ligated. It is seldom used with infusion therapy, as it is associated with substantial complications, and at best only results in transient regression, as new tumour collateral circulation rapidly develops (Kin *et al*, 1988; Nagasue *et al*, 1976; Plengvanit *et al*, 1972; Wang *et al*, 1994).

Blood flow modification depends on the type of agent employed. Size variation will affect the depth of vascular bed occluded, and degradation characteristics of embolic particles will result in flow modification varying from mild occlusion to hypoxia. Co-administration of embolic starch microspheres, and cytotoxic drug, may result in the drug being trapped in a relatively stationary fluid column, allowing greater exposure time to the surrounding tissues. Cytotoxic drugs have different optimal exposure times for antitumour activity. Suitable embolic material is then best selected depending on the optimal occlusion time required. Starch microspheres may provide added benefit, by causing redistribution of blood flow towards hypovascular tumours, as they preferentially embolise arteriolar high flow areas (**See Table 1.9**) (Civalleri *et al*, 1985; Dakhil *et al*, 1982; de Takats *et al*, 1994; Murray, 1992; Soulen, 1994).

Radioembolisation, with or without radiosensitizing chemotherapy, is a new technique that delivers microspheres with high doses of ionizing radiation to the tumour compartment while maintaining radiation exposure of the normal liver to a tolerable level. It can be regarded as a form of brachytherapy, and it also has been termed selective internal radiotherapy (Sharma *et al*, 2007).

Table 1.9

Agents that modify tumour blood flow

Embolic agents				
Agent	**Material**	**Size (μm)**	**Degradation time**	**Reference**
Ivalon	Polyvinyl alcohol	150-500	1 wk - 6 mths	(Zou, 1989)
Albumin Microspheres	Albumin + drug + angiotensin II	15-40	1.5-11.7 days	(Goldberg *et al*, 1991c) (Anderson *et al*, 1991) (Benita *et al*, 1984) (Goldberg *et al*, 1990) (Goldberg *et al*, 1991b) (Goldberg *et al*, 1991a)
Collagen	Microfibrillar collagen	20-250	2-6 wks	(Daniels *et al*, 1987) (Sternlicht *et al*, 1989) (Cho *et al*, 1989)
Gelfoam	Gelatin sponge	1-2 mm (block) 40-50 (powder)	48-72 hrs	(Matsui *et al*, 1986) (Uchida *et al*, 1990)
Degradable Starch Microspheres	Polymerized starch	45	20-30 mins	(Civalleri *et al*, 1985) (Civalleri *et al*, 1989)
SMANCS	Styrene-maleic acid conjugated neocarzinostatin	15.5 kDa	1-7 days	(Maeda *et al*, 1984) (Maeda *et al*, 1992)
SIR-spheres	Resin microspheres containing yttrium-90	20-60	Non-degradable	(Blanchard *et al*, 1989) (Burton *et al*, 1989)

Vasoactive agents			
Agent	**Maximum constriction**	**Tumour/Normal perfusion change**	**References**
Angiotensin II	Within 1 to 2 mins	↑ 300%	(Sasaki *et al*, 1985) (Burke *et al*, 2001) (Goldberg *et al*, 1991a; Goldberg *et al*, 1991b)
Vasopressin	Within 1 to 15 mins	↑ 30%	(Hemingway *et al*, 1991) (Dworkin *et al*, 1995) (Dworkin *et al*, 1997)

Other agents		
Agent	**Properties**	**References**
Lipiodol/ Ethiodol	Iodinated poppy seed oil. Selective retention in tumour for weeks to months. No antitumour activity by itself. Not embolic. Acts as delivery system.	(Iwai *et al*, 1984) (Hind *et al*, 1992) (Miller *et al*, 1987) (Konno, 1990)

Data adapted from: An update on regional treatment of liver tumours (Civalleri, 1995).

(iv) Ethiodol

It has been demonstrated that the lymphangiographic oil Ethiodol (an iodinated poppy seed oil also known as Lipiodol), is selectively retained in liver tumours for weeks to months after hepatic intra-arterial infusion, in contrast to normal liver parenchyma, where it is cleared in days (Bhattacharya *et al*, 1996; Bhattacharya *et al*, 1994; Hind *et al*, 1992; Iwai *et al*, 1984; Konno, 1990; Miller *et al*, 1987; Rubin *et al*, 1995b).

Ethiodol is thought to have no antitumour activity itself, and although it is not truly an embolic agent, it is possible that it may cause temporary microembolic phenomenon, that could potentiate drug efficacy. However, it is more plausible that Ethiodol is simply a vehicle for drug delivery, with unique sustained release characteristics that may be related to its density and the selective retention in tumours, which probably occurs because of poor tumour venous and lymphatic drainage. Ethiodol penetrates into liver tumour and endothelial cells, possibly by pinocytosis (Bhattacharya *et al*, 1996). In culture, Ethiodol has been seen to significantly enhance the uptake of doxorubicin by hepatoblastoma cells (Towu *et al*, 2002). Preliminary evaluation of an FUDR containing chemotherapeutic Ethiodol emulsion, as a method to further dose intensify therapy without associated toxicity, demonstrated that the emulsion behaved as a depot or sustained-release preparation. This emulsion was a clinically effective vehicle for delivering FUDR to hepatic-metastatic colorectal tumours (Rubin *et al*, 1995b).

(v) Clinical practice

As previously discusses, because of the poor outcomes associated with metastatic colorectal cancer, HAI chemotherapy has been explored as an alternative treatment strategy for patients with liver-only metastases (Cohen & Kemeny, 2003). HAI therapy was first

reported in 1964, and objective response with clinical benefit was observed in 10 of 16 patients with gastrointestinal tumours metastatic to the liver, treated with continuous HAI therapy (Sullivan *et al*, 1964).

Early studies of HAI therapy used hepatic arterial catheters placed surgically or percutaneously and attached to an external infusion pump. Treatment required prolonged inpatient stays and was complicated by frequent catheter-associated thrombosis, bleeding, infection, and catheter tip migration (Ensminger, 2002). Another method was attachment of the catheter to a subcutaneous port, which could then be accessed intermittently or continuously for treatment, however these ports also had a high failure rate, particularly with regard to hepatic arterial thrombosis (Wickremesekera *et al*, 2000).

Development of a totally implantable infusion pump eliminated many of the previous difficulties associated with HAI therapy. The implantable infusion pump is no larger than a pacemaker, and can hold 30 to 50 mL of fluid and deliver the chemotherapeutic agent at a slow fixed rate over two-weeks. Continuous infusion decreases the rate of thrombosis, and filling the pump every two weeks allows for more convenient ambulatory treatment. Most pumps also contain a side port for direct infusion into the catheter, allowing reservoir bypass as needed. A study demonstrated that the implantable pump provided 115 days of chemotherapy administration, compared with 31 days for surgically, and 25 days for percutaneously placed catheters attached to an external infusion device (Yasuda *et al*, 1990). A retrospective study compared outcomes of pumps versus ports found a lower therapy-relevant complication rate (30% versus 47%) and a higher complication-free survival time (12.2 versus 7.3 months) in favour of pumps (Heinrich *et al*, 2003).

Table 1.10

Randomised trials of HAI versus systemic therapy for unresectable liver metastases

Study group/ Reference	Arm	Number	% Treated	Crossover to HAI	Response (%)	Median survival (months)	*P* value
MSKCC/ (Kemeny *et al*, 1987)	HAI FUDR IV FUDR	48 51	94 94	Yes	50 20	17 12	0.424
NCI/ (Chang *et al*, 1987)	HAI FUDR IV FUDR	32 32	66 91	No	62 17	17 12	0.27
NCOG/ (Hohn *et al*, 1989)	HAI FUDR IV FUDR	67 76	75 86	Yes	42 10	16.8 17.2	> 0.05
City of Hope/ (Wagman *et al*, 1990)	HAI FUDR IV 5FU	31 10	100 100	Yes	55 20	13.8 11.6	0.55
NCCTG/ (Martin *et al*, 1990)	HAI FUDR IV 5FU/LV	39 35	85 103	No	48 21	12.6 10.5	0.53
French/ (Rougier *et al*, 1992)	HAI FUDR IV 5FU or supportive care	81 82	87 50 (5FU)	No	43 9	15 11	< 0.02
English/ (Allen-Mersh *et al*, 1994)	HAI FUDR IV 5FU or supportive care	51 49	96 20 (5FU)	No	- -	14.5 8.1	0.03
German/ (Lorenz & Muller, 2000)	HAI FUDR HAI 5FU/LV IV 5FU/LV	54 57 57	69 70 91	Yes	43 45 20	12.7 18.7 17.6	> 0.05
MRC/EORTC/ (Kerr *et al*, 2003)	HAI 5FU/LV IV 5FU/LV	145 145	66 87	No	22 19	14.7 14.8	0.79
CALGB/ (Kemeny *et al*, 2006)	HAI FUDR IV 5FU/LV	68 67	87 87	No	47 24	24.4 20	0.0034

Data adapted from: An update on hepatic arterial infusion chemotherapy for colorectal cancer (Cohen & Kemeny, 2003); Management of liver metastases from colorectal cancer (Kemeny, 2006).

Early phase 2 trials of HAI therapy with 5FU or FUDR for colorectal liver metastases demonstrated promising results, with response rates between 29% to 88% and improved survival compared with historical controls (Balch *et al*, 1983; Niederhuber *et al*, 1984; Oberfield *et al*, 1979; Weiss *et al*, 1983). Ten randomised phase 3 trials comparing HAI with systemic chemotherapy have been published (see **Table 1.10**). Five trials compared HAI using FUDR with either IV FUDR (Chang *et al*, 1987; Hohn *et al*, 1989; Kemeny *et al*, 1987), IV 5FU (Wagman *et al*, 1990), or IV 5FU/LV (Mayo regimen) (Martin *et al*, 1990). Two European trials compared HAI using FUDR with a control arm

of IV 5FU or best supportive care, based on the treating physician's choice (Allen-Mersh *et al*, 1994; Rougier *et al*, 1992). These seven trials demonstrated higher response rates for HAI therapy compared with systemic chemotherapy (42 to 62% versus 9 to 21%). Trends toward longer times to progression and overall survival were seen in most of these trials for the HAI arms, however only the two European trials demonstrated significant improvement in median survival [French, 15 versus 11 months, $P < 0.02$ (Rougier *et al*, 1992); English, 14.5 versus 8.1 months, $P = 0.03$ (Allen-Mersh *et al*, 1994)]. However, since modern systemic chemotherapy has shown proven benefit over best supportive care for metastatic colorectal cancer, these two trials would not be considered to have appropriate control arms today (Simmonds, 2000).

Investigators have observed the reasons why the superior response rates seen with HAI therapy did not translate into improved survival in most of these earlier trials. These include, (1) many trials were too small; (2) technical problems with pump placement and unexpectedly high rates of extra-hepatic disease discovered at laparotomy, which led to a substantial number of patients who never received regional therapy; (3) lack of experience in certain centres and the lack of a strict, predetermined dose-reduction schema in several trials may have led to greater toxicities and fewer cycles of regional therapy; and (4) a number of trials allowed crossover to HAI at the time of progression on systemic chemotherapy. These factors potentially diluted results that might have demonstrated a survival benefit based on an intention to treat analysis (Kemeny, 2006).

Based on the premise that the individual trials were underpowered to detect a survival benefit, two meta-analyses of these seven original trials were conducted, and more than 600 patients were included. The Meta-Analysis Group in Cancer confirmed the

higher response rate seen with HAI (41 versus 14%), and a 27% relative survival advantage was seen for HAI ($P = 0.0009$) compared with controls. However, exclusion of the European trials that included best supportive care, decreased the survival advantage to 19% ($P = 0.14$) (Meta-Analysis-Group-In-Cancer, 1996). A second meta-analysis demonstrated a 12.5% one-year ($P = 0.002$) and a 7.5% two-year ($P = 0.026$) absolute survival difference in favour of HAI over systemic chemotherapy, which persisted even when the European studies were excluded (Harmantas *et al*, 1996).

Following the meta-analyses, three other randomised trials of HAI have been reported. The German Cooperative Group randomised 168 patients with unresectable liver metastases from colorectal cancer to HAI using FUDR, HAI using 5FU/LV, or IV 5FU/LV. Response rates were higher in the two HAI arms, with no differences in time to progression or overall survival among the arms. However, only 70% of patients in the HAI arms actually received the assigned treatment, and 51% of patients crossed over to other arms. There was a lower rate of extra-hepatic progression (13% versus 41%) and a higher rate of moderately severe systemic toxicity (68% versus 30%) for HAI 5FU compared with HAI FUDR, and these observations were consistent with the known lower hepatic extraction of 5FU compared with FUDR (Lorenz & Muller, 2000).

The Medical Research Council (MRC) and the European Organization for the Research and Treatment of Cancer (EORTC) groups compared HAI 5FU/LV with the de Gramont regimen of IV 5FU/LV. Crossover from the IV to the HAI arm was not allowed. It should be noted that both the German and MRC trials utilized subcutaneous ports rather than implantable pumps and had significant catheter-related problems. Of 290 patients randomised, 221 (76%) received treatment as assigned, including only 63% assigned to

HAI therapy. Response rates were assessed in 183 patients at a single time point (12 weeks) and were nearly identical (22% for HAI, 19% for IV 5FU/LV). No differences between the arms were noted for toxicity, and progression-free and overall survival. Of patients randomly assigned to receive HAI therapy, 37% did not commence the assigned treatment whereas the corresponding figure for the systemic arm was 13%. Factors which contributed to poor outcome and compliance for HAI therapy include, (1) randomization before hepatic arteriography, which subsequently showed 19% abnormal hepatic vasculature; (2) 29% patients who were started on HAI received fewer than six cycles due to catheter failure; (3) there was a delay before the commencement of HAI, mainly due to the wait for laparotomy; (4) around half of the entire HAI group, including patients who did not start or received fewer than six cycles, switched to the systemic treatment; and (5) the use of 5FU for HAI therapy instead of FUDR (Chan & Kerr, 2004; Kerr *et al*, 2003).

The Cancer and Leukaemia Group B (CALGB) trial 9481 compared systemic 5FU/LV (the Mayo clinic regimen, which was considered a standard of care at the time of the trial design) with HAI using FUDR/LV and dexamethasone, which was a regimen that had produced high response rates (78%) and low toxicity (3% biliary sclerosis) in a prior phase 2 study (Kemeny *et al*, 1994). No crossover was permitted. Only 135 out of an accrual goal of 340 patients were randomised, in part because of delays caused by a temporary halt in manufacture and production of FUDR and implantable pumps. The majority of patients had >30% liver involvement (70%), synchronous metastases (78%), and were chemotherapy naïve (97%). The response rate (47% versus 24%, $P = 0.021$) and time to hepatic progression (9.8 versus 7.3 months, $P = 0.034$) were better in the HAI arm, but overall time to progression was similar (5.3 versus 6.8 months, $P = 0.8$), and time to extra-hepatic progression better in the systemic arm (7.7 versus 14.8 months, $P = 0.029$).

Median survival time was better in the HAI therapy arm (24.4 versus 20 months, P = 0.0034). Quality of life measurements showed improved physical functioning in the HAI group at the three and six-month follow-up assessments. Toxicity included moderately severe neutropaenia (2% and 45%; $P < 0.01$), stomatitis (0% and 24%; $P < 0.01$), and bilirubin elevation (18.6% and 0%; $P < 0.01$) in the HAI and systemic treatment groups respectively. In addition, greater proportion of men versus women receiving HAI experienced biliary toxicity (37% and 15%; $P = 0.05$) (Kemeny *et al*, 2006).

Differences between the CALGB trial and the European trials (German and MRC) might explain the variation in the outcomes. The CALGB study used pumps instead of ports, and HAI therapy included FUDR with dexamethasone to decrease toxicity. Survival was based on intent to treat in all three studies, and the actual number of patients treated was much lower in the European studies (66% in the German study and 63% in the MRC study, versus 86% in the CALGB study). The CALGB study did demonstrate that regional therapy alone can improve survival over systemic 5FU/LV with a survival similar to that seen with newer agents. Randomised studies of HAI therapy versus the new therapies have not been conducted, and in the future, studies comparing HAI therapy, or HAI plus new agents, versus the new agents alone would be appropriate.

As second-line monotherapy in patients refractory to systemic chemotherapy, HAI-based therapy has produced much higher response rates in several small studies (Cyjon *et al*, 2001; Kemeny *et al*, 1993). A trial using HAI FUDR/LV/dexamethasone in both chemotherapy-naive and previously treated patients produced response rates of 72% and 52%, and median survivals of 23 and 13.5 months, respectively (Kemeny *et al*, 1994). HAI FUDR/dexamethasone plus mitomycin administered through the pump sideport,

produced a 70% response rate in previously treated patients, with a median survival of 20 months from the start of HAI therapy after progression on systemic 5FU/LV (Kemeny *et al*, 2005a).

Reports of HAI as second-line therapy in combination with newer systemic regimens have also produced encouraging results. A phase 1 study of HAI therapy using FUDR combined with systemic irinotecan in previously treated patients (45% had previously received irinotecan) reported a response rate of 74%, a time to disease progression of 8.1 months, and a median survival of 20 months (Kemeny *et al*, 2001). A total of 13 of the 16 patients with prior irinotecan exposure responded to this regimen. Systemic oxaliplatin plus 5FU/LV or oxaliplatin plus irinotecan with concurrent HAI therapy using FUDR/dexamethasone in 36 previously treated patients (74% had received prior irinotecan) produced response rates of 86%, with a median survival of 36 months, and a one-year survival of 80% (Kemeny *et al*, 2005b). These and other small phase 1-2 studies using newer agents such as oxaliplatin for HAI therapy (Ducreux *et al*, 2005), suggest that a benefit may be derived from HAI and systemic therapy as second-line therapy, and randomised studies exploring the use of HAI plus systemic therapy versus newer agents alone in the second-line setting have been suggested (Kemeny, 2006).

Transarterial chemoembolisation or TACE, or embolisation without chemotherapy, have both been developed mostly as successful treatments for hepatocellular cancer, and have had more limited application for treatment of colorectal cancer metastases. This treatment is applied via a percutaneous catheter advanced into the hepatic artery and is associated with less technical complications than HAI therapy. The embolisation treatment leads to the acute ischaemic necrosis of tumours by blocking arterial flow. This results in

the death of most, but not all, cells within a tumour. Tumours with slowly dividing cells may be treated successfully with multiple embolisations over many months. Faster growing tumours may recur quickly between treatments and may not respond well overall to this approach (Kokudo & Makuuchi, 2004).

In a review of nineteen non-randomised phase 1 and 2 studies, which evaluated chemoembolisation for colorectal cancer metastases in 324 patients, utilizing lipid particles, Gelfoam, or collagen in combination with multiple chemotherapy regimens, response rates varied from 25% to 100% depending on the criteria used for measuring response, and median survival ranged between 7 and 23 months (Tellez *et al*, 1998). These results have not been adequately compared to other forms of regional therapy for the liver. Therefore, chemoembolisation has not been recommended as standard therapy for colorectal hepatic metastases and is considered investigational (Bartlett et al, 2006).

An alternative approach to infusional chemotherapy is infusional radiotherapy using ^{90}yttrium microspheres. These microspheres are infused into the common hepatic artery via a percutaneous or surgically placed catheter, and they preferentially localize to the tumour vasculature and emit therapeutic radiation. Two commercially available ^{90}Y-microspheres exist for this selective internal radiation therapy (SIRT). SIR-Spheres[1] (made of resin), and Thera-Spheres (made of glass). SIR-Spheres have been used in combination with infusional FUDR.

[1] ^{90}Yttrium is a high-energy pure beta emitting isotope with no primary gamma emission. The maximum energy of the beta particles is 2.27MeV with a mean of 0.93MeV. The maximum range of emissions in tissue is 11 mm with a mean of 2.5 mm. The half-life is 64.1 hours. In therapeutic use 94% of the radiation is delivered in 11 days.

A phase 3 randomised trial in 71 previously treated patients with unresectable bilobar disease, demonstrated an improved response rate (reduction in tumour volume, 50% vs. 24%, $P = 0.03$) and time to progression (9.7 versus. 15.9 months, $P = 0.001$) in favour of HIA FUDR plus SIR-Spheres, compared to HIA therapy using FUDR alone(Gray *et al*, 2001).

A randomised phase 2 study in twenty-one patients with previously untreated advanced colorectal liver metastases, with or without extra-hepatic metastases, demonstrated an improved survival from 12.8 to 29.4 months ($P = 0.02$) with SIR-Spheres plus systemic 5FU/LV compared to systemic therapy alone (Van Hazel *et al*, 2004).

A recent phase 1 study of SIR-Spheres therapy with modified FOLFOX4 systemic chemotherapy was conducted in 22 patients with inoperable liver metastases from colorectal cancer who had not previously received chemotherapy for metastatic disease. Partial responses were demonstrated in 18 patients and stable disease in two patients. Two patients underwent partial hepatic resection following protocol therapy. Median progression-free survival was 9.3 months, and median time to progression in the liver was 12.3 months (Sharma *et al*, 2007). Toxicity with this approach is secondary to the escape of radioactive microspheres from the liver leading to damage of other organs, such as the stomach or duodenum, lung, and bone marrow, however overall this therapy appears to be well tolerated.

Isolated hepatic perfusion or IHP is a surgical technique where the liver vasculature is completely isolated from the rest of the body and perfused with chemotherapy. The dose of chemotherapy is limited only by hepatotoxicity as no drug can leak out of the perfusion

circuit during the perfusion (Elaraj & Alexander, 2004). Early phase 1 and 2 studies in patients with unresectable disease have demonstrated response rates between 59% and 74% and median survival of 27 to 29 months, and development of this modality is ongoing (Bartlett *et al*, 2001; J. Rothbarth, 2003).

In late 2006, an international panel of experts produced a consensus statement regarding guidelines for therapy defined as "intra-arterial approaches" for unresectable colorectal hepatic metastatic disease. The current guidelines are as follows (Bartlett *et al*, 2006):

- Although numerous successful approaches exist for the regional therapy of hepatic metastases from colorectal cancer, these approaches should not be considered outside of investigational protocols.
- Given the effectiveness of systemic chemotherapy, regional chemotherapy should be used in conjunction with systemic chemotherapy.
- Too little data exist to determine an overall advantage of one form of regional therapy over another; however, chemoembolisation is the least appealing option and can no longer be recommended.
- Clinical trials comparing regional therapy plus systemic chemotherapy to systemic chemotherapy alone should be performed to define the role for each approach in the management of patients with hepatic metastases from colorectal cancer.

1.33 Therapy post resection

A. Systemic therapy

As previously discussed, because there is a high risk of relapse following resection of hepatic metastatic colorectal disease, many oncologists recommend adjuvant chemotherapy following hepatectomy. There is however, a lack of data supporting such practice. Recently, a randomised study addressed the utility of systemic therapy after liver resection. This report represents the first publication of an adequately powered, prospective, randomised, phase 3 trial comparing systemic chemotherapy after surgery to surgery alone (Alberts, 2006).

A total of 173 patients of a planned 200 patients with completely resected (R0) hepatic metastases from colorectal cancer were randomised over a period of 10 years on the multicentre FFCD 9002 trial, to surgery alone and observation (87 patients) or to surgery followed by 6 months of systemic adjuvant chemotherapy with a 5FU/LV monthly regimen (86 patients). After a median follow-up of 87 months, the five-year disease-free survival rate, after adjustment for major prognostic factors, was 33.5% for patients in the chemotherapy group and 26.7% for patients in the control group (Cox multivariate analysis: odds ratio for recurrence or death = 0.66; $P = 0.028$). The median disease-free survival was 24.4 months versus 17.6 months, respectively. With regard to secondary outcome measures, a trend towards increased overall survival was observed (five-year overall survival: chemotherapy group, 51.1% versus control group, 41.1%; odds ratio for death = 0.73; $P = 0.13$) (Portier *et al*, 2006).

The chemotherapy used in this trial is considered inferior to currently available regimens containing potentially more active agents such as oxaliplatin, irinotecan, bevacizumab, or cetuximab. The fact that this trial showed benefit with 5FU/LV, based on the predefined end point of this trial, provides a proof of concept of adjuvant chemotherapy in this patient population. It has been suggested that this trial should be validated through additional trials, using more modern systemic approaches. This will be accomplished, in part, by the European Organisation for Research and Treatment of Cancer trial 40983, which randomised patients with potentially resectable liver-only metastases to surgery alone, or 3 months of FOLFOX4 before surgery, and 3 months of FOLFOX4 after surgery. This trial recently completed its accrual of approximately 300 patients with overall survival as the primary end point (Alberts, 2006).

In late 2006, an international panel of experts produced a consensus statement regarding guidelines for adjuvant systemic therapy following hepatic resection for colorectal cancer. The current guidelines are as follows (Bartlett *et al*, 2006):

- The rationale for use of chemotherapy for initially resectable hepatic colorectal metastases, by extrapolation from response rates seen in treating metastatic colorectal cancer, probably justifies it being offered to selected patients. This rational is further supported by the fact that most patients with hepatic colorectal metastases also have concomitant extra-hepatic disease that would theoretically benefit from systemic chemotherapy.
- Both adjuvant and advanced-disease regimens can reasonably be offered, depending on the patient's previous treatment and response, performance status, organ function, and other factors.

- The optimal duration of adjuvant systemic chemotherapy following liver resection is unknown, but most oncologists utilize 4 to 6 months of systemic chemotherapy.
- If bevacizumab is employed as neoadjuvant/adjuvant therapy, the recommendation is to discontinue this drug approximately 8 weeks prior to surgery and/or to wait 8 weeks following surgery due to possible issues with wound-healing and other complications.
- Enrolment in clinical trials is strongly encouraged to better define the efficacy and toxicity of adjuvant therapy following hepatic resection.

B. Regional therapy

(i) HAI

HAI chemotherapy has also been used in adjuvant setting after liver resection. Four large randomized trials addressed the question of whether adjuvant therapy with HAI is useful after liver resection. At MSKCC, 156 patients were randomised after liver resection to HAI therapy using FUDR/dexamethasone plus systemic 5FU with or without LV, or systemic chemotherapy alone. The endpoint was two-year survival. Patients were stratified according to the number of liver metastases (i.e. 1, 2-4, > 4) and type of chemotherapy (none, 5FU with or without levamisole, or 5FU/LV). Numerous other parameters, including molecular markers, were comparable in both treatment groups. The study found that the two-year survival rate was increased with HAI plus systemic chemotherapy to 86%, versus 72% for systemic therapy alone ($P = 0.03$) (Kemeny *et al*, 1999).

An update of this trial with a median follow-up time of ten years, revealed a ten-year survival rate of 41% for the HAI plus systemic therapy group versus 27% for the systemic therapy alone group. Overall median survivals were 68.4 versus 58.8 months for the HAI plus systemic group versus systemic alone. Median time to hepatic recurrence was not reached in the combination arm, but was 32.5 months for patients receiving systemic therapy alone ($P \leq 0.01$). Median progression-free survival was 31 versus 17 months for HAI plus systemic therapy versus systemic therapy alone ($P = 0.02$) (Kemeny & Gonen, 2005).

An Intergroup (ECOG and SWOG) trial of adjuvant therapy for patients with one to three potentially resectable colorectal metastases undergoing hepatic resection, preoperatively randomised either to receive no further therapy (control arm, 56 patients) or postoperative HAI therapy using FUDR combined with IV 5FU (chemotherapy arm, 53 patients). After exclusion of patients identified as ineligible for the planned treatment at the time of surgery, there were 45 control patients and 30 on the chemotherapy arm. The study was powered to evaluate improvement in time to recurrence and hepatic disease-free survival, not overall survival. The four-year recurrence-free rate was 25% for the control arm and 46% for the chemotherapy group ($P = 0.04$). The four-year liver recurrence-free rate was 43% in the control group and 67% in the chemotherapy group ($P = 0.03$). The median survival of the 75 assessable patients was 49 months for the control arm and 63.7 months for the chemotherapy arm ($P = 0.60$). The median survival of all 109 patients was 47 months for the control arm compared with 34 months for the chemotherapy arm ($P = 0.19$). .The study concluded that adjuvant intra-arterial and intravenous chemotherapy was beneficial in prolonging time to recurrence and pre-venting hepatic recurrence after hepatic resection of colorectal cancer (Kemeny *et al*, 2002).

In a German prospective, multicentre, randomised trial of hepatic resection alone versus hepatic resection plus adjuvant HAI therapy using 5FU/LV, patients were stratified according to number of liver metastases (i.e. 1-2, 3-6) and the site of the primary tumour (colon or upper rectum, mid or lower rectum). A total of 113 patients were assigned to each group. Only 87 (77%) were actually treated in the HAI plus systemic therapy group for various reasons including extra-hepatic disease and technical complications. Chemotherapy data was available for 73 (64.6%) of the 113 patients randomised, and only 34 (30%) patients completed the assigned protocol, possibly due to the use of ports and 5FU therapy. No survival advantage was seen. In the secondary analysis comparing the "actually treated" patients (n = 87) to those receiving no therapy (n = 114), median survival was 44.8 versus 39.7 months, respectively. Median time to progression in the liver doubled in the group receiving HAI 5FU/LV (44.8 versus 23.3 months), and median time to any progression was 20 versus 12.6 months, respectively (Lorenz *et al*, 1998; Lorenz *et al*, 1999).

A prospective, randomised study from Greece used intra-operative randomisation to regional liver therapy plus systemic therapy versus systemic therapy alone. The regional therapy group received HIA mitomycin, 5FU/LV, and interleukin-2 (Proleukin) plus the same drugs by a systemic route. The systemic group received the same drugs without HAI therapy. A total of 143 patients were randomised, 5 died during the postoperative period, and 16 were lost to follow-up, leaving 122 in the follow-up groups. The two-year survival rates were 92% and 75%, and five-year survival rates were 73% and 60%, in the regional-plus-systemic versus systemic-alone groups, respectively ($P = 0.004$). Five-year freedom

from hepatic recurrence was also increased in the regional-plus-systemic group (82% versus 49%, $P < 0.001$) (Lygidakis *et al*, 2001).

A small study randomised 19 patients who underwent curative hepatectomy for metastatic colorectal carcinoma to continuous infusion 5FU HAI for six weeks. There was an increase in three-year disease-free survival of 66.7% for the regional group versus 20% for the control group ($P = 0.045$). The five-year survival rate was 77.8% for the regional group versus 50% for the control group ($P = 0.2686$) (Tono *et al*, 2000).

In a Japanese non-randomised study 30 of 58 patients who had radical resection of metastatic colorectal carcinoma, chose 5FU HAI plus oral UFT systemic therapy versus oral UFT systemic therapy alone after surgery. The five-year survival was increased for the HAI plus systemic therapy group (59% versus 27%; $P = 0.00001$). There was also a reduction in hepatic recurrence, 7% for HAI plus systemic therapy versus 57% for systemic therapy alone ($P = 0.00001$) (Kusunoki *et al*, 2000). In another small Japanese non-randomised study, 10 of 38 patients received HAI 5FU therapy for 3 weeks versus no further treatment after hepatic resection. The four-year survival rate was 100% versus 47% for the HAI and control groups, respectively ($P< 0.05$) (Asahara *et al*, 1998).

A meta-analysis performed in 2005 reviewed use of HAI for the delivery of chemotherapeutic agents to treat residual microscopic disease after curative hepatic resection for colorectal cancer metastases. Prospective clinical trials comparing hepatic arterial chemotherapy after curative hepatic resection for colorectal cancer metastases against a control arm were included. Non–English-language publications were excluded. The outcome measure was survival difference at one and two years after surgery. Seven

studies met the inclusion criteria, and all except one were randomised trials (Kemeny *et al*, 2002; Kemeny *et al*, 1999; Kusunoki *et al*, 2000; Lorenz *et al*, 1998; Lygidakis *et al*, 2001; Rudroff *et al*, 1999; Tono *et al*, 2000).

The survival difference in months (positive values favoring the treatment arm) was 1.8 at one year and 9.6 at two years. Neither was statistically significant (at two years; $P = 0.11$). Based on these findings, routine adjuvant HAI after curative resection for colorectal cancer of the liver was not recommended by the reviewers, however given the trend toward a survival benefit at two years, further study was recommended (Clancy *et al*, 2005). It should be noted that a more recent review looking at these same studies and reporting three and five-year survivals, suggested different results with improved survival for HAI therapy post hepatic resection (Kemeny, 2006).

(ii) Portal vein infusion

The goal of portal vein infusion in the post-resectional setting is to treat microscopic disease, which travels through the portal system and lodges in the portal venules. The advantage of arterial infusion may not be relevant in this scenario. Postoperative portal vein infusion of 5FU has shown modest activity as adjuvant treatment for colorectal cancer after curative resection of the primary (Elias *et al*, 2004a).

A meta-analysis was performed in 1997 to assess the effects on recurrence and survival of administering 5FU-based chemotherapy by portal vein infusion after colorectal cancer surgery. Data from ten randomised trials involving about 4000 patients with Duke's stage A, B and C tumours, were available for analysis. This review concluded that portal vein infusion of 5FU (with or without other cytotoxic drugs) for about 1 week after

surgery in patients with colorectal cancer may produce an absolute improvement in five-year survival of a few percent, and although encouraging, this finding was not statistically secure, and additional evidence from randomised trials involving several thousand more patients was needed.. Of interest, it should be noted that survival among patients with stage D (271 patients were reclassified at laparotomy) disease in the portal vein infusion group was slightly better than survival among control patients (11% versus 6%) (Liver-Infusion-Meta-analysis-Group, 1997).

The EORTC conducted a randomised trial and included 1235 patients after resection of colorectal cancer, and compared adjuvant portal vein infusion 5FU and heparin with no further treatment after surgery. No difference were observed in disease-free (67% versus 65%) or overall five-year survival (73% versus 72%) (Rougier *et al*, 1998a).

The UK AXIS trial randomised 3583 patients with presumed colorectal cancer, to surgery with or without 7 days of portal vein infusion with 5FU and heparin. In addition, patients with rectal cancer could be randomised to radiotherapy or no radiotherapy, to be given either before or after surgery. No overall benefit of portal vein infusion was established in AXIS when colonic and rectal cancers were considered together, however a greater treatment benefit was suggested for patients with colonic cancer than with rectal cancer, with respect to disease-free survival (hazard ratio, 0·79 versus 1·03; $P = 0{\cdot}07$), and there was also a non-significant trend with respect to overall survival (hazard ratio 0·87 versus 1·03; $P = 0{\cdot}17$). Furthermore, this differential treatment effect according to site of cancer in AXIS was strongly supported by a meta-analysis incorporating previous trials (included in the 1997 meta-analysis). Combining the data gave hazard ratios of 0·82 and 1·00 for colonic and rectal tumours respectively (test for interaction, $P = 0{\cdot}024$), equating

to an absolute survival benefit for patients with colonic cancer of 5·8% (The-AXIS-collaborators, 2003).

Two recent phase 2 studies in the United States evaluated the value of portal vein infusion with FUDR as an adjuvant to surgically treated hepatic colorectal metastases. Fifty-one patients who underwent complete resection and/or ablation were prospectively enrolled on two sequential trials, which included either alternating (22 patients) or concurrent (29 patients) regional portal vein infusion FUDR and systemic 5FU/LV chemotherapy. The mean number of lesions resected was three (range, one to 11 lesions). One and three-year overall survival rates were 92.7% and 41.8%, respectively. The one and three-year disease-free survival rates were 64.5% and 19%, respectively. The median overall survival time was 30.4 months and the median progression-free survival time was 14.5 months. The site of first recurrence was hepatic in 35.9% of patients. Treatment was terminated early in 24 patients (17 patients progressed, two refused treatment, and five had non-hepatic toxicities). Fifty-five percent of patients received 75% to 100% of the planned FUDR courses, and 72% received greater than 50% of the planned FUDR dose. Only four patients required dose reductions of FUDR because of moderately severe hepatic toxicity. No patient required biliary stenting or had discontinuation of portal vein infusion because of hepatic toxicity. The study concluded that the delivery of portal vein infusion FUDR and 5FU/LV can be performed with a high percentage of expected drug delivery and a low drug-induced hepatic toxicity rate, while achieving acceptable overall and disease-free survival (Faynsod *et al*, 2005).

The value of combined hepatic artery ligation and portal vein infusion has also been explored. The EORTC conducted a multicentre, prospective, randomised clinical trial in

74 patients with colorectal liver metastases, to study the efficacy of hepatic artery ligation, with and without 5FU portal vein infusion. Objective response rates were 20% versus 3%, respectively. Complications of hepatic artery ligation were relatively high, including four hepatic failures. Median survival for both groups was 12 months. Median time to progression for both groups was 6 months. As such, this study did not show any advantage of delivery using the portal route in addition to hepatic artery ligation in terms of progression or survival (Gerard *et al*, 1991).

A small prospective study randomised 39 patients with colorectal liver metastases to either hepatic artery occlusion for 16 hours followed by 5FU portal vein infusion for 5 days every sixth week (18 patients) or HAI therapy using 5FU plus IV LV for 2 days every second week (21 patients). The HAI group had longer mean (19 versus 13 months; $P = 0.0147$) and median survival (18 versus 12 months), increased objective response rates (81% versus 44%), and improved median time to progression (7 versus 4 months). The study concluded that 5FU HIA therapy produced better survival than hepatic artery occlusion followed by portal vein infusion (Naredi *et al*, 2003).

1.34 Other therapies

A. Radiation therapy

Radiation therapy has historically not been an effective modality for the treatment of colorectal cancer, because the liver is quite sensitive to radiation, and when doses greater than 3,500 cGy are used, radiation toxicity is common. Recently, new strategies in administering radiation therapy have made such treatment more effective. These include three-dimensional (3D) conformal radiation with or without simultaneous administration of

radio-sensitizing chemotherapy agents, antibody-directed radioablation, radioembolisation (see ^{90}yttrium microspheres discussed above), and intra-operative interstitial radiation.

Conformal 3D treatment planning, in which beams can enter the patient from almost any angle, can substantially reduce irradiation to the normal liver (Lawrence *et al*, 1990; Ten Haken *et al*, 1991). In one study, 22 patients with localized unresectable colorectal cancer metastatic to the liver, 14 of whom had progressed after prior chemotherapy, were treated with HAI FUDR combined with up 48 to 72.6 Gy. An objective response rate of 50% was reported, and the overall median survival was 20 months (Robertson *et al*, 1995). A recent phase 3 trial in 128 patients (47 with colorectal cancer) used 60.7 Gy of radiation with HAI FUDR. These patients had unresectable hepatic lesions that were too large for treatment by RFA. Approximately 60% of the colorectal cancer patients responded. The median survival was 15.8 months for the whole group and 17.2 months for the colorectal group. Moderately severe toxicity was seen in 21% and 9% of patients, consisting mostly of liver function abnormalities, upper gastrointestinal bleeding, and haematologic complications (Ben-Josef *et al*, 2005).

High-dose localized radiation can be delivered to part of the liver by using a single large dose of radiation. This technique has sometimes been referred to as "stereotactic" radiation, although current methods of liver treatment lack the precision in setup and tumour localisation typical of stereotactic irradiation to other organ sites such as brain. Doses of 8 to 30 Gy have been delivered in a single or few fractions to small solitary tumours (Blomgren *et al*, 1995). In a phase 1 study of 37 patients with 60 tumours (4 primary liver tumours and 56 metastases, with a median tumour size of 10 cm), local tumour control was achieved in 81% at 18 months after therapy (Herfarth *et al*, 2001).

A single high dose of radiation can be administered to localized hepatic metastases by employing a high-dose-rate iridium-192 afterloader placed at the time of laparotomy. A relative disadvantage of this approach is that sources can be placed only at the time of a laparotomy (Thomas *et al*, 1993). As discussed above, radiation can be combined with either systemic or regional chemotherapy and the fluoropyrimidines have been shown to be effective radiation sensitizers in both the laboratory and clinical settings (Bruso *et al*, 1990; Byfield *et al*, 1982; Heimburger *et al*, 1991; Van Hazel *et al*, 2004).

2.0 RATIONAL, HYPOTHESIS AND AIMS

2.1 Rationale

As previously discussed, in developed countries such as the United States and Western Europe, the most common malignant tumours of the liver are metastatic in origin, and most common neoplasms will have spread to the liver by the time of death. Hepatic metastases from colorectal carcinoma represent the majority of metastatic liver tumours. Although hepatic resection for colorectal liver metastases offers the best chance for cure and long-term survival, it has been estimated that only 10% to 25% (or about 100,000 of such patients per year worldwide) will be candidates for resection.

For the majority of patients (or more than 300,000 per year worldwide) with non-resectable colorectal liver metastases, the standard of care has been systemic therapy. However, despite recent advances in chemotherapy, five-year survivors are rare and systemic therapy has been considered palliative. In contrast to systemic therapy, patients with colorectal liver metastases who undergo hepatic resection for cure in modern times can expect five-year survival of at least 30%. However, even with the use of aggressive modern regimens of preoperative therapy, only about half of patients with initially unresectable disease will demonstrate an objective response, and at best 40% will become amenable to hepatic resection with or without ablation, with five-year survival equivalent to that of initially resectable disease. For patients with metastatic colorectal cancer, liver involvement is a major source of morbidity, and eventually leads to death in the vast majority of such individuals (Alexander, 2005; Kemeny, 2006; Khatri *et al*, 2005; Leonard *et al*, 2005).

Furthermore, resistance to chemotherapy limits the effectiveness of current cancer therapies, including those used to treat colorectal cancer. Drug resistance can be intrinsic or acquired during treatment and is thought to cause treatment failure in over 90% of patients with metastatic cancer, while drug resistant micrometastatic tumour cells may also reduce the impact of adjuvant chemotherapy treatment after surgery. The identification of panels of biomarkers that not only identify those patients most likely to benefit from chemotherapy treatment, but also which chemotherapies to use, would be a major advance. Overcoming drug resistance remains one of the main challenges of current cancer research (Longley *et al*, 2006).

There is therefore a need for additional effective therapies for hepatic-metastatic disease secondary to colorectal and other common cancers, both for cure and palliation. Locoregional therapy is attractive because it allows for tumour-directed dose intensification without associated increase in systemic toxicity. This may translate into improved efficacy in the liver and may be combined with systemic or other targeted therapy for control of extra-hepatic disease. In addition, the use of hepatic sustained-release or depot preparations may obviate the need for continuous infusion via expensive implantable pumps, and may also allow treatment of patients who are unfit for surgery, or who have more advanced disease with decreased performance status. Lipiodol/Ethiodol demonstrates ideal properties as a vehicle for development of experimental depot or sustained-release preparations for novel HAI therapy (Rubin & Dookeran 1997).

This project will determine the value of (1) Ethiodol as a vehicle for creation of novel depot or sustained-release preparations using lipophillic/oil-soluble anticancer agents, and (2) experimental HAI therapy with these novel Ethiodol-based depot preparations in animal models.

2.2 Hypothesis

Anticancer agents soluble in Ethiodol will (1) demonstrate depot or sustained-release characteristics; (2) demonstrate effective antitumour activity; and (3) eradicate hepatic-metastatic disease when administered as regional HAI therapy.

2.3 Specific aims

- Select a variety of novel oil-soluble anticancer agents and determine (a) maximum solubility in Ethiodol and (b) sustained-release characteristics *in vitro*
- Test Ethiodol based preparations for (a) antitumour activity *in vitro* and *in vivo*, and (b) systemic toxicity *in vivo*
- Establish an experimental animal hepatic-metastatic model that will allow evaluation of Ethiodol-based HAI therapeutic preparations
- Compare efficacy of Ethiodol-based HAI agents with systemic therapies

3.0 METHODS, RESULTS & DISCUSSION

3.1 9-aminocamptothecin

A. Background

As previously discussed, camptothecin, a plant alkaloid extract from the bark and wood of the Chinese tree *Camptotheca acuminata*, has been shown to possess anti-neoplastic activity. Analogs of camptothecin belong to a family of anticancer agents with a unique mechanism of action that is based on reversible inhibition of DNA topoisomerase I (Eng *et al*, 1988; Hsiang & Liu, 1988; Wall *et al*, 1966). The poor solubility of camptothecin, conferred by the unusually weak basicity of its quinoline nitrogen atom, precluded direct parenteral administration to patients. Instead, the less active water-soluble carboxylate salt of camptothecin was used for the initial phase 1 clinical trials performed in the early 1970s (de Jonge *et al*, 1999b; Garcia-Carbonero & Supko, 2002; Ulukan & Swaan, 2002).

Despite the cytotoxicity of this compound, further development was halted because of a number of severe and unpredictable side effects observed in early clinical trials, including myelosuppression, vomiting, diarrhoea and severe haemorrhagic cystitis (Gottlieb *et al*, 1970; Moertel *et al*, 1972; Muggia *et al*, 1972). The subsequent search for less toxic analogs of camptothecin resulted in the discovery of several semisynthetic

derivatives, including the water soluble irinotecan and topotecan, and water insoluble 9-nitrocamptothecin and 9-amino-20(*S*)-camptothecin or 9-aminocamptothecin[1] (9AC).

In aqueous solutions, camptothecins are unstable and undergo a rapid, pH-dependent, non-enzymatic hydrolysis of the terminal lactone ring to form the more water-soluble, ring-opened carboxylate form. The presence of the intact terminal lactone ring is thought to be essential for the topoisomerase I inhibition. The closed lactone ring predominates at acidic pH, whereas in human plasma the equilibrium between these two species greatly favours formation of the carboxylate form, partly because of the physiologic pH and the preferential binding of this form to albumin (Burke & Mi, 1994; Fassberg & Stella, 1992; Hertzberg *et al*, 1989; Takimoto *et al*, 1994). It has been reported that the carboxylate forms of camptothecin and 9AC bind to human serum albumin with 200-fold greater affinity than the lactone, resulting in a predominance of the carboxylate in the presence of this blood protein *in vitro* (Burke & Mi, 1994; Champoux, 1978; Stivers *et al*, 1997).

The DNA topoisomerases are nuclear enzymes that reduce the torsional stress of supercoiled DNA. This action enables selected regions of DNA to become sufficiently

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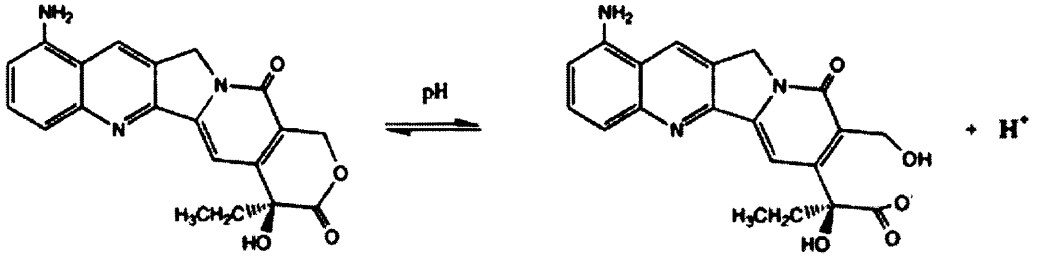
[1] Chemical structure and pH dependent conversion of 9-amino-20(*S*)-camptothecin lactone and carboxylate.



[de Jonge MJA, Verweij J, Loos WJ, Dallaire BK, Sparreboom A (1999b) Clinical pharmacokinetics of encapsulated oral 9-aminocamptothecin in plasma and saliva. *Clin Pharmacol Ther* **65:** 491-499]

exposed and relaxed to facilitate essential cellular processes such as DNA replication, recombination, and transcription to occur. Topoisomerase I binds covalently to double-stranded DNA through a reversible reaction creating a single-strand break. This so-called "cleavable complex" facilitates the relaxation of torsional strain in supercoiled DNA, either by allowing passage of the intact single strand through the nick, or by free rotation of the DNA about the uncleaved strand. Once the torsional strain has been relieved, the enzyme rejoins the cleaved strand of DNA and dissociates from the relaxed double helix (Champoux, 1978; Gupta *et al*, 1995; Stivers *et al*, 1997). Importantly, higher expression levels of topoisomerase I have also been detected in colon tumours (McLeod *et al*, 1994).

The camptothecins bind to and stabilize the normally transient DNA-topoisomerase I cleavable complex. Although the drug does not affect the initial cleavage action of topoisomerase I, the religation step is inhibited, leading to the accumulation of single-stranded breaks in the DNA. These lesions are not in themselves toxic to the cell, because the strands readily religate on drug removal. However, collision of the DNA replication fork with the ternary drug-enzyme-DNA complex produces an irreversible double-strand break that ultimately leads to cell death. The camptothecins are therefore S-phase[1] specific drugs, because ongoing DNA synthesis is a necessary condition to induce the above sequence of events leading to cytotoxicity. This has important implications for the clinical use of these agents, because optimal therapeutic efficacy of S-phase specific cytotoxic

[1] In 1951, investigators studying the division of plant root cells, separated the process into four phases eventually referred to as *GAP1*, *synthetic phase*, *GAP2*, and *mitosis*. The shorthand that emerged from this descriptive work (i.e. G_1, S-phase, G_2 and M-phase or mitosis) has been the lens through which all subsequent dividing cells have been observed, and the four successive phases are referred to collectively as the *cell cycle* [Howard A, Pelc SR (1951) Nuclear incorporation of P 32 as demonstrated by autoradiographs. *Exp Cell Res* **2:** 178-187].

drugs generally requires prolonged exposure of the tumour to concentrations exceeding a minimum threshold (Hsiang & Liu, 1988; Hsiang *et al*, 1985; Tsao, 1993).

9AC is a camptothecin analogue with potent antitumour activity against human colon cancer xenograft models. Pharmacokinetic studies performed during clinical trials using continuous IV therapy have revealed that < 10% of the total drug was present in plasma as the active lactone form. There is also evidence that 9AC is subject to significant hepatic metabolism and biliary excretion appears to be the primary route of drug elimination (Giovanella *et al*, 1989; Grossman *et al*, 1998; Supko & Malspeis, 1993).

The most frequently encountered dose-limiting toxicity of systemic 9AC is myelosuppression with neutropaenia, although thrombocytopaenia and diarrhoea may also prove to be dose-limiting in a minority of patients. Other commonly encountered toxicities include nausea and vomiting, mucositis, anaemia, fatigue, and alopecia. Phase 2 studies using 72-hour IV infusion schedules have been conducted in patients with various types of malignancies with disappointing results, and objective response rates were 0% against colon cancer. One partial response was noted in 13 patients with metastatic colorectal cancer treated with oral colloid dispersion, polyethylene glycol formulated 9AC. In addition, dose-limiting haematological toxicity has precluded achieving plasma concentrations of 9AC lactone in humans comparable with the levels provided by doses affording optimal activity against human tumour xenografts in nude mice (de Jonge *et al*, 1999a; Erickson-Miller *et al*, 1997; Herben *et al*, 1999; Leonard B. Saltz, 1997; Siu *et al*, 1998; Vey *et al*, 1999).

B. Rationale for selection

The myelosuppression associated with systemic 9AC administration, together with water insolubility, need for continuous administration, unique requirement for maintenance of the lactone form for drug activity, and hepatobiliary excretion, suggest that 9AC may be an ideal novel chemotherapeutic candidate for development of a sustained-release preparation. Regional therapy with 9AC depot-preparations may allow the realization of antitumour potential, promised by the encouraging results of preclinical 9AC studies with colon tumours, with the additional benefit of decreased systemic toxicity.

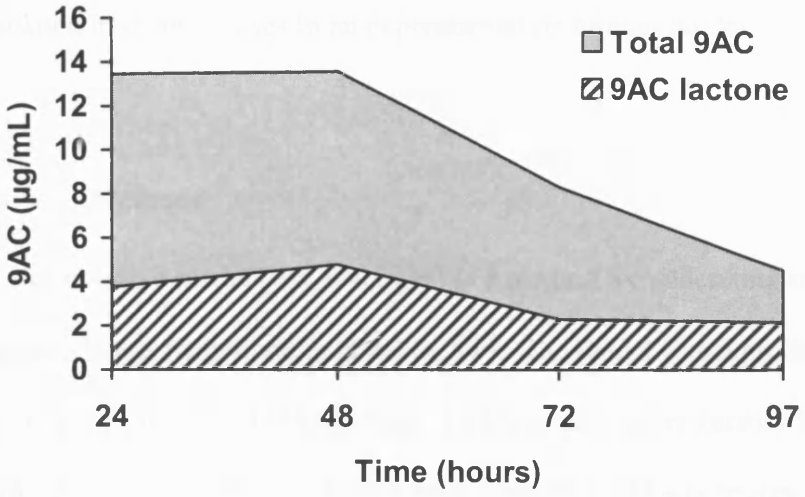


Figure 3.1 Release of 9AC lactone from Ethiodol over time.[1]

Furthermore, it has also been demonstrated that Ethiodol acts as a delivery vehicle for 9AC. A preparation of 5mg/mL 9AC in Ethiodol demonstrated release of 9AC from

[1] 0.5 mL 9AC in Ethiodol at concentration of 5mg/mL was overlayed with one mL of fresh plasma, and 9AC lactone and total 9AC were measured at given timepoints by sampling plasma and HPLC analysis [Data adapted from: Rubin J, Brumfield A, Dookeran K, Lotze M (1995a) Tumour-targeted delivery of sustained, high dose 9-aminocamptothecin. *Proc AACR* **36:** 452].

the Ethiodol in a sustained fashion beyond five days, reaching concentrations of 10 μg/mL in plasma, of which approximately 50% of the drug (as detected by high performance liquid chromatography or HPLC) (Takimoto *et al*, 1994) was the active lactone form (see **Figure 3.1**). The released drug also demonstrated both *in vitro* and *in vivo* antitumour activity (Rubin *et al*, 1995a).

3.11 Aims

This study sought to determine whether locoregional 9AC in Ethiodol was a more effective antitumour preparation than systemically administered aqueous 9AC, as treatment for isolated liver metastases in an experimental rat tumour model.

3.12 Methods

An in-house model was developed as a method for generating multiple hepatic metastases in rats (described in detail below). The model was reproducible and useful for evaluating the efficacy of HAI therapy of various anticancer agents. In the hands of a skilled operator, and after experience with more than 300 procedures, perioperative mortality was low (< 1%). When seen, it is secondary to postoperative bleeding. Meticulous technique and the use of an operating microscope made concomitant extra-hepatic metastases rare in our hands. The need for splenectomy was also obviated by direct injection of tumour cells into the portal vein. Syngeneic cell lines and animal models were chosen to facilitate uniform conditions for response, and this was considered particularly important for experiments involving immunotherapy (such as interleukin-2, described later) (Lafreniere & Rosenberg, 1986).

Other models of hepatic metastases have been previously described previously. The liver has been directly inoculated with tumour and investigators have also implanted small pieces of tumour into an incision made in the left lateral lobe. Others have injected tumour cell suspensions beneath the capsule of the liver (Ackerman, 1974; Kemeny *et al*, 1992; van Hillegersberg *et al*, 1992). Although these models reportedly generated measurable tumour in a reproducible fashion, in our laboratory, results of these models were variable and not reproducible. In addition, the relevance of direct inoculation of tumour into the liver to experimental study of hepatic-metastatic cancer is unclear, since as previously discussed hepatic metastases are usually generated by portal vein spread. Hence these models were rejected.

Another model demonstrated that intra-splenic injection of tumour cell suspensions followed by splenectomy consistently generated multiple hepatic metastases in mice. Others used this technique to evaluate the antitumour activity of IL2 and lymphokine-activated killer cells in rats. Seeding of the liver via the portal vein, which was accomplished by this model, was probably more akin to the mechanism by which gastrointestinal tumours metastasise. However, the effect of splenectomy on immune reactivity may limit the applicability of this model in ways that have not been elucidated. In addition, this model has also been associated with a 20% incidence of concomitant extra-hepatic metastases (Lafreniere & Rosenberg, 1986; Schwarz & Hiserodt, 1990). Hence these models were also rejected.

Other models similar to ours have been described without specific technical detail and as far as we were aware, have not been used to evaluate HIA therapy. In addition, the incidence of perioperative mortality and extra-hepatic tumour growth were not reported.

Another model, similar to ours, employed an ileal mesenteric vein for infusion of tumour. However, this was technically more demanding in our hands due to the small size of this vein, and for reasons that are unclear, as many as 12% of injected rats failed to develop measurable hepatic tumours in this model (Dworkin *et al*, 1995; Liu *et al*, 1995). Hence these models were also rejected.

A. Rats

Syngeneic Fisher (F344) rats [Taconic Farms, Germantown, NY] were housed and fed in accordance with University of Pittsburgh research guidelines. They were allowed to acclimatize for at least 2 weeks prior to use in these studies. They were exposed to alternating 12 hour light and dark cycles. Animals used in these experiments weighed approximately 350 grams.

B. Tumour cell lines

B16, a spontaneously occurring murine melanoma, and MC38, a colon adenocarcinoma, are both tumour cell lines syngeneic to C57BL/6 mice (Lafreniere & Rosenberg, 1986). TSA is a breast adenocarcinoma cell line syngeneic to BALB/C mice (Nanni *et al*, 1983). MADB106 (W. Chambers, Pittsburgh, PA) is a cultured breast adenocarcinoma cell line syngeneic to F344 rats (Barlozzari, 1985). All tumour cell lines were cultured in complete medium consisting of RPMI1640 supplemented with 10% foetal calf serum [GIBCO, Grand Island, NY], 100 mg/mL streptomycin [GIBCO], 100 U/mL penicillin [GIBCO], 3% glutamine [GIBCO], 1 mM sodium pyruvate [GIBCO], 0.1 mM non-essential amino acids [GIBCO], and 5 X 10^{-5} M 2-mercaptoethanol [Sigma Labs., St. Louis, MO].

C. *In vitro* studies

(i) Evaluation of 9AC in Ethiodol drug-efficacy in a cell culture model

The sustained release antitumour activity of 9AC [Pharmacia, Columbus, OH] in Ethiodol [Savage Labs., Melville, NY] was confirmed using a previously detailed method (Rubin *et al*, 1995b), prior to commencing *in vivo* studies. MC38 and MADB106 cell lines were cultured on cell culture inserts in six well plates at a seeding dose of 5×10^4 cells. Once growth was established, 200 μg 9AC in 40 μl Ethiodol was inserted into the test wells. The same amount of Ethiodol only was inserted into control wells. Cell death (as determined by trypan blue exclusion) in the test wells was indicative of release of active 9AC. The cell culture insert was then replaced with an insert with newly cultured cells. Half the media in the wells was also replaced with new medium daily. This was done for a period of 10 days which was thought to approximate the maximum limit of the planned period of drug exposure for *in vivo* experiments, and was based on data from prior evaluation of release of 9AC lactone from Ethiodol, which indicated that the active drug was released beyond five days (Rubin *et al*, 1995a).

(ii) Evaluation of aqueous 9AC drug-efficacy in a cell culture model

To evaluate and compare the value of 9AC **without** Ethiodol, a water soluble formulation called colloidal dispersion (CD) 9AC [Pharmacia, Columbus, OH] was obtained. In order to simulate the sustained-release properties of 9AC/Ethiodol, and provide aqueous 9AC for a period of almost 10 days, which was thought to approximate the maximum limit of the planned period of drug exposure for *in vivo* experiments, CD/9AC was delivered via a mini-osmotic pump [Alzet Corp., Palo Alto, Ca]. The *in vivo* experimental plan was to make use of this mini-osmotic pump to deliver sustained drug delivery via the

intra-peritoneal (IP) route as a control group. The effectiveness of the mini-osmotic pump for sustained-release of active aqueous CD/9AC was tested by inserting the pump containing 60 μg CD/9AC in 200 μl water (this was the maximum dose available as specified by restrictions due to CD/9AC formulation and pump volume requirements), into a 50 cm^2 culture flask with 5 x 10^4 MADB106 cells established in culture (since the pump was to large to fit adequately in a six well plate reservoir with cell culture insert). Cell death was indicative of release of active drug. The mini-osmotic pump was then transferred to a flask with newly cultured cells and new media and antitumour effect was studied for a total period of ten days.

D. *In vivo* studies

(i) Titration of 9AC in Ethiodol drug-efficacy in an engraftment model

1 x 10^6 MADB106 tumour cells were admixed with 200 μl Ethiodol only, or plus 9AC in doses of 1000 μg, 500 μg or 250 μg and injected into the abdominal flank of F344 rats. Tumour growth was measured with callipers over a 6 month period. Animals with tumours larger than 2 x 2 cm in size were sacrificed.

(ii) Generation of hepatic metastases

MADB106 tumour cell suspensions were injected subcutaneously into the flanks of F344 rats and underwent serial passage (i.e. tumour cells were passed on to animals sequentially after harvest to maintain viability and not maintained for long periods in cell culture). Tumours were harvested under aseptic conditions and single-cell suspensions were prepared by mincing the tumours into 2 mm fragments in Hank's balanced salt solution (HBSS) [GIBCO]. These fragments were then digested for two to four hours at 37° C in

RPMI1640 containing 1 mg/mL type IV collagenase [Sigma Labs, St. Louis, MO]. An alternate cell preparation method involved mechanical cell fragmentation with scraping of tumour tissue with a scalpel blade to produce a suspension. The resulting cell suspensions from either method were passed through 100 μm Nytex [Lawshe Industrial Co. Inc., Bethesda, MD], washed three times in HBSS, and cryopreserved. Tumour cells were thawed when needed and cultured in monolayers in complete medium as described above. Tumour cells were harvested with a solution of 0.05% trypsin [GIBCO], washed 3 times with RPMI1640, and stored on ice prior to inoculation. Tumour cell preparations were tested for viability prior to infusion by trypan blue exclusion.

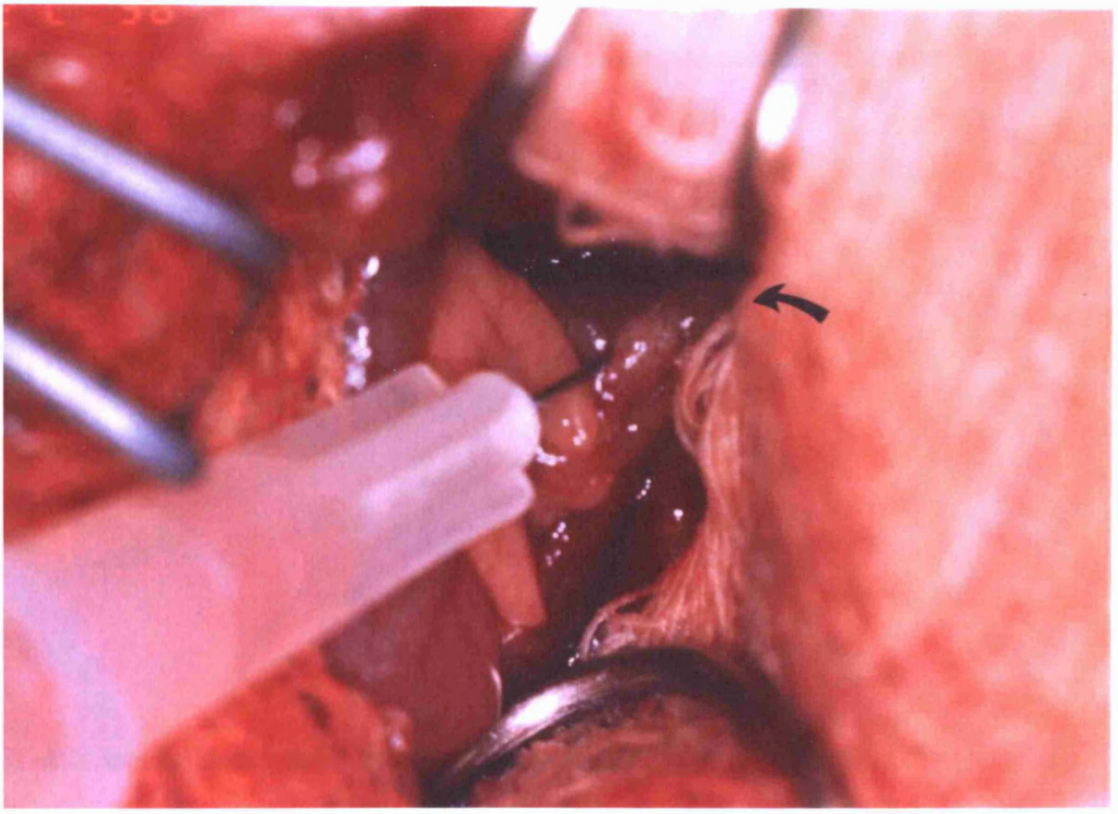


Figure 3.2 **Photograph of direct intra-portal injection of tumour cells. Arrow indicates portal vein cannulation with 30 gauge needle without extravasation using operative microscope.**

F344 rats, anesthetised with methoxyflurane [Pitman-Moore, Mundelein, IL], were shaved and cleaned with 70% ethanol [Fisher Scientific, Pittsburgh, PA]. Using aseptic technique, a 1 cm upper midline abdominal incision was made. The median lobe of the liver, the stomach, and the intestines were retracted within the abdominal cavity in order to visualise the portal vein. Using an operating microscope, 2 x 10^5 tumour cells suspended in approximately 150-200 μL of RPMI1640 were directly injected into the portal vein through a 30 gauge needle [Becton-Dickinson & Co., Franklin Lakes, NJ] (see **Figure 3.2**). Portal vein haemorrhage and haematoma formation were assiduously avoided by applying pressure to the injection site with a sterile cotton-tipped applicator for two minutes after injection. The wound was sutured in 2 layers with 4-0 Dexon [Davis & Geck, Manati, PR].

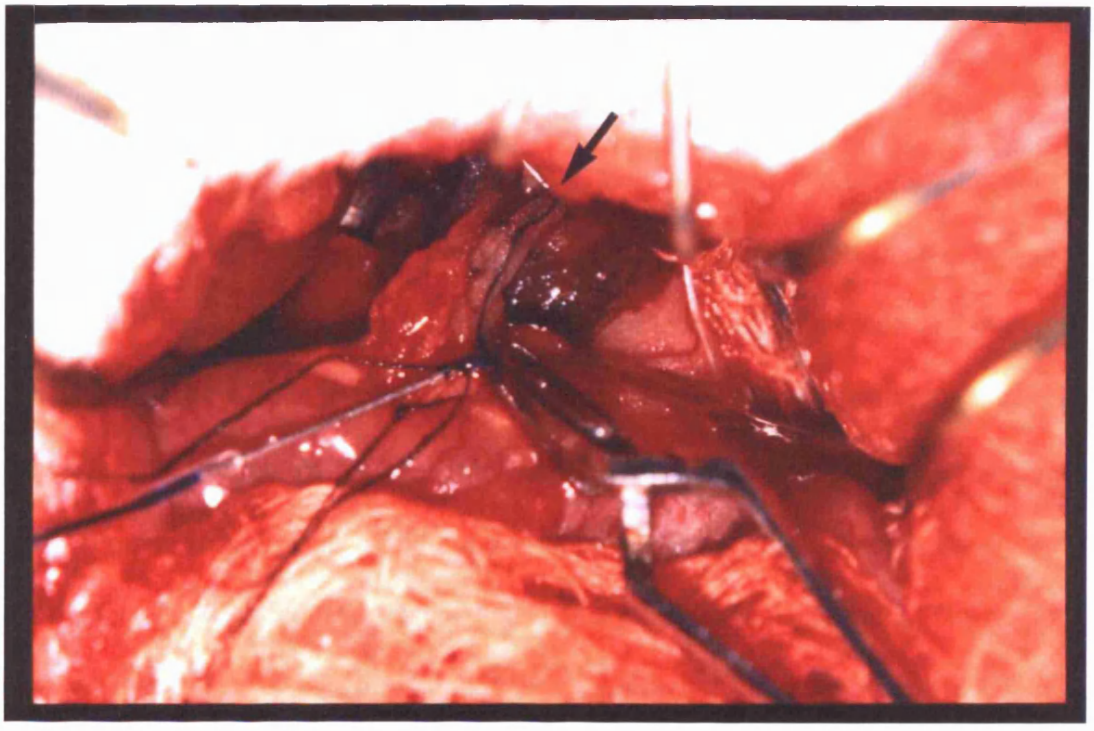


Figure 3.3 **Photograph demonstration of intra-arterial injection with methylene blue dye. Arrow indicates methylene blue dye injected through cannulated gastroduodenal artery into the proper hepatic artery.**

(iii) Hepatic intra-arterial therapy

Six to seven days after tumour inoculation, rats anesthetised with methoxyflurane and intraperitoneal Nembutal [Abbott Laboratories, North Chicago, IL], 0.09 ml/100 mg body weight, underwent repeat laparotomy. A disposable high temperature cautery device [Aaron Medical Inc., St. Petersburg, FL] was used for haemostasis. The spigelian lobe of the liver was released and reflected cephalad (see **Figure 3.3**), in order to expose the common bile duct, portal vein and both the hepatic and gastroduodenal arteries. Using an operating microscope and fine-tipped forceps, the gastroduodenal artery was isolated and ligated distally before its bifurcation into the superior and inferior pancreaticoduodenal arteries. Care was taken to avoid injury to the gastroduodenal vein. The common hepatic artery was clamped proximally with a small vascular clamp in order to prevent retrograde flow of intra-arterial therapy.

A blunt-tipped 30 gauge needle, attached to 30 gauge PE-10 Intramedic polyethylene tubing [Clay Adams, Parsippany, NJ] was inserted into the gastroduodenal artery through a small arteriotomy and secured with 7-0 silk suture [Ethicon Inc., Somerville, NJ] placed around the artery and cannula. Ethiodol intra-arterial therapy was infused through the cannula after testing the arteriotomy for leakage by first flushing with 100 μL of 0.9% saline [Sigma Labs.]. Prior to removing the cannula and securing the gastroduodenal artery, the cannula was flushed with 0.9% saline to ensure that no drug remained in the cannula. The hepatic artery clamp was then removed. The abdominal incision was closed as described above and the rats were returned to their cages after having fully recovered.

(iv) Liver metastases treatment groups

All groups were treated six to seven days post inoculation as follows. Group (a) received bolus HIA 9AC (60 μg) in 60 μL of Ethiodol. Group (b) received bolus HIA aqueous CD/9AC (60 μg) in 200 μL water. Group (c) received bolus HIA Ethiodol only (60 μL). Group (d) received CD/9AC (60 μg) in 200 μL water, via mini-osmotic pump pumping at a rate of 1 μL/hr for almost seven to ten days intraperitoneally (IP).

(v) Tumour evaluation

Rats were sacrificed at ten to twelve days post drug treatment using carbon dioxide suffocation and their livers were carefully harvested. The number of metastases on the liver surface was counted by direct inspection without knowledge of the prior treatment. Tumours appeared as pearl white surface lesions contrasted against the darker background of the liver (see **Figure 3.4**). Based on our previous observations and a prior report[1], this method was determined to be the most sensitive and reliable for evaluation of tumour progression in this model. No other method of tumour evaluation was therefore employed.

[1] The method of hepatic tumour-evaluation was adapted from a prior report in which tumours were counted in a blinded fashion without knowledge of the prior treatment. Hepatic metastases could be reliably enumerated when less than 250 in number, however when too numerous to count, they were assigned an arbitrary value of 250, since it was not possible to reliably count metastases approaching and greater than 250 per liver. This method was validated in more than 3000 procedures and the significance of differences in numbers of liver metastases among test groups was determined by the Wilcoxon rank sum test [Lafreniere R, Rosenberg SA (1986) A novel approach to the generation and identification of experimental hepatic metastases in a murine model. *Journal of the National Cancer Institute* **76:** 309-22].

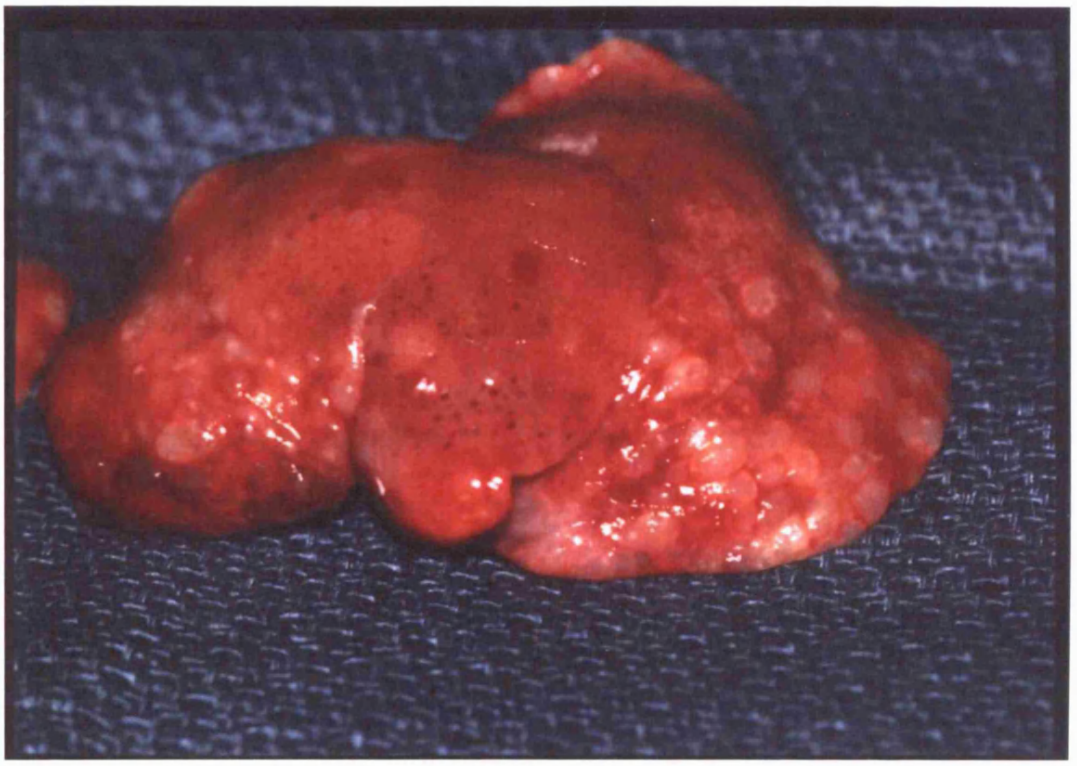


Figure 3.4 **Photograph of liver of F344 rat demonstrating multiple metastases as white tumour nodules against the parenchymal background.**

E. Statistical analysis

Due to the method of enumeration employed, the number of metastases for any test group was considered ordinal level data (i.e. the data is arranged in some order or rank, however, the differences between the data values cannot be determined or is meaningless) (Lind *et al*, 2008). As such, due to small sample size and ordinal level data, a non-parametric statistical method was considered appropriate for detection of significant differences between groups. The Wilcoxon Rank-Sum Test for two independent medians, is used in the situation where two independent random samples size n_1 and n_2 are from two populations, with ordinal variables and medians m_1 and m_2. The *null hypothesis* is that the medians m_1 and m_2 in the two populations are equal. Statistical analysis was therefore performed using the Wilcoxon Rank-Sum Test and all *P* values were two-tailed (Daly *et*

al, 1991; Wilcoxon, 1945). Calculations were performed with Microsoft Excel software with MegaStat.

3.13 Results

A. Measurable hepatic metastases are reproducibly generated

Multiple hepatic metastases were consistently produced after portal vein injection (see **Table 3.1**). Operative mortality was low as this model was being developed. When it occurred, it was invariably secondary to early postoperative bleeding. Rats became progressively ill over a period of 28 to 42 days. Upon sacrifice a full examination was made of all thoracic and abdominal viscera to determine the extent of metastatic disease. Abdominal exploration revealed large numbers of hepatic metastatic tumours throughout the substance of the liver. Tumours were evenly distributed between all lobes of the liver. Only rarely did tumours become established at the site of portal vein inoculation. Other extra-hepatic metastases were not evident. Eventually rats observed for almost six weeks manifested hair loss, ruffled fur, abdominal distension, jaundice, or lethargy as a result of tumour progression.

Table 3.1

Direct portal vein infusion of tumour cells produces multiple hepatic metastases

[1]Experiment Number	Number of rats	[2]Number of counted metastases	Mortality
1	3	150, ≥250, ≥250	0
2	5	≥250, ≥250, ≥250, ≥250, ≥250	0
3	4	148, ≥250, ≥250, ≥250	0
4	4	≥250, ≥250, ≥250, ≥250	0
5	4	180, ≥250, ≥250, ≥250	0
6	4	105, 102, ≥250, ≥250	0

[1]Series of six consecutive experiments, however method had been developed and optimised in over 300 rats; [2]Tumours were counted to a maximum of 250, method adapted from: A novel approach to the generation and identification of experimental hepatic metastases in a murine model (Lafreniere & Rosenberg, 1986). Untreated F344 rats inoculated with 2 x 10^5 MADB106 cells at day 0 and hepatic tumours counted at day 21.

The rate development of hepatic metastases was dependant on the number of inoculated tumour cells (see **Figure 3.5**). When livers of animals inoculated with tumour were sectioned in the sagittal plane in 3-4 mm strips, tumours were seen throughout the sectioned surface.

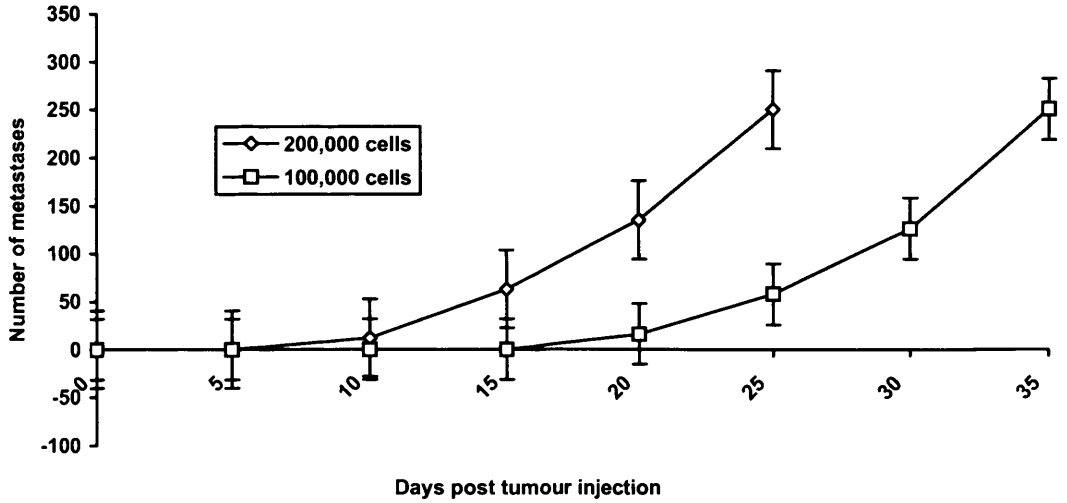


Figure 3.5 **Development of hepatic metastases over time in model. Generation of metastases was dependent on the number of inoculated tumour cells. 10^5 or 2 x 10^5 MADB106 cells injected day 0 and rats scarificed every 5 days, and tumours enumerated (n=3/group; 3 experiments).**

For control rats, treated with gastroduodenal artery (GDA) ligation only at second laparotomy one week after tumour inoculation, evaluation of total tumour content as a percentage of normal liver parenchyma replaced, appeared comparable to counting the surface tumours (see **Table 3.2**).

Table 3.2

Comparison of surface count of hepatic metastases to % parenchyma replaced by tumour in control rats

Experiment Number	Number of rats	Median number of counted metastases	Mean % parenchymal replacement
1	4	250	62
2	5	250	58
3	3	250	75

Tumour inoculated rats were treated with GDA ligation only at seven days post inoculation, and sacrificed 14 days post treatment (day 21 post inoculation). At sacrifice, rats were all in good condition with normal activity and had no evidence of jaundice. After enumeration of surface metastases, livers were sectioned in the sagittal plane in 3-4 mm strips, and % of tumour replacement seen throughout the sectioned surface of each strip was estimated. Estimates were then totalled for each liver, and the average for each experimental group was determined.

B. Hepatic intra-arterial infusions are well tolerated

Hepatic intra-arterial infusions of 100 to 200 μL Ethiodol were well tolerated in non-inoculated animals. No animals manifested systemic toxicity at these doses. Animals treated with 200 μL of Ethiodol experienced minimal weight loss comparable to those treated with GDA ligation only (median values 92.6% vs. 91.8% respectively) at seven days post treatment, and animals from both groups recovered their pre-treatment body weights and proceeded to gain weight in the 7-14 day interval after treatment.

Hence at sacrifice both animals treated with HIA Ethiodol and GDA ligation only, had body weights above initial pre-treatment values. Bilirubin levels remained within normal range for both animals treated with HIA Ethiodol and GDA ligation only (< 1.2 mg/dL). Mild elevation of liver enzymes (< twice increase over normal values for SGOT, SGPT, GGT) were seen at day one post treatment with Ethiodol, with values normalising at seven days post intervention, consistent with transient mild hepatocellular dysfunction (Kew, 2000).

For animals treated with Ethiodol, evaluation of tumour content as a percentage of normal liver parenchyma replaced was also comparable to counting the surface tumours (see **Table 3.3**).

Table 3.3
Comparison of surface count of hepatic metastases to % parenchyma replaced by tumour in rats treated with Ethiodol only

Experiment Number	Number of rats	Median number of counted metastases	Mean % parenchymal replacement
1	4	232	60
2	4	250	54
3	3	216	75

Tumour inoculated rats treated with 200 µl HAI Ethiodol only seven days post inoculation and sacrificed 14 days post treatment (day 21 post inoculation). At sacrifice, rats were all in good condition with normal activity and had no evidence of jaundice. Methods as outlined in Table 3.2.

There was also no difference in the number of surface metastases counted between animals treated with Ethiodol HAI or GDA ligation only. (see **Table 3.4**).

Table 3.4

Comparison of median hepatic tumour surface counts for rats treated with GDA ligation only or Ethiodol HAI only

Experiment Number	Median Number liver metastases	
	GDA ligation (n)	Ethiodol HAI (n)
1	250 (3)	216 (3)
2	250 (5)	250 (3)
3	224 (4)	232 (4)
4	227 (4)	233 (6)
Methods as described in Tables 3.2 and 3.3 above. Wilcoxon Rank-Sum Test: $p > 0.05$ for comparison of GDA ligation only and HAI Ethiodol only groups		

C. 9AC in Ethiodol demonstrates effective antitumour activity *in vitro*

9AC in Ethiodol demonstrated effective antitumour activity as a depot or sustained-release preparation in *in vitro* evaluation. Cells in established culture on inserts, placed in combined culture with 9AC/Ethiodol as previously described in section 3.12.C (i), experienced almost complete cell death at two days post insertion into the combined culture. Replacement of the inserts in combined culture at three day intervals (with other inserts with cells in established culture) demonstrated similar results over a period of ten days. This evaluation demonstrated sustained release of active 9AC drug from Ethiodol, with cytotoxicity, over a ten day test period (see **Figure 3.6**). Since the active 9AC lactone is quickly degraded to the inactive carboxylate form at physiologic pH, it was considered unlikely that the antitumour effects were secondary to residual carboxylate 9AC in the combined culture. Cells in combined culture with Ethiodol only control, demonstrated viable culture pattern. Cell death was confirmed with trypan blue exclusion.

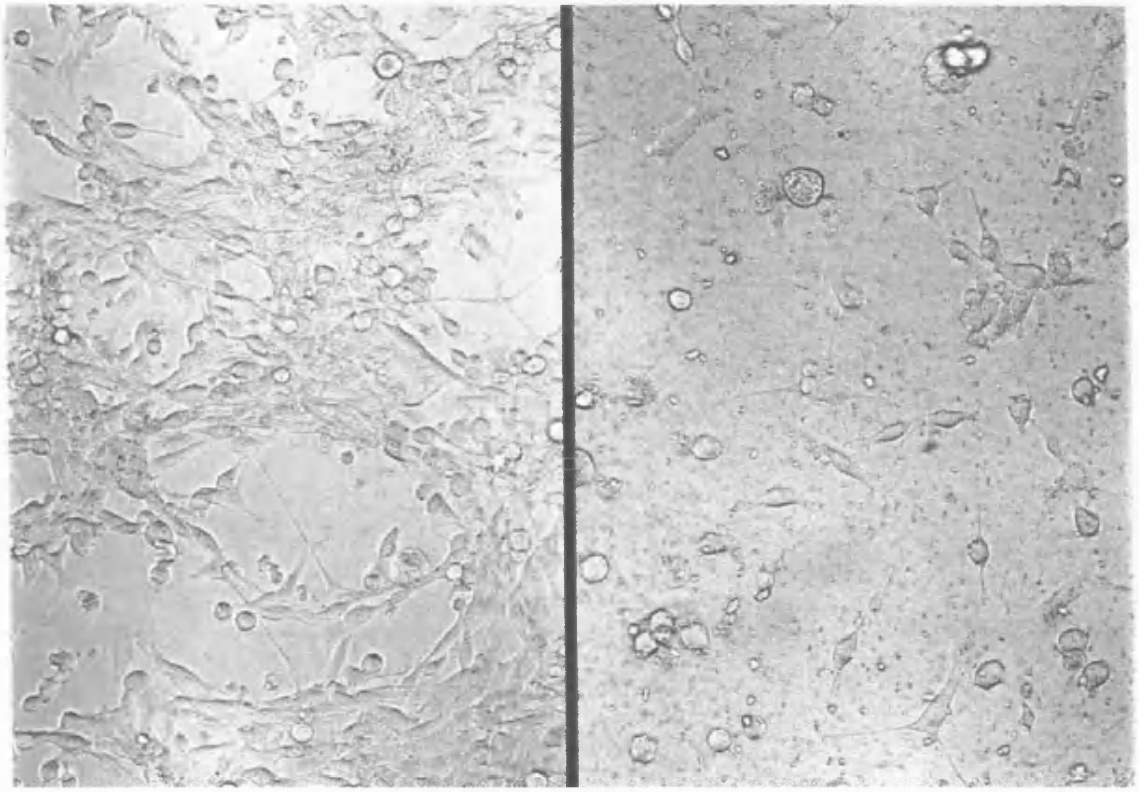


Figure 3.6 **Photomicrograph (x 40 magnification) of culture appearance and cytotoxicity (right panel) of MC38 cells placed in combined culture with 9AC in Ethiodol. Left panel shows MC38 in co-culture with Ethiodol only control with viable culture pattern.**

D. Mini-osmotic pump releases CD/9AC with effective antitumour activity *in vitro*

Aqueous CD/9AC released from the mini-osmotic pump was also effective in causing cell death. Cells in established culture in 50 cm^2 flask were exposed at three day intervals to the same primed pump over a 10 day test period. Cultures experienced almost total cell death at two days post pump insertion. This confirmed continuous release of active drug for the duration of the test period.

E. 9AC in Ethiodol is effective in preventing tumour engraftment

Tumour engraftment, as described in section 3.12.D (i), occurred only in rats treated with Ethiodol only. Engraftment was effectively prevented at all test concentrations of 9AC in Ethiodol (see **Figure 3.7**). Statistical tests were not applied to this model.

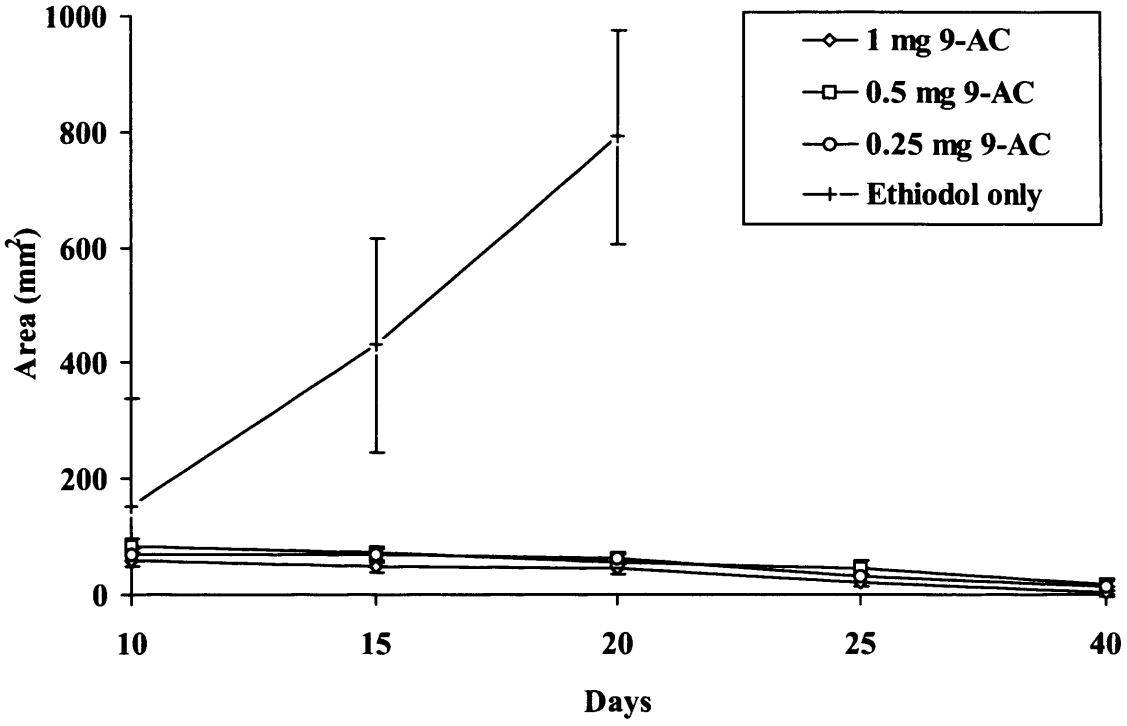


Figure 3.7 **Engraftment model in F344 rats. 9AC in Ethiodol effectively prevented engraftment in all concentrations evaluated. In contrast, the Ethiodol only control demonstrated no antitumour effect with sustained tumour growth (n=3/group; 3 experiments).**

F. HAI 9AC in Ethiodol is well tolerated in rats without tumour

Doses of 9AC of up to 1 mg admixed in 200 μL Ethiodol were well tolerated as HAI therapy in rats without tumour. At one week post treatment, rats treated with 0.25 or 0.5 mg per 200 μL Ethiodol maintained ≥ 97% weight compared with similar Ethiodol only treated animals. Comparison of liver histology for 9AC in Ethiodol and Ethiodol only groups was also within normal limits. There was no evidence of cirrhosis, or portal tract fibrosis, with viable hepatocytes with rare necrotic cells (see **Figure 3.8**).

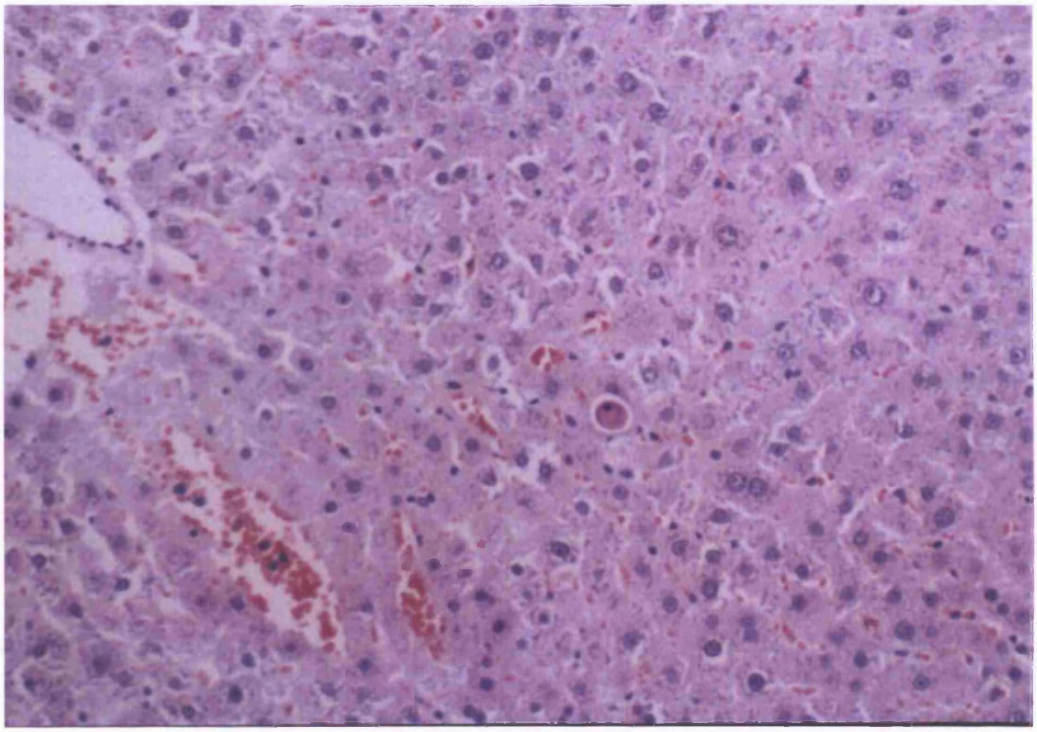


Figure 3.8 **Photomicrograph (x 40 magnification, H&E stain) of histological section from liver of a rat treated with HAI 9AC in Ethiodol, demonstrating normal architecture.**

G. Treatment of liver metastases: HAI 9AC in Ethiodol is superior to CD/9AC

There were no significant difference in the median number of metastases between groups treated with HAI CD/9AC and those treated with HAI Ethiodol only or systemic IP CD/9AC (see **Table 3.5**). There was significant reduction in the median number of metastases for the HAI 9AC in Ethiodol group compared with those treated with HAI CD/9AC, HAI Ethiodol only or systemic IP CD/9AC. Median number of metastases was also significantly reduced for the systemic IP CD/9AC group when compared to HAI Ethiodol only group (see **Figures 3.9, 3.10, 3.11, 3.12**). HAI 9AC/Ethiodol was well tolerated. In contrast, systemic continuous infusion of aqueous CD/9AC via mini-osmotic pump IP was associated with anorexia, vomiting, weight loss and ruffled fur with hair loss.

Table 3.5

Comparison of median numbers of liver metastases for rats treated with HAI 9AC/Ethiodol, HAI CD/9AC, HAI Ethiodol only or IP CD/9AC

Treatment group	Median number liver metastases (n)		
	Experiment 1	**Experiment 2**	**Experiment 3**
(a) HAI 9AC/Ethiodol	13 (5)	17.5 (4)	19 (5)
(b) HAI CD/9AC	183 (5)	194.5 (4)	189 (5)
(c) HAI Ethiodol only	215 (4)	177.5 (4)	248.5 (4)
(d) IP CD/9AC	NA	NA	138.5 (4)
HIA hRIL-2 in Ethiodol	74 (4)*	38 (5)*	21 (5)*

Wilcoxon Rank-Sum Tests:

Group (a) versus (b); $P < 0.00001$
Group (a) versus (c); $P < 0.00001$
Group (a) versus (d); $P = 0.023$

Group (b) versus (c); $P = 0.1413$
Group (b) versus (d); $P = 0.098$

Group (c) versus (d); $P = 0.0267$

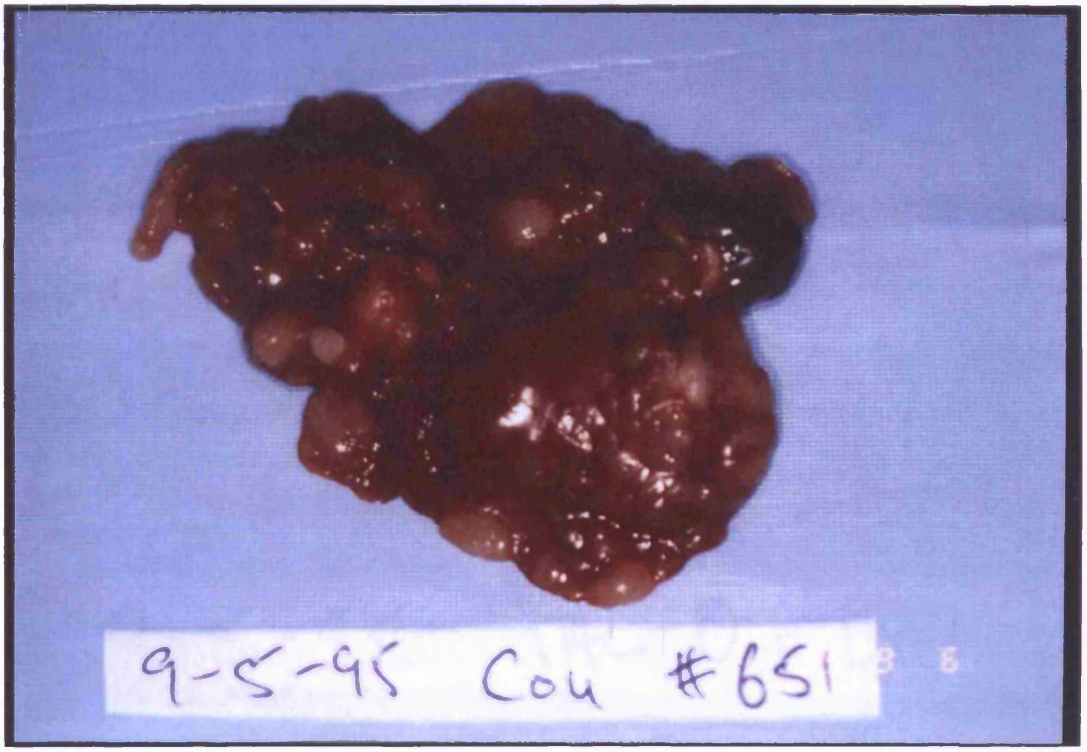


Figure 3.9 **Photograph of rat liver: HAI Ethiodol only treatment group.**

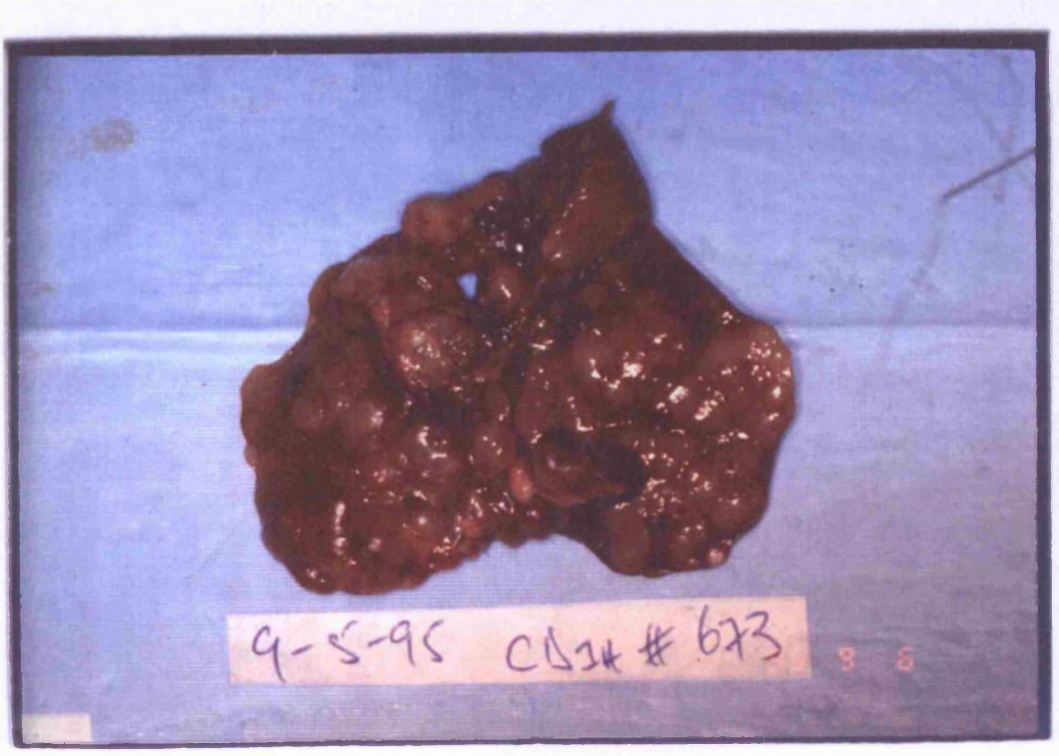


Figure 3.10 **Photograph of rat liver: HAI CD/9AC treatment group.**

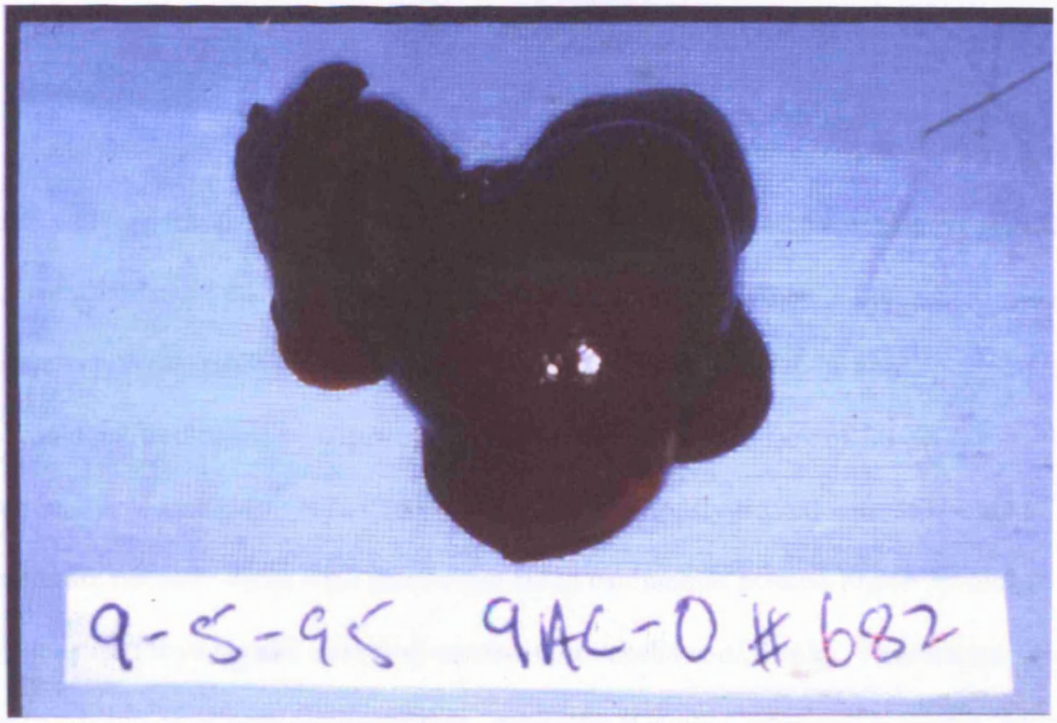


Figure 3.11 **Photograph of rat liver: HAI 9AC/Ethiodol treatment group.**

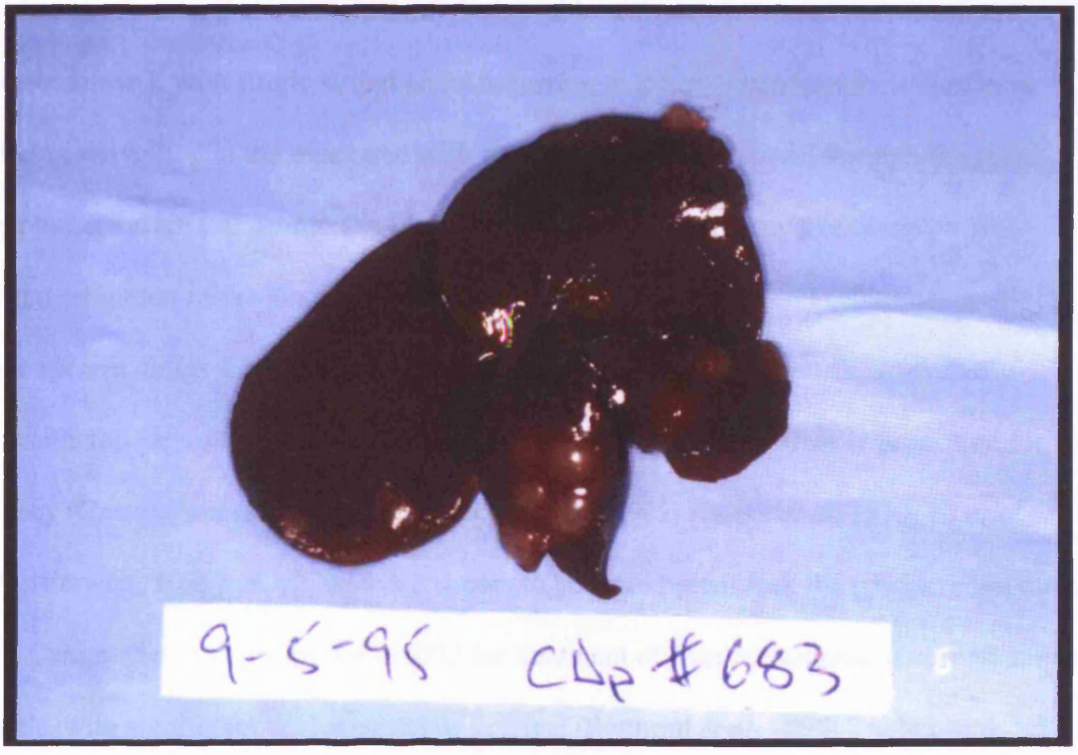


Figure 3.12 **Photograph of rat liver: IP CD/9AC treatment group.**

3.14 Discussion

Sustained-release or depot preparations allow for a change in approach to therapy for patients with hepatic-metastatic disease. This may obviate the need for expensive infusion pump with implantation. Same day surgery treatments are performed in the radiology suite with one to two day patient hospitalisation. This treatment may even be applied to patients who would not traditional be eligible for HAI therapy because of lack of fitness for operation. New agents that are effective in patients previously treated with 5FU-based regimens are needed. It has been shown that colon carcinomas possess higher levels of topoisomerase I than normal intestinal mucosa (Giovanella *et al*, 1989). This feature may be taken advantage of with topoisomerase I inhibitors (Garcia-Carbonero & Supko, 2002).

Topoisomerase I is an essential nuclear enzyme that relaxes supercoiled duplex DNA enabling replication and transcription. A cleavable complex is formed between DNA and topoisomerase I, with single strand nicks occurring in the phosphodiesterase backbone, allowing swivelling at the nicks and with passage of the intact strand through the nicks. Camptothecins are topoisomerase I inhibitors that bind to the cleavable complex and prevent religation of the single strand DNA breaks. Topoisomerase I inhibitors are S-phase specific drugs that result in inhibition of RNA synthesis. This effect is rapidly reversible following drug removal, suggesting that prolonged exposure is important for efficacy (Creemers *et al*, 1994; Horwitz & Horwitz, 1973; Kessel *et al*, 1972; Li *et al*, 1972; Verweij, 1995). 9AC has been shown to be more potent than the mother compound 20-S-Camptothecin and superior to 5FU for treatment of liver metastases in murine animal models, with significant prolongation of survival (Potmesil *et al*, 1995; Takimoto & Thomas, 2000).

These experiments demonstrate that bolus HAI CD/9AC given in identical fashion to 9AC in Ethiodol has no appreciable antitumour effect, and this may be due in part to rapid conversion to the inactive acid form of the drug at physiological pH (Ulukan & Swaan, 2002). *In vitro* studies confirmed the antitumour activity of both 9AC in Ethiodol and CD/9AC via mini-osmotic pump for periods of at least 10 days, demonstrating that both preparations delivered drug as intended. This period was equal to the planned drug exposure time *in vivo*. Titration experiments demonstrated the potency of 9AC in Ethiodol in preventing engraftment of 1 x 10^6 cells in doses as small as 250 μg, in rats observed for up to 6 months. This experiment guided the choice of dose for HAI therapy.

To advance to clinical testing, 9AC in Ethiodol would have to be more effective as locoregional treatment for hepatic metastases than comparable systemic treatment, since HAI therapy will be more complex and expensive to institute that systemic therapy. To address this problem, the mini-osmotic pump was used for systemic IP continuous infusion of the same dose of aqueous CD/9AC. The results indicate that although comparable systemic treatment results in a reduction in the number of liver metastases (median 138) when compared to Ethiodol only (median 250) treated controls, this reduction is not as great as that for 9AC in Ethiodol (median 18), which was significantly better ($P < 0.01$). Systemic therapy was also associated with substantial clinical systemic toxicity which has been a problem generally with the Camptothecins, and is one reason for the long delay in their clinical evolution (Pommier, 2006). The significant reduction in hepatic metastases in the 9AC in Ethiodol group was not associated with toxicity and the treatment was well tolerated.

These results suggest that locoregional therapy of hepatic neoplasms with sustained release 9AC in Ethiodol would be associated with clinical efficacy far exceeding that associated with its systemic administration (Dookeran *et al*, 1997).

3.2 Human recombinant interleukin-2

A. Background

Initially identified as a *T-cell growth factor*, interleukin-2 or IL2 has been shown to be a 15-kD polypeptide made up of 153 amino acids, the first 20 of which form a signal sequence that is proteolytically cleaved during secretion. Natural IL2 is glycosylated, although the attachment of sugar moieties is not essential for biologic activity. The molecule has cysteine residues at positions 58, 105, and 125, the first two of which form an intra-molecular disulfide bridge. The third cysteine is not essential for biologic activity and can be replaced with alternative amino acids to minimize polymerisation. Crystallographic analysis indicates that IL2 is a spherical molecule comprised of six α-helical regions. The biologic effects of IL2 are the result of the binding of the lymphokine to specific surface receptors. The biologic effect of IL2 most relevant to use as an antitumour agent, is the ability to enhance the cytolytic activity of antigen-specific cytotoxic T lymphocytes and natural killer (NK) cells. The cells responsible for HLA[1] unrestricted killing in response to IL2 have been termed *lymphokine-activated killer* (LAK) *cells*. LAK cells appear to be a mixture of activated NK cells and CD3+/CD8+ cytotoxic T cells, the relative contributions of which depend on the duration of culture in IL2 and

[1] The human leukocyte antigen system (HLA) is the name of the human major histocompatibility complex (MHC), and is located on chromosome six. The MHC is a region of highly conserved polymorphic genes, and the products of these genes are on the cell surface of a wide array of cell types. MHC genes play a pivotal role in immune response, and antigen specific T lymphocytes only recognize antigens as small pepetides bound to MHC molecules. There are two types of cell surface MHC molecules, class I and II. Lymphocytes are usually restricted to one of these two classes, and antigens associated with class I are recognized by CD8+ T cells, whereas antigens associated with class II are recognized by CD4+ T cells [Restifo NP, Wunderlich JR (2005) Cancer immunology. In *Cancer: principles and practice of oncology. Philadelphia, PA: Lippincott Williams & Wilkins*, DeVita VT, Hellman S, Rosenberg SA (eds), 7 edn, pp 139–161.].

whether human peripheral blood lymphocytes or murine spleen suspensions are used as an LAK cell source (Lotze *et al*, 1981b; Mier & Atkins, 2005; Taniguchi & Minami, 1993).

Human recombinant interleukin-2 (hRIL2) has had modest success as treatment for advanced melanoma and renal cell carcinoma. However, there has been more limited success as treatment for other types of advanced cancer. The usefulness of high-dose IL2 therapy has been limited by toxicity, many features of which resemble bacterial sepsis. Side effects are dose dependent and largely predictable, and rapidly reversible. Common side effects include fever, chills, lethargy, diarrhoea, nausea, anaemia, thrombocytopaenia, eosinophilia, diffuse erythroderma, hepatic dysfunction, and confusion. Myocarditis also occurs in approximately 5% of patients. IL2 therapy also commonly produces a "capillary leak syndrome," leading to fluid retention, hypotension, early adult respiratory distress syndrome, prerenal azotemia, and occasionally myocardial infarction. As a consequence of these side effects, few patients are able to receive all of the proposed therapy. IL2 has also been shown to produce a neutrophil chemotactic defect that predisposes patients to infection with gram-positive and occasionally gram-negative bacteria. The considerable toxicity of the high-dose IL2 regimens has continued to limit application to highly selected patients with excellent performance status and adequate organ function treated at medical centres with considerable experience with this approach. A myriad of strategies to overcome toxicity and ineffectiveness have been evaluated. Examples include the concomitant administration of activated lymphoid cells, co-administration of other cytokines, novel schedules of infusion, and co-administration of chemotherapy or radiation therapy. These approaches have been associated with only a modicum of success (Mier & Atkins, 2005).

Long-term follow-up data for patients with melanoma and renal cell cancer treated with high-dose bolus IL2 have confirmed the earlier findings of response durability, with median duration for complete responses yet to be reached and few relapses observed in patients free of disease for longer than 30 months. In addition, several patients have remained free of disease in excess of ten years since initiating treatment. These data suggest that high-dose IL2 treatment may actually have led to the cure of some patients with these advanced malignancies previously considered incurable (Atkins *et al*, 2000; Fisher *et al*, 2000). Furthermore, results from a randomised trial of high-dose versus low-dose IL2 for treatment of patients with metastatic renal cell cancer suggested that IL2 was more clinically active at maximal doses (Yang *et al*, 2003).

B. Rationale for selection

Therefore, insufficiently high concentrations of IL2 within tumours may in part underlie this apparent resistance to therapy. The generation of higher concentrations by intravenous administration is proscribed by its short half life and the multi-organ toxicity that limits its dose intensification, in addition to previously described factors. Tumour-targeted therapy with a depot or sustained-release preparation of hRIL2 represents a rational strategy for overcoming this problem (Rubin & Dookeran, 1997; Rubin *et al*, 1995b).

3.21 Aims

The aims of this study were to evaluate the solubility of hRIL2 in Ethiodol, the kinetics of its release into aqueous medium, and its subsequent biological activity, and effectiveness as treatment for isolated liver metastases in an experimental rat tumour model.

3.22 Methods

A. Cytokine assays

hRIL2 [Chiron Corporation, Emeryville, CA], was reconstituted by adding 3.6 mL of Ethiodol or 3.6 mL of RPMI1640, to each vial containing 1.2 mg (1.8 X 10^7 IU). In order to determine whether hRIL2 dissolved in Ethiodol was free to diffuse into an aqueous medium, 1 mL of Ethiodol containing a known amount of hRIL2 was overlaid[1] with one mL of RPMI1640 in cryogenic vials [Corning Glassware, Corning, NY]. The medium was sampled sequentially from different vials that had been left to incubate at 37°C for varying periods of time. In other assays of hRIL2 release from Ethiodol, the overlying aqueous medium was repetitively sampled from the same vial at different points in the time and the aspirated volume (0.5 mL) was replaced each time with fresh medium.

These supernatants were assayed for hRIL2 by phytohaemagglutinin (PHA) blast assay. Briefly, peripheral blood mononuclear cells (PBMC) were cultured at 5 x 10^5 cells/mL in AIM V [GIBCO] containing 10 μg/mL PHA [Sigma Labs, St. Louis, MO] for 3 days after which the cells were harvested, washed, and incubated with serial dilutions of supernatant or known concentrations of hRIL2 for 48 hours in 96 well plates, 5 x 10^5 cells/mL, 100 μL/well. The cells were pulsed with 1 μCi of [^{3}H]thymidine [NEN/Dupont, Boston, MA] per well and were harvested 18 hours later with a Skatron Titertek System [Skatron AS, Lierbyen, Norway]. [^{3}H]thymidine incorporation was determined with a liquid scintillation counter [Pharmacia, Gaitherburg, MD] and was used as an index of

[1] Since Ethiodol and water are immiscible, it is possible to develop two distinct layers when both reagents are placed in the same container. Since Ethidol is oil, and is denser than water, it will naturally occupy the lower part of the container, when placed together. If Ethiodol is placed first into a container, then the aqueous layer can be gently laid over the oil (i.e. overlaid) without disturbing it.

DNA synthesis. Results were expressed as mean counts per minute per culture. Counts per minute generated by serial dilutions of experimental samples were compared to a standard curve to quantitate the amount of hRIL2 (Lotze *et al*, 1981a).

B. Tumour cell lines

B16, a spontaneously occurring murine melanoma, and MC38, a colon adenocarcinoma, are both tumour cell lines syngeneic to C57BL/6 mice. B16 is of interest since it is commonly used with success in immunotherapy experiments involving IL2 in murine models of liver metastases (Lafreniere & Rosenberg, 1986). MADB106 is a breast adenocarcinoma cell line syngeneic to F344 rats (see above, section 3.12 B). YAC1 [ATTC, Rockville, MD] is a MHC-negative, NK cell sensitive, murine cell line. These cell lines were cultured in complete medium consisting of RPMI-1640, 100μg/mL streptomycin, 100 U/mL penicillin, 3% glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 5 x 10^{-5} M 2-mercaptoethanol and 5% foetal calf serum prior to use in these studies. The lymphokine-activated killer (LAK) cell-sensitive target Daudi and NK cell-sensitive target K562, used in cytotoxicity assays, were similarly cultured in complete medium.

C. LAK cell generation

PBMC from normal human donors were isolated on Ficoll-Hypaque gradients, as described previously (Strausser & Rosenberg, 1978). They were cultured in known concentrations of hRIL2 at 37°C with 2 x10^6 cells/mL of complete medium for four days. Cell culture was performed in six well plates using cell-culture inserts so that cells did not come into direct contact with Ethiodol. The cells were then harvested and tested in four

hour sodium ^{51}Cr-chromate (or ^{51}Cr)[1] release assays against the Daudi cell line (Lotze *et al*, 1981a; Miller & Dunkley, 1974; Strausser & Rosenberg, 1978).

D. Cytotoxicity assays

Cell targets were labelled for 60 minutes in 0.1 mL of complete medium with 100 μCi Na^{51}Cr [NEN/Dupont]. Cells were washed three times and added at 5 x 10^3 cells/well to various numbers of effectors in round-bottomed microtiter plates [Becton Dickinson]. Supernatants were manually harvested and counted using a γ-counter [Pharmacia]. Maximum release was produced by incubation of targets with 0.01% Triton-X [Sigma Labs]. Spontaneous release was produced by incubation of targets with medium alone. All determinations were made in triplicate. The percent specific lysis was calculated by the formula (experimental cpm – spontaneous cpm x 100%) / (maximum cpm – spontaneous cpm). A lytic unit can be defined as the number of effector cells capable of causing the death of a fixed percentage of radiolabeled target cells. This effector cell number is expressed as lytic units per fixed number of PBMC, so that the lytic unit value increases with increased effector cell cytotoxic activity. Lytic units[2] in this study were

[1] ^{51}Cr is a chromium radioisotope with a half-life of nearly 27 days, and is used in the ^{51}Cr release micro-cytotoxicity assay. This assay determines the *in vitro* lytic activity of effector cells by measuring the amount of radioactive chromium released.

[2] 51Chromium release-derived cytotoxicity data yield curvilinear plots when the x axis displays the effector/target ratio and the y axis displays the percentage of cytotoxicity. To facilitate data analysis, several biomathematical models (simple linear regression, exponential fit, and Von Krogh) have been used to express these cytotoxicity curves as a single numerical value, termed the lytic unit. Other than using raw cytotoxicity data, the lytic unit has been the most common method of data presentation in human and animal tumor immune studies involving natural killer cells, lymphokine-activated killer cells, and cytotoxic T cells. Unfortunately, the models for determining lytic unit values incorporate assumptions and methods of calculation that can result in inaccurate model-predicted cytotoxicity in comparison with the actual observed cytotoxicity data. Even when the model is accurate in predicting cytotoxicity values (i.e., the nonlinear regression-calculated three-parameter Von Krogh model), comparisons between donors of minimally different or highly different cytotoxicity are still fraught with potential error due to statistically verifiable

defined as the number of cells/10^6 mononuclear cells capable of causing 20% lysis of 5 x 10^3 targets (Bryant *et al*, 1992; Pollock *et al*, 1990).

E. Animal model of tumour engraftment

C57BL/6 mice, F344 rats, and athymic (nu/nu) mice were housed and fed in accordance with University of Pittsburgh research guidelines. Mice used in these experiments were approximately twelve weeks of age and rats weighed approximately 350 grams. Tumour cell injections were carried out using freshly prepared suspensions in a total volume of 200 µL of Ethiodol containing hRIL2. All injections were performed subcutaneously in the lower abdomen. Tumour engraftment was assessed by measuring tumour area using callipers. Animals were sacrificed when tumours became ulcerated or exceeded 2 cm in diameter. Control animals were inoculated with tumour in either Ethiodol, RPMI1640, aqueous hRIL2, incomplete Freund's adjuvant[1], or polyethylene glycol (PEG)/IL2 [Chiron Corp.]. PEG/IL2 was made with hRIL2 linked to a 6 to 7-kD esterified PEG moiety. It has a MW of 160kD, is water soluble, and is heavily hydrated (Katre *et al*, 1987).

F. Immunohistochemistry

In some experiments of tumour engraftment, tumours or inoculation sites were excised, snap frozen in OCT Compound[2] [Tissue Tek, Elkhart, IN], and stored at -70°C for

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violations of assumptions of parallelism [Pollock RE, Zimmerman SO, Fuchshuber P, Lotzova E (1990) Lytic units reconsidered: pitfalls in calculation and usage. *J Clin Lab Anal* **4:** 274-82].

[1] A water-in-oil emulsion that stimulates the T-cell immune response to antigens and may be used in various types of cancer vaccines.

[2] The optimum cutting temperature (or O.C.T.) formulation of water-soluble glycols and resins provides a convenient specimen matrix for cryostat sectioning at temperatures of -10°C and below. It leaves no residue on slides during staining procedure, eliminating undesirable background staining.

immunohistochemical evaluation. Tissue sections (8μm) were prepared on glass slides and stored over night at 5°C. Specimens were evaluated with a 3-step avidin-biotin immunoperoxidase technique, as previously described (Rubin *et al*, 1989). Briefly, tissue sections, fixed in acetone, were sequentially incubated at room temperature with a primary mouse anti-rat monoclonal antibody for one hour, biotinylated goat anti-mouse antibody [TAGO Inc. Labs, Burlingame, CA] and an avidin-biotin-peroxidase complex [Vector Labs, Burlingame, CA] each for 30 minutes. Slides were washed with 0.05 M tris buffered saline after each incubation. Goat serum [GIBCO] was used to block non-specific staining. Tissue sections were stained with 3,3'-diaminobenzidine [Sigma Labs] and hydrogen peroxide, and counter stained with Mayer's haematoxylin. The primary antibodies used in this study included a mouse anti-rat macrophage marker anti-CD3, anti-CD4, and anti CD8 [Pharmingen, San Diego, CA] diluted 1:10 with distilled water.

G. *In vivo* cell depletion

These experiments were performed in order to dissect the immunological mechanism responsible (i.e. explore whether NK cells or macrophages were involved) for observed hRIL2 mediated inhibition of tumour engraftment. Anti-asialo GM1 has been shown to eliminate NK activity *in vitro* (Habu *et al*, 1981). Mice were treated with intraperitoneal injections of anti-asialo GM1 [Wako Bioproducts, Richmond, VA] reconstituted with RPMI1640. Each mouse received 20 μL every five days for three weeks. Rabbit serum [Wako Bioproducts] was used as a control. The efficacy of NK-cell depletion was monitored by evaluating splenocytes for lytic activity against YAC1 cells, 18 hours after treating mice with 100 μg of polyinosinic: polycytidylic acid [Sigma Labs]. Treatment with anti-asialo GM1 was associated with suppression of NK-cell activity as measured in this assay. In order to inhibit macrophage activity, mice were treated with

carrageenan [Sigma Labs], 0.5 mL (5 mg/mL) intraperitoneally (Bartocci *et al*, 1987), beginning one day prior to tumour injection and then every other day afterwards for 2 weeks.

H. Animal model of hepatic metastases

The model of hepatic metastases has been described in detail (see above, section 3.12 D). Briefly, F344 rats anesthetised with methoxyflurane underwent laparotomy and intraportal injection of 2 x 10^5 MADB106 tumour cells suspended in 200 μL of RPMI1640. Six days later, they underwent repeat laparotomy and gastroduodenal arterial injection of Ethiodol, 0.1 mL, containing 6 x 10^5 IU of hRIL2 administered through 30 gauge PE-10 Intramedic polyethylene tubing. Proximal control of the common hepatic artery and distal control of the gastroduodenal artery prevented retrograde flow of Ethiodol. Following the infusion, the gastroduodenal artery was ligated. Control rats were injected intra-arterially with either 0.1 mL of Ethiodol, or 6 x 10^5 IU of aqueous hRIL2. Rats were sacrificed 10 to 12 days after therapy and their livers were harvested. The number of metastases on the liver surface was then enumerated as previously outlined. The number of metastases in each group was compared using the non-parametric Wilcoxon Rank-Sum Test as previously described.

3.23 Results

A. hRIL2 is soluble in Ethiodol

It was demonstrated that hRIL2 dissolves in Ethiodol. This was facilitated by passing the mixture through increasingly smaller needles, which helped to dissolve small particles of hRIL2. Concentrations of 0.4 mg/mL (6 X 10^6 IU/mL) can be obtained in this way.

B. Ethiodol serves as a sustained-release preparation for hRIL2

The suitability of Ethiodol as a vehicle to deliver hRIL2 to sites of tumour depends not only on its drug solubility, but also on release of active hRIL2. The rate at which cytokines dissolved in Ethiodol will diffuse into an aqueous solution depends upon many variables including temperature, surface area surrounding the oil-water interface, and the relative avidity of the particular cytokine for oil or water. The diffusion kinetics of hRIL2 from Ethiodol was evaluated by first overlaying the oil with aqueous medium. The concentration of hRIL2 in the aqueous supernatant was noted to equilibrate with that of Ethiodol over a period of four days. The equilibrium strongly favoured the oil phase by a factor of 10,000. This was consistent with the hydrophobic nature of hRIL2. The rate at which hRIL2 would be liberated from Ethiodol *in vivo* depends not only on the aforementioned variables, but on the rate of diffusion through interstitial fluid surrounding the oil. This would be determined in part by tumour blood supply and interstitial fluid pressure within the tumour. In order to more accurately reflect the kinetics of cytokine release in a dynamic tumour microenvironment, the aqueous supernatant overlying cytokine-containing oil was serially sampled and replenished with medium. Despite the repetitive loss of hRIL2 from the aqueous phase by 50%, its concentration was maintained by diffusion from Ethiodol (see **Figure 3.13**). These results indicate that Ethiodol serves as a sustained-release preparation for hRIL2.

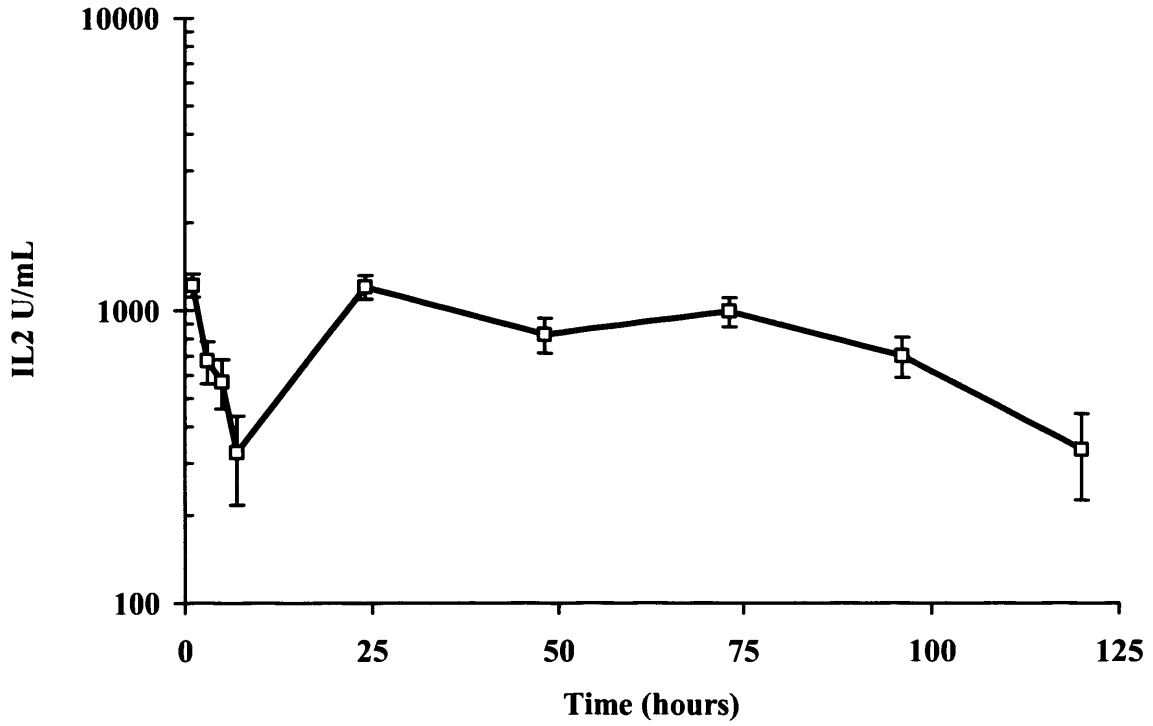

Figure 3.13 **Sustained-release of hRIL2 from Ethiodol *in vitro*. hRIL2 in Ethiodol placed in wells and overlaid with plasma. Plasma sampled at given time points, and replaced with equal volume plasma. hRIL2 concentration determined with PHA blast assay, and compared to standard curves for known concentrations hRIL2 (n=3/group; 3 experiments).**

C. hRIL2 release from Ethiodol retains biological activity

It is conceivable that admixture of cytokines with iodinated oil may produce physical and chemical interactions, which may alters the cytokines and result in modification of biological activity. We compared the LAK activity generated in four-day cultures of PBMC with hRIL2, against that generated by hRIL2 delivered in Ethiodol. Ethiodol did not appear to have a significant adverse effect on the immunomodulatory activity of hRIL2 although the lytic activity generated by hRIL2 was consistently lower when delivered in Ethiodol. Underlying this difference is the observation that Ethiodol-bound hRIL2 diffused into aqueous medium over a period of several days (see **Figure 3.13** above), which implies that the hRIL2 equilibrium favoured Ethiodol. Consequently, PBMC

cultured with hRIL2 delivered in Ethiodol were probably exposed to lower concentrations of this cytokine (see **Figures 3.14 and 3.15**).

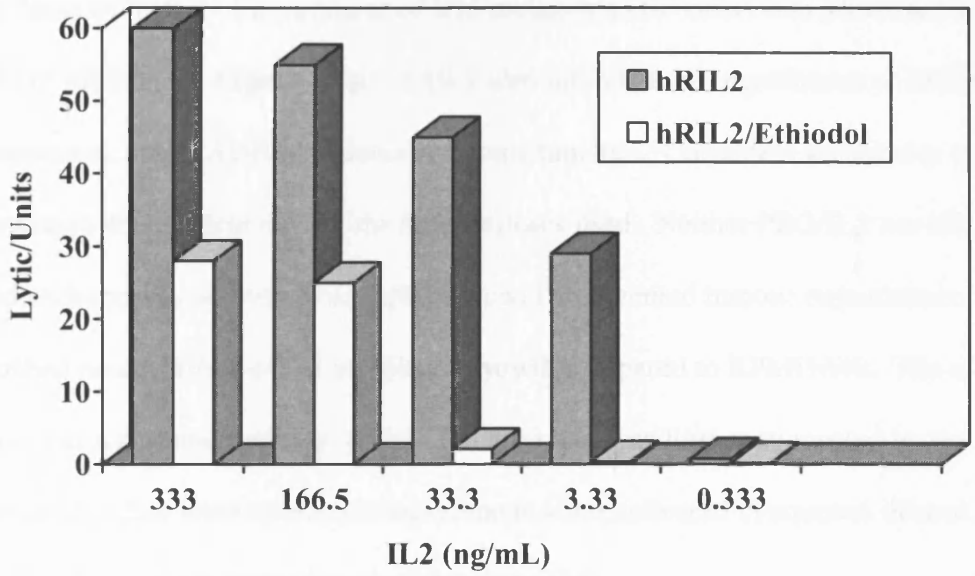

Figure 3.14 **Activity of hRIL2 released from Ethiodol (I). PBMC were cultured with hRIL2 or hRIL2 in Ethiodol for four days and tested against Daudi cells for cytolytic activity. One lytic unit was based upon 20% lysis per 10^6 cells (n=3/group; 3 experiments).**

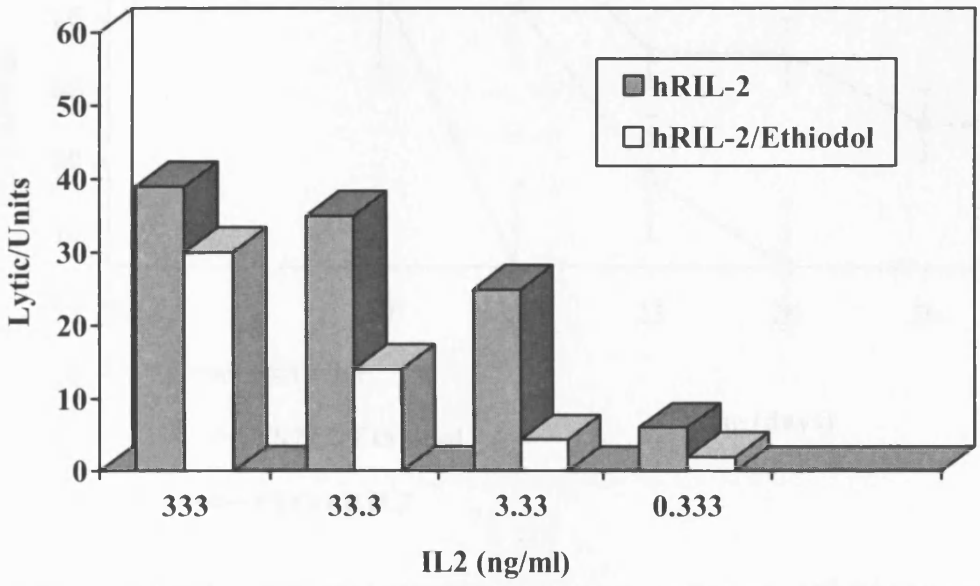

Figure 3.15 **Activity of hRIL2 released from Ethiodol (II). PBMC were cultured with hRIL2 or hRIL2 in Ethiodol for four days and tested against K562 cells for cytolytic activity. One lytic unit was based upon 20% lysis per 10^6 cells (n=3/group; 3 experiments).**

D. Tumour admixture with hRIL2 and Ethiodol inhibits engraftment

C57BL/6 mice were inoculated with tumour admixed with 0.2 mL of Ethiodol and varying doses of hRIL2. Engraftment of B16 melanoma (10^5 cells) was prevented by 1.2 X 10^6 IU of hRIL2 (see **Figure 3.16**). hRIL2 also inhibited the engraftment of MC38 adenocarcinoma and MADB106 adenocarcinoma tumours. Consistent antitumour activity in these assays was evident only at the highest doses used. Neither PEG/IL2 nor hRIL2 admixed with the oily adjuvant incomplete Freund's prevented tumour engraftment. Ethiodol had no consistent effect on tumour growth compared to RPMI1640. The efficacy of Ethiodol as a sustained-release vehicle for cytokines was further suggested by the inability of hRIL2 to prevent tumour engraftment when delivered in aqueous diluent. Statistical tests were not applied to engraftment models.

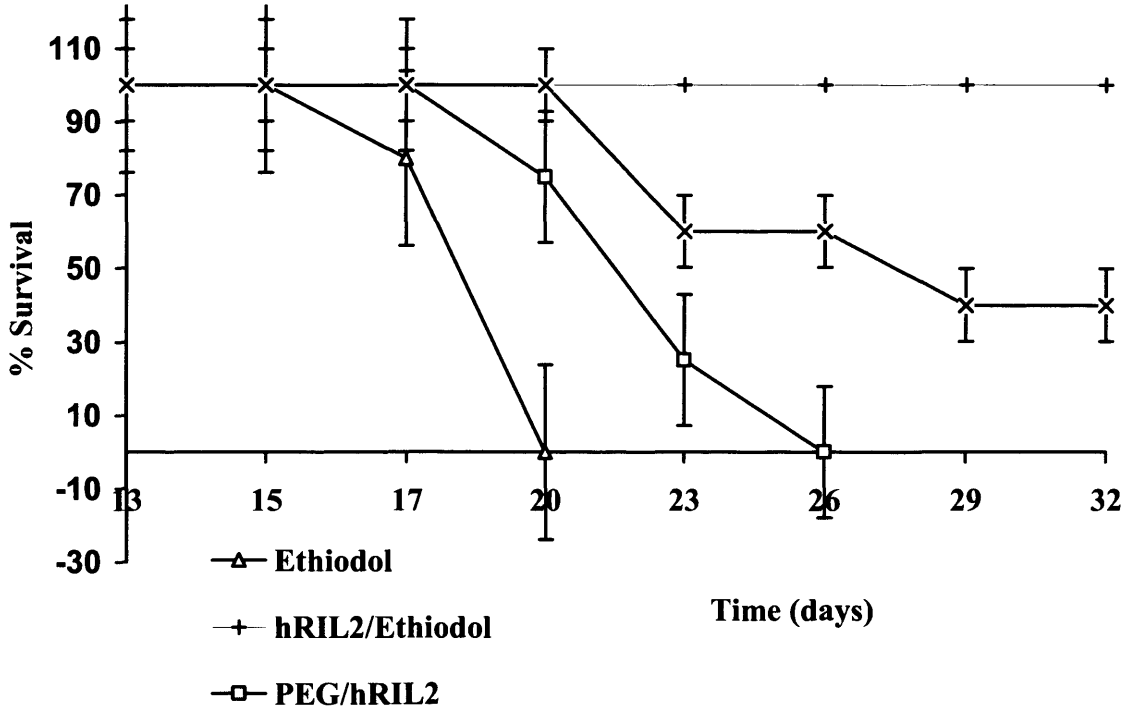

Figure 3.16 **hRIL2 in Ethiodol prevents tumour engraftment. 10^5 B16 tumour cells were injected subcutaneously into the flanks of C57/BL6 mice admixed with Ethiodol only, hRIL2 in Ethiodol, PEG/hRIL2, or Incomplete Freund's. Test groups administered the same dose hRIL2 (1.2 x 10^6 IU) in same volume (200 μl) (n=3/group; 3 experiments).**

E. Macrophages mediate the antitumour effects of hRIL2 in Ethiodol

In order to dissect the immunological mechanism responsible for hRIL-2 mediated inhibition of tumour establishment, mice were depleted of NK and LAK cell precursors or depleted of macrophages prior to inoculation with 10^5 B16 melanoma cells subcutaneously with 1.2 X 10^6 IU hRIL2 in 0.2 mL of Ethiodol. Tumour outgrowth was inhibited by hRIL2 in Ethiodol in mice treated with antibody to asialo GM1. Splenocytes from these mice had no activity against YAC1 in four hour cytotoxicity assays. Tumour engraftment was inhibited, as well, in nu/nu mice. However, treatment of mice with carrageenan abrogated the antitumour activity of hRIL-2 in Ethiodol (see **Figure 3.17**). Immunohistochemical evaluation of resected tumours was consistent with these observations. Inoculation sites were infiltrated by significant numbers of macrophages. A far lesser degree of infiltration was observed at sites of Ethiodol or hRIL2 injection. T cells were conspicuously absent from these sites. There was no inflammatory infiltrate at sites of tumour inoculated with RPMI1640.

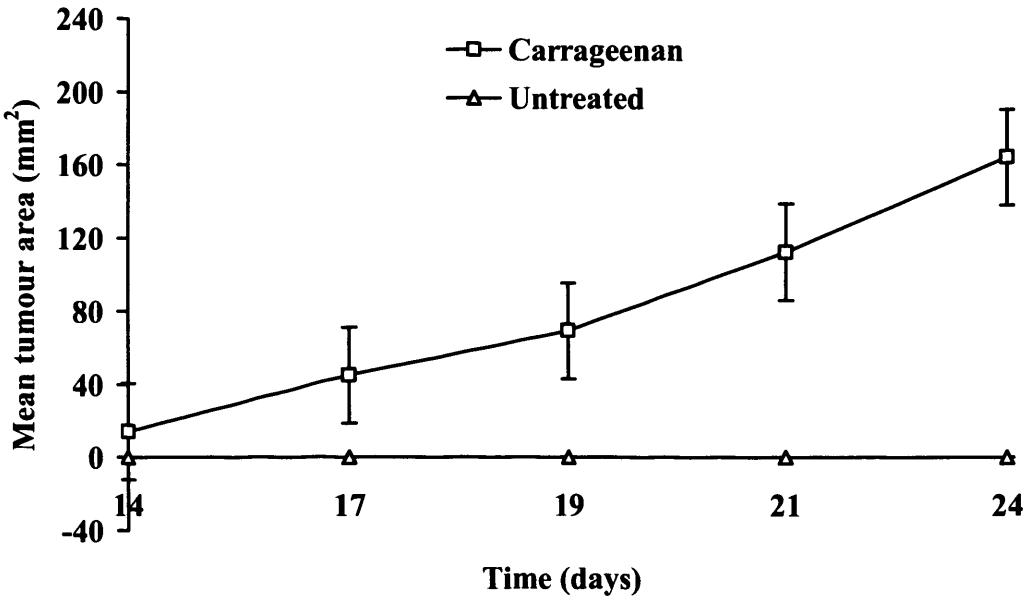

Figure 3.17 **Carrageenan facilitates engraftment of B16 tumour and abrogates the antitumour effect of hRIL2 in Ethiodol, suggesting that macrophages are involved in the process (n=3/group; 3 experiments).**

F. HAI hRIL2 in Ethiodol is superior to Ethiodol or hRIL2

Rats bearing hepatic-metastatic adenocarcinoma were treated with HAI therapy of Ethiodol, hRIL2, and hRIL2 in Ethiodol according to the methods outlined above. Rats treated with gastroduodenal artery ligation after infusion of aqueous hRIL2 or Ethiodol alone consistently developed large numbers of sizable hepatic metastases by the time they were sacrificed ten days after therapy. These control animals usually manifested hair loss, ruffled fur, abdominal distension, jaundice, or lethargy. In contrast, rats treated with hRIL2 in Ethiodol remained well throughout the period of observation. Evaluation of livers ten days after therapy revealed marked differences in treatment groups. Livers of rats treated with aqueous hRIL2 or Ethiodol alone had more numerous metastases on initial evaluation at first laparotomy throughout all lobes, compared to rats treated with hRIL2 in Ethiodol. The number of liver metastases was significantly reduced among animals treated with HIA hRIL2 in Ethiodol, compared to those treated with Ethiodol alone or aqueous hRIL2 alone (see **Table 3.6; Figures 3.18, 3.19, 3.20**).

Table 3.6

Comparison of median numbers of liver metastases for rats treated with HAI hRIL2/Ethiodol, HAI Ethiodol only or HAI hRIL2 only

Treatment	Median Number liver metastases (n)		
	Experiment 1	Experiment 2	Experiment 3
(a) HIA hRIL2 in Ethiodol	83 (4)	24 (5)	18 (5)
(b) HIA Ethiodol only	250 (4)	250 (4)	177.5 (4)
(c) HIA hRIL2 only	NA	86 (4)	111.5 (4)
Wilcoxon Rank-Sum Tests: Group (a) versus (b); $P < 0.00001$ Group (a) versus (c); $P = 0.0057$ Group (b) versus (c); $P = 0.0225$			

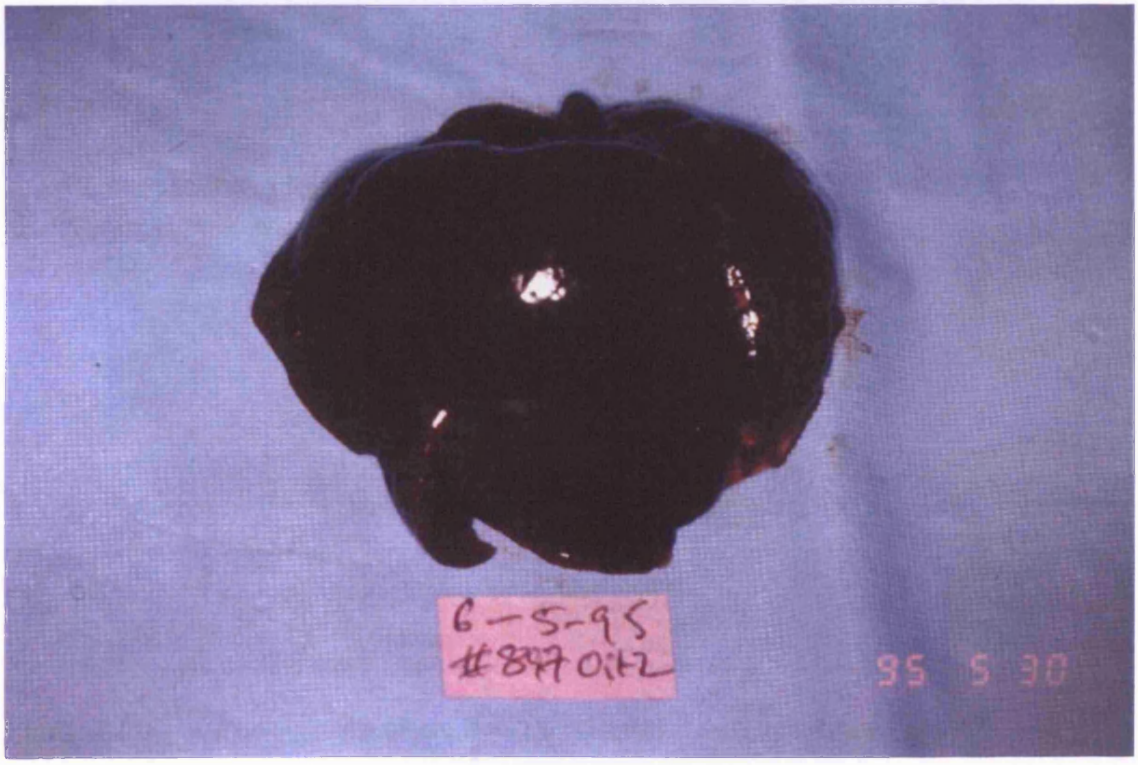

Figure 3.18 **Photograph of rat liver: HAI hRIL2 in Ethiodol treatment group.**

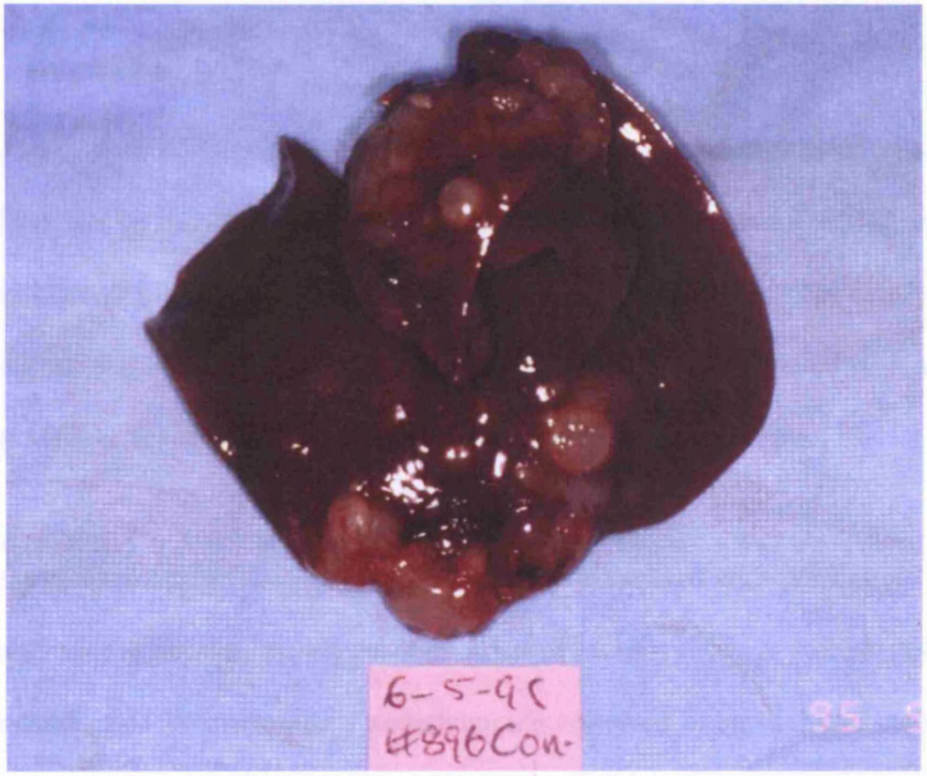

Figure 3.19 **Photograph of rat liver: HAI Ethiodol only treatment group.**

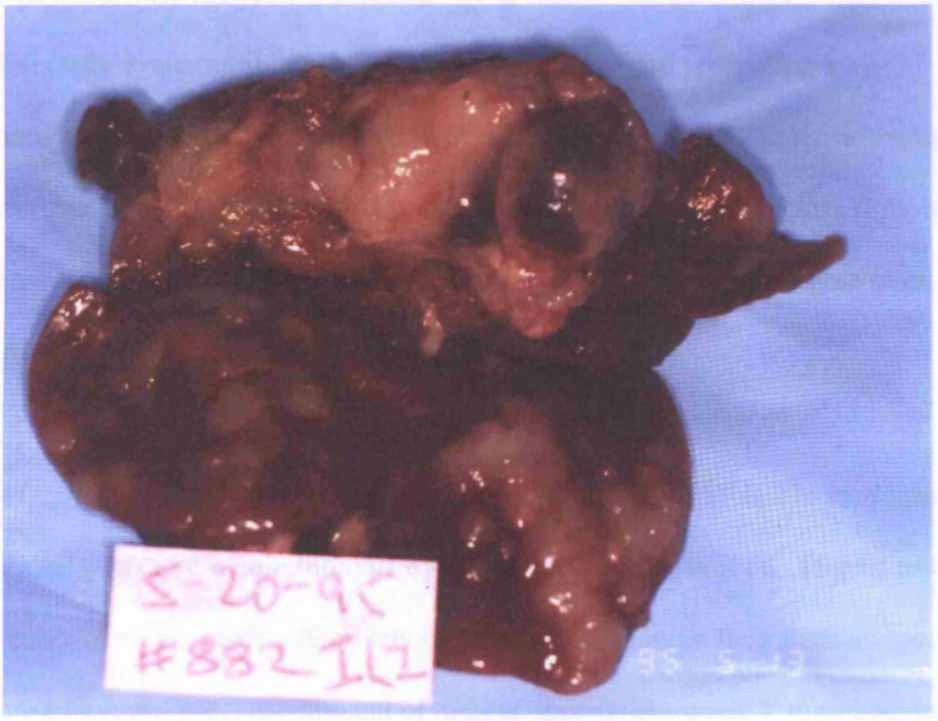

Figure 3.20 **Photograph of rat liver: HAI hRIL2 only treatment group.**

3.24 Discussion

This study demonstrates that Ethiodol can be used as a sustained-release vehicle for biologically active hRIL2. When delivered subcutaneously with Ethiodol, hRIL2 inhibits tumour engraftment in a dose-dependant manner. Macrophages appear to be central to the observed antitumour effect. HAI of Ethiodol containing hRIL2 is associated with marked antitumour activity against established hepatic-metastatic adenocarcinoma.

The systematic administration of hRIL2 has been associated with clinically significant tumour regression in only small numbers of patients (Mier & Atkins, 2005). Our understanding of the mechanisms underlying this observed insensitivity of several tumours to hRIL2 therapy remains unclear. There are probably many ways that tumours escape immune recognition. One hypothesis implicates an inadequate helper T-cell response resulting from ineffective presentation of tumour antigen. hRIL2 may overcome immune tolerance to susceptible tumours by enhancing antigen processing and presentation, possibly through the induction of other cytokines such as interferon-alpha or tumour necrosis factor-alpha. The observed association between MHC class II molecules and response to therapy with hRIL2 is concordant with this hypothesis (Rubin *et al*, 1989; Rubin *et al*, 1995b).

Another paradigm attributes the limited clinical efficacy of hRIL2 to inadequate concentrations generated within tumours by its systemic administration. Higher intra-tumoural concentrations might effectively bypass the requirement for a tumour-specific Th1 response through direct recruitment of tumour-specific cytotoxic T lymphocytes to sites of tumour. This is corroborated by the clear dose-response relationship for cytokines demonstrated in certain murine tumour models and evidence from clinical trials

(Rosenberg *et al*, 1985; Tepper *et al*, 1989; Yang *et al*, 2003). The use of higher systemic doses of hRIL2 as a strategy to overcome this in humans is proscribed by systemic toxicity.

Locoregional administration of cytokines has been suggested as an alternative approach for generating higher intra-tumoural concentrations without associated toxicity. Experience with this technique has been limited. Intra-lesional injection of hRIL2 is associated with regression of superficial bladder tumours (Pizza *et al*, 1984). Local and systemic immune activation has been observed after peri-tumoural injection of hRIL-2 in patients with advanced head and neck cancer (Whiteside *et al*, 1993).

HAI administration of hRIL2, although not extensively studied, has been associated with regression of metastatic leimyosarcoma, ocular melanoma, and primary hepatocellular carcinoma. The clinical applicability of these infusional approaches, however, is limited by the need for operative placement of vascular catheters and cumbersome infusions for variable periods of time. In addition, the regional drug advantage over systemic infusion is limited by unfavourable first pass pharmacokinetics (Mavligit *et al*, 1990; Yamamoto *et al*, 1993).

The results of this study suggests that Ethiodol may serve as a sustained-release vehicle for delivering hRIL2, as well as other cytokines, selectively to tumours by several possible routes. The demonstrated ability of Ethiodol to target tumours after HAI, may obviate the need for indwelling arterial catheters. The sustained-release of hRIL2 from Ethiodol within tumours would generate high concentrations locally, without concomitant systemic toxicity. This would exceed the pharmakokinetic advantage of regional

perfusion. Repeated doses could easily be administered through temporary arteriography catheters positioned transfemorally.

The ability of Ethiodol to deliver and sustain high concentrations of hRIL2 at sites of subcutaneous inoculation also suggests an alternative to cytokine-gene modified tumour vaccines. Ethiodol based delivery of cytokines may be equivalent to cytokine-gene transduction of tumours and may actually obviate the need for costly and time consuming cell culture and gene transduction prior to inoculation (Iwazawa *et al*, 2000). Another potential advantage of cytokine delivery via Ethiodol as a base or depot reagent would be the consistency with which large amounts of a selected cytokine can be delivered, unencumbered by the vicissitudes of gene transduction. The apparent superiority of Ethiodol over PEG or incomplete Freund's adjuvant in our models of tumour engraftment may be explained in several ways. PEG/IL2 and hRIL2 alone are water soluble preparations, and probably diffuse rapidly from the site of inoculation. However, Ethiodol remains at the site of subcutaneous inoculation, as suggested by the persistence of a palpable bleb at that site. This enables it to serve more effectively as a depot preparation for cytokines. It is possible that both Ethiodol and hRIL2 recruit macrophages or dendritic cells, which may lead to enhanced presentation of tumour antigens. The observation that carrageenan abrogates the antitumour effects of hRIL2 in this engraftment model suggest that these cells play a major role in the antitumour effects observed.

3.3 Nitric oxide donors

A. Background

Nitric oxide (NO) is a multifunctional gaseous molecule and a highly reactive free radical. It is synthesized from L-arginine, NADPH[1] and oxygen by NO synthase (NOS). As a signaling molecule, NO regulates various physiological and pathophysiological processes including vascular functions (angiogenesis, blood flow, vascular permeability, leucocyte–endothelial interaction, platelet aggregation and microlymphatic flow), neurological functions (neurotransmission and development of the nervous system) and, at relatively high concentration, cytotoxic functions (cytostasis and cytolysis). Importantly, various studies have shown that NO can both promote and inhibit tumour progression and metastasis. The effects of NO in tumours seem to depend on the activity and localization of NOS isoforms, concentration and duration of NO exposure, and cellular sensitivity to NO (Bonavida *et al*, 2006; Fukumura *et al*, 2006; Xu *et al*, 2002b).

The role of NO in tumour transformation and progression is complex. Nitric oxide synthases (NOSs) are ubiquitously expressed in malignant tumours. NO regulates several physiological processes through the soluble-guanylyl-cyclase–cGMP pathway and S-nitrosylation, and has cytotoxic and genotoxic effects at high concentrations. Tumour-cell-derived NO promotes tumour progression by induction of tumour-cell invasion, proliferation and the expression of angiogenic factors. The inducible isoform of NOS (iNOS), which produces high concentrations of NO, mediates neoplastic transformation in oncogene and chemical induced tumourigenesis models, although conflicting results are reported in the literature. Conversely, the transfection of iNOS-expressing constructs into NO-sensitive tumour cells inhibits tumour growth and metastasis. Host stromal-cell-

[1] Nicotinamide adenine dinucleotide phosphate, reduced form.

derived NO, which is synthesized by iNOS, inhibits growth of NO-sensitive tumours but promotes growth of NO-resistant tumours (Ekmekcioglu *et al*, 2005; Friebe & Koesling, 2003; Lala & Chakraborty, 2001; Stamler *et al*, 2001).

NO that is predominantly synthesized by endothelial NOS (eNOS) in vascular endothelial cells promotes angiogenesis directly and functions both upstream and downstream of angiogenic stimuli. In addition, NO mediates recruitment of perivascular cells and, therefore, remodeling and maturation of blood vessels. NO that is synthesized by eNOS promotes tumour progression through the maintenance of blood flow, induction of vascular hyperpermeability and reduction of leucocyte–endothelial interactions. Induction of NO signaling can induce direct tumour-cell cytotoxicity or sensitise tumour cells to other treatments such as radiation. Conversely, blockade of NO signaling can inhibit neoplastic transformation, tumour angiogenesis and blood flow. Expression, activity and localisation of NOS isoforms, concentration and duration of NO exposure, and cellular sensitivity to NO are important determinants of NO function (Carmeliet & Jain, 2000; Fukumura & Jain, 1998; Fukumura *et al*, 2006).

Various direct and indirect mechanisms have been proposed for the antitumour properties of NO. Mechanisms include direct damage of DNA, inhibition of DNA synthesis and inhibition of the rate-limiting enzyme ribonucleotide reductase. Reduced activity of cis-aconitase and loss of a large fraction of the iron pool, have also been suggested as possible mechanisms. Importantly, NO-generation can impact mitochondrial physiology leading to reduction of O_2 consumption and damage to complexes in the mitochondrial electron transport chain, and induction of apoptosis. Several laboratories

have demonstrated that NO-releasing agents can kill tumour cells, and as a consequence there have been attempts deliver NO to cells (Xu *et al*, 2002b).

B. Rationale for selection

It was therefore hypothesized that experimental depot or sustained-release NO modulators, which donate NO at high levels, might be effective locoregional antitumour agents.

3.31 Aims

This study evaluated the solubility of several experimental NO donors in Ethiodol, the kinetics of release into aqueous medium and subsequent biological activity, and effectiveness as treatment for isolated liver metastases in an experimental rat tumour model.

3.32 Methods

A. Determination of NO donor solubility in Ethiodol

NO modulators were tested for solubility by mixing (agitation with Vortex) with Ethiodol. The volume of Ethiodol progressively increased with 0.5mL aliquots as needed and the mixture was filtered through progressively smaller needle sizes to help disintegrate particles. Mixtures that were truly suspensions would precipitate if left standing, and were eliminated from further evaluation.

B. Determination of *in vitro* nitrite release from NO donors in Ethiodol

200 μL of each NO donor reagent in Ethiodol was placed into six well plates containing 4 mL complete culture medium only (see above, section 3.22A). Two mL media was removed from each well and replaced daily with new media. Nitrite concentrations in supernatants functioned as reflections of NO production (Dinapoli *et al*, 1996; Stuehr & Nathan, 1989), and samples were assayed for nitrite using the Greiss reaction[1]. In brief, an equal volume of Greiss reagent [0.5% sulfanilamide, 0.05% *N*-(1-naphthyl) ethylenediamide dihydrochloride, and 2.5% phosphoric acid] was added to an equal volume of sample and incubated at room temperature for ten minutes. The absorbance at 550 nm was measured, and nitrite concentrations were determined by using sodium nitrite in newly prepared culture medium as reference standard.

C. Tumour cell lines

TSA is a spontaneously occurring metastatic breast adenocarcinoma cell line syngeneic to BALB/C mice. TSA is a robust cell line that displays a remarkable morphologic heterogeneity in culture and this was considered of value for *in vitro* experiments evaluating novel NO donors (Nanni *et al*, 1983). MADB106 is a breast adenocarcinoma cell line syngeneic to F344 rats (see above, section 3.12.A). These cell lines were cultured in complete medium consisting of RPMI1640, 100 μg/mL

[1] One means to investigate NO formation is to measure nitrite (NO_2^-), which is one of two primary, stable and nonvolatile breakdown products of NO. This assay relies on a diazotization reaction that was originally described by Griess in 1879, however through the years many modifications to the original reaction have been described. The Griess Reagent system is based on the chemical reaction, which uses sulfanilamide and N-1-napthylethylenediamine dihydrochloride under acidic (phosphoric acid) conditions. This system detects nitrite in a variety of biological and experimental liquid matrices such as plasma, serum, urine and tissue culture medium [Griess P (1879) Bemerkungen zu der abhandlung der HH Weselsky und Benedikt "Ueber einige azoverbindungen." *Chem Ber* **12:** 426].

streptomycin, 100 U/mL penicillin, 3% glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 5 X 10^{-5} M 2-mercaptoethanol and 5% foetal calf serum prior to use in these studies.

D. Determination of *in vitro* cytotoxicity

TSA cells (5 x 10^4) and MADB106 cells (5 x 10^4) were incubated in six well plates and 200 µL of each experimental NO modulator/donor in Ethiodol (including sodium nitroprusside) was added using well inserts at day one, into the culture wells for combined culture evaluation (see **Figure 3.21**). Cells were examined at 48 hrs for appearance, adhesion, growth pattern and cell death as determined with trypan blue exclusion.

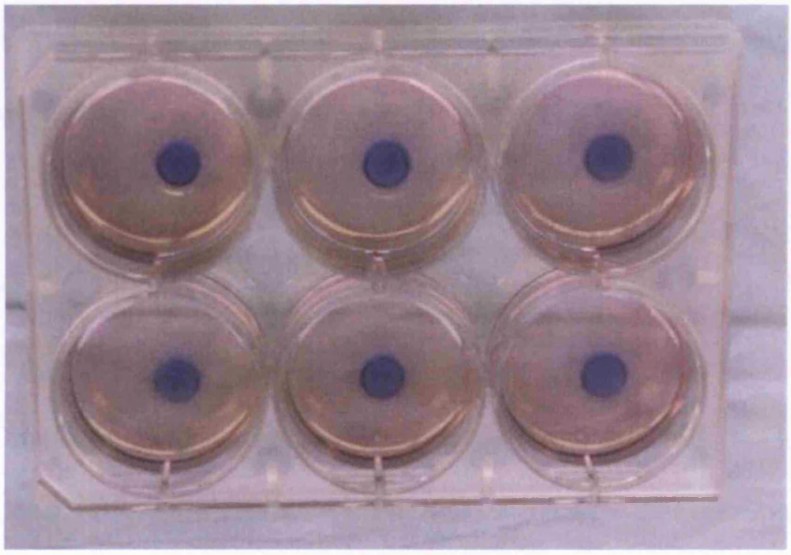

Figure 3.21 **Photograph of example of MADB106 cells in co-culture with PTIO in Ethiodol. PTIO has natural blue colour, and was placed in sterile well inserts and co-cultured with tumour cells for 2 days.**

E. Animal model of tumour engraftment

TSA tumour cells (2 x 10^5) were admixed with 200µL of NO donor in Ethiodol and injected subcutaneously into the flank area of BALB/C mice (Jackson Laboratory, Bar Harbor, ME). Animals were housed and fed in accordance with University of Pittsburgh

research guidelines. Tumour area was measured three times per week using callipers, and animals were sacrificed when ascites or ulceration occurred or when tumours were larger than 2 x 2 cm^2 in area. Control animals were inoculated with tumour in Ethiodol, RPMI1640, or incomplete Freund's adjuvant (see above, section 3.22.E). Statistical tests were not applied to engraftment models.

F. Animal model of hepatic metastases

Briefly, as outlined above (sections 3.12.D and 3.22.H) F344 rats approximately 250-300gms weight were given 2 x 10^5 MADB106 breast adenocarcinoma cells by direct intraportal injection on day zero. On day six the gastroduodenal artery was cannulated and either 50 µl of NOR1 in Ethiodol, or 40, 60 or 80 µl of sodium nitroprusside in Ethiodol, or Ethiodol only was administered. Rats are sacrificed on day sixteen and the number of liver metastases were enumerated and compared.

3.33 Results

A. NO donors are soluble in Ethiodol

Several NO modulators/donors dissolve in Ethiodol (see **Table 3.7**). SNAP, NOR1, NOR2, NOR3, and NOR4 are experimental NO donors. PTIO is an experimental NO scavenger which can abrogate the effects of NO donors. Sodium nitroprusside is a clinically available preparation which is used as a rapid acting vasodilator and produces hypotension. In addition to producing NO, sodium nitroprusside also produces cyanide ions which are toxic. The effects of cyanide can be abrogated with administration of thiosulfate.

B. *In vitro* nitrite release from NO donors in Ethiodol

The NO donors SNAP, NOR1, NOR2, NOR3 and NOR4, release NO (as determined by the Greiss reaction for stable nitrite product) from Ethiodol in a sustained fashion over time (see **Figure 3.22**). Sodium nitroprusside also released NO from Ethiodol in a sustained fashion over time.

Larger aliquots of sodium nitroprusside in Ethiodol released proportionally larger amounts of NO. There is an early release peak during one to two days, and NO production increases steadily over the next five to six days, and then appears to peak between seven to nine days. However, NO production continues as long as eleven days after creating the preparation, demonstrating good sustained-release characteristics (see **Figure 3.23**).

Table 3.7

Solubility for NO modulators in Ethiodol

NO Modulator **short name / (function and source) / [chemical name]**	**Maximum Solubility (mg/mL)**
SNAP (NO donor; Alexis Biochemicals) [S-Nitroso-N-acetyl-D,L-penicillamine]	16.67
NOR1 (NO donor; Alexis Biochemicals) [(±)-(E)-Methyl-2-[(E)-hydroxyimino]-5-nitro-6-methoxy-3-hexeneamide]	6.67
NOR2 (NO donor; Alexis Biochemicals) [(±)-(E)-Methyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide]	6.67
NOR3 (NO donor; Alexis Biochemicals) [(±)-(E)-Ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexeneamide]	6.67
NOR4 (NO donor; Alexis Biochemicals) [(±)-(E)-Ethyl-2'-[(E)-hydroxyimino]-5-nitro-3-hexenecarbamoylpyridine]	6.67
PTIO (NO scavenger; Alexis Biochemicals) [2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide]	20
Nitropress (NO donor; Abbott Labs. Illinois) [Sodium nitroprusside]	10

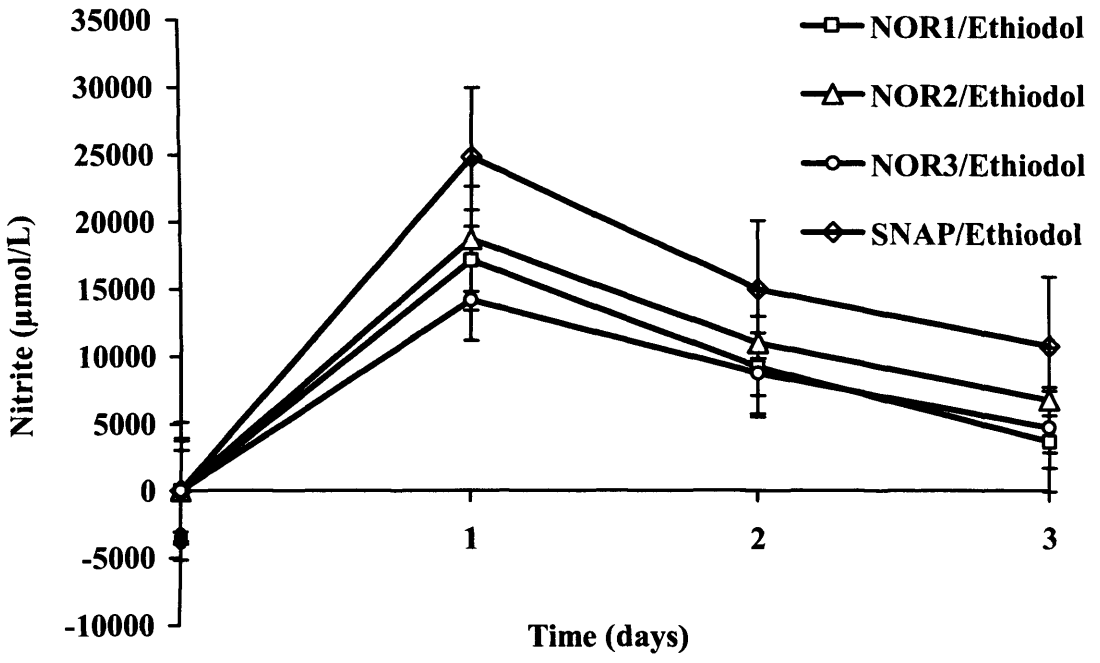

Figure 3.22 Nitrite release over time from NO donors in Ethiodol. 200 µL of each NO donor in Ethiodol (n=2) at concentrations given in Table 3.7, placed in 6 well plates with 4 mL complete medium, and 2 mL media sampled from wells and replaced daily with new media. Nitrite concentrations in supernatants were assayed with the Greiss reaction system as a measure of stable NO product (3 experiments).

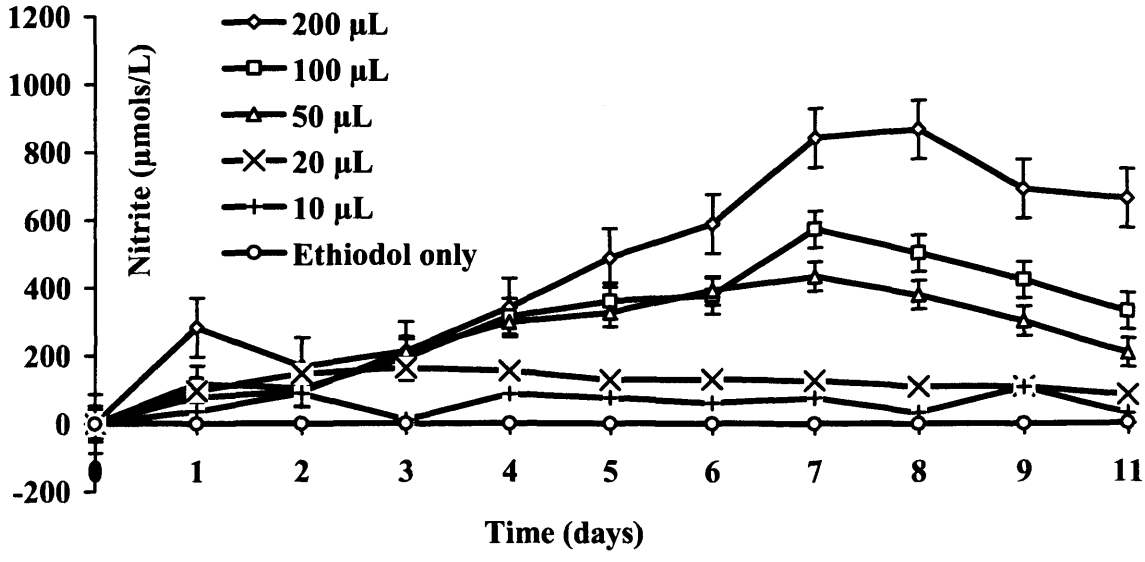

Figure 3.23 Nitrite release over time from NO donor sodium nitroprusside in Ethiodol. 10-200 µL aliquots (n=2) at concentration given in Table 3.7, were evaluated with method above. Nitrite concentrations in supernatants assayed with Greiss reaction system (3 experiments).

C. NO released from Ethiodol has *in vitro* biologic activity

NO released from donors NOR1, NOR2, NOR3 and SNAP in Ethiodol, was effective in causing TSA tumour cell death and non-viable appearance of combined cultures (see **Figures 3.24, 3.25, 3.26, 3.27, 3.28**).

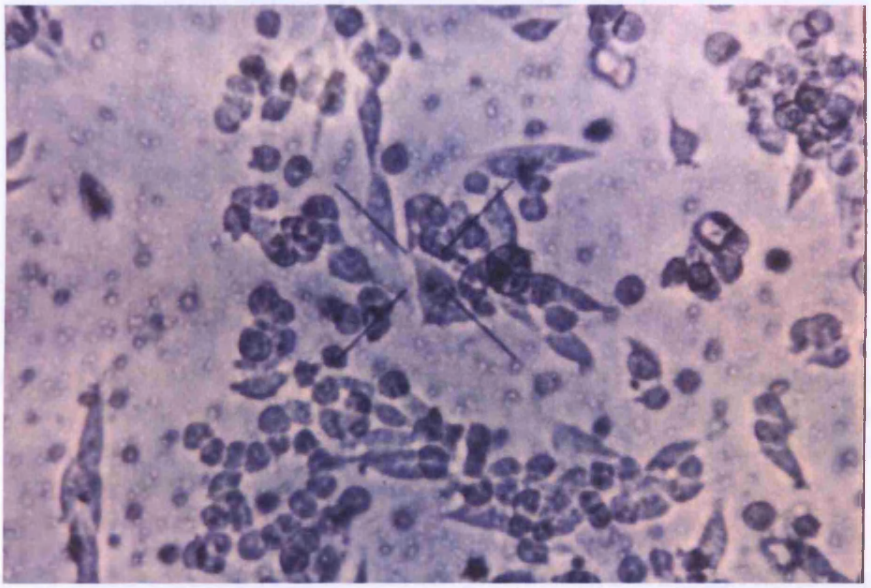

Figure 3.24 **Photomicrograph (x 40 magnification) of TSA cells co-cultured with 200 µL Ethiodol only for 2 days. Cells and culture pattern both appeared viable (n=3/group; 3 experiments).**

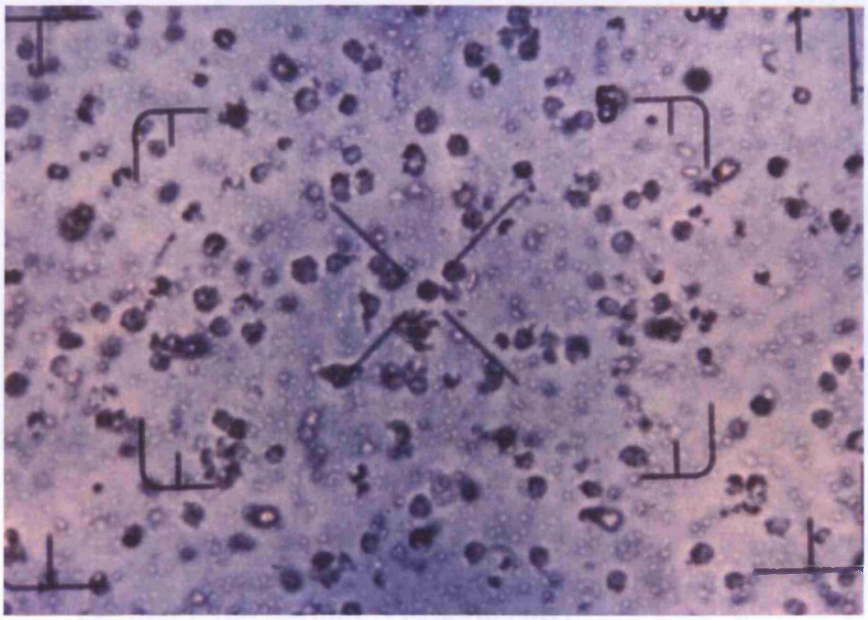

Figure 3.25 **Photomicrograph (x 40 magnification) of TSA cells co-cultured with 1.3 mg NOR1 in 200 µL Ethiodol for 2 days. Cells and culture pattern appeared non-viable, and trypan blue exclusion confirmed cell death (n=3/group; 3 experiments).**

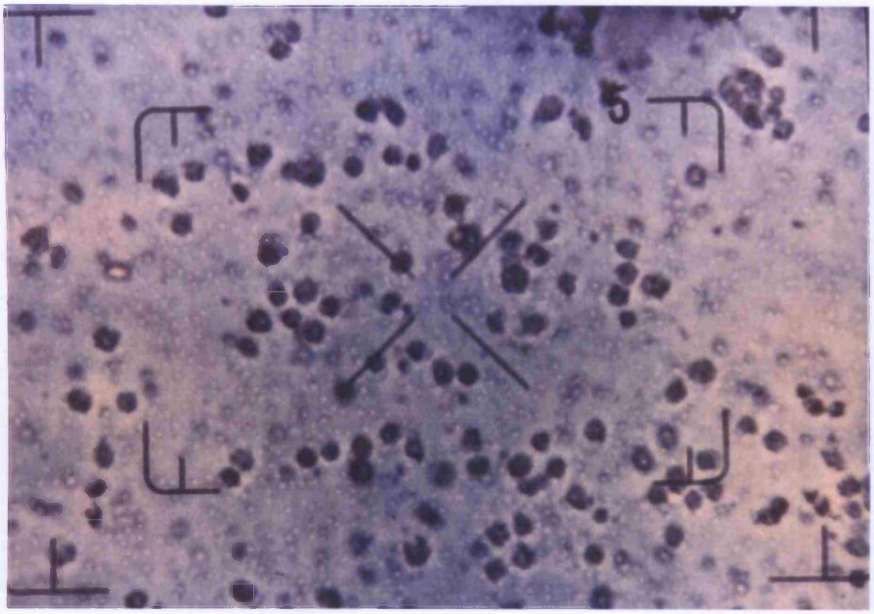

Figure 3.26 **Photomicrograph (x 40 magnification) of TSA cells co-cultured with 1.3 mg NOR2 in 200 µL Ethiodol for 2 days. Cells and culture pattern appeared non-viable, and trypan blue exclusion confirmed cell death (n=3/group; 3 experiments).**

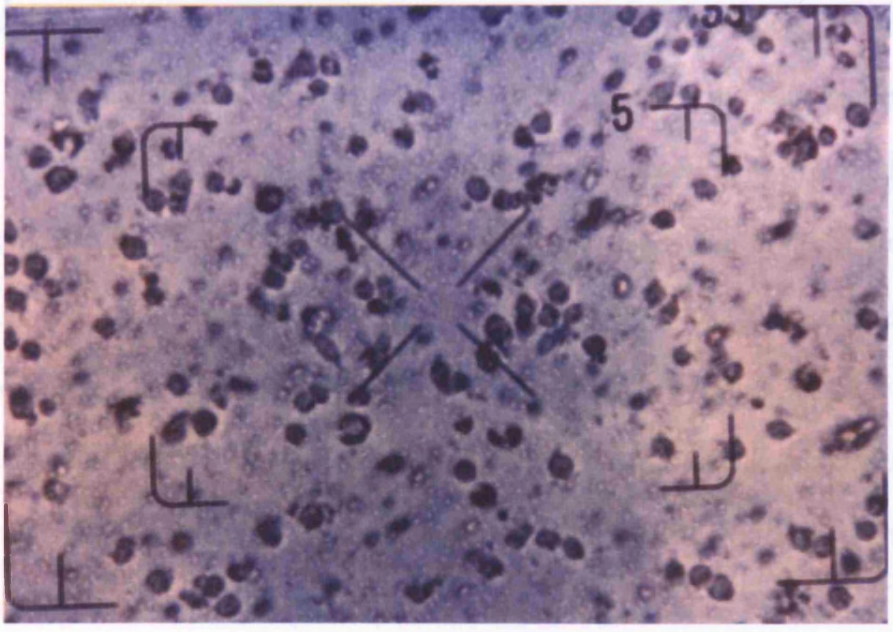

Figure 3.27 **Photomicrograph (x 40 magnification) of TSA cells co-cultured with 1.3 mg NOR3 in 200 µL Ethiodol for 2 days. Cells and culture pattern appeared non-viable, and trypan blue exclusion confirmed cell death (n=3/group; 3 experiments).**

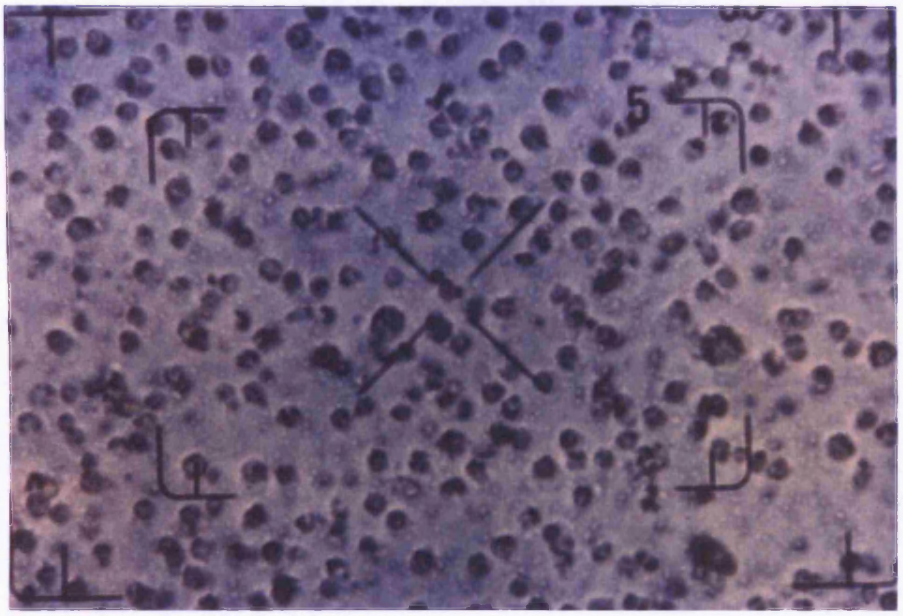

Figure 3.28 **Photomicrograph (x 40 magnification) of TSA cells co-cultured with 8.3 mg SNAP in 200 µL Ethiodol for 2 days. Cells and culture pattern appeared non-viable, and trypan blue exclusion confirmed cell death (n=3/group; 3 experiments).**

Sodium nitroprusside in Ethiodol was similarly effective in producing cell death with abnormal culture pattern in co-cultures with MADB106 tumour cells (see **Figures 3.29, 3.30, 3.31, 3.32**). At concentrations of 1000 µg of sodium nitroprusside in Ethiodol, almost total cell death with no culture and adherence was seen, however decreasing concentrations of sodium nitroprusside in Ethiodol to 500 µg and 250 µg, appeared to result in reduced cell death with some culture and adherence (**Figures 3.30, 3.31, 3.32**). Further evaluation of MADB106 tumour cell viability and culture, with decreased concentrations of 100 and 50 µg sodium nitroprusside in Ethiodol, demonstrated few viable tumour cells with minimal culture and adherence (see **Table 3.8**).

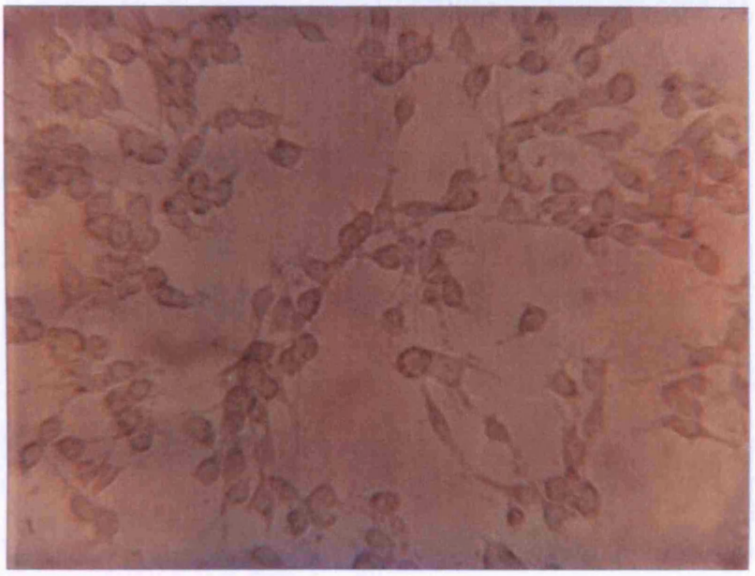

Figure 3.29 **Photomicrograph (x 40 magnification) of MADB106 cells co-cultured with 200 µL Ethiodol only for 2 days. Cells and culture pattern appeared viable (n=3/group; 3 experiments).**

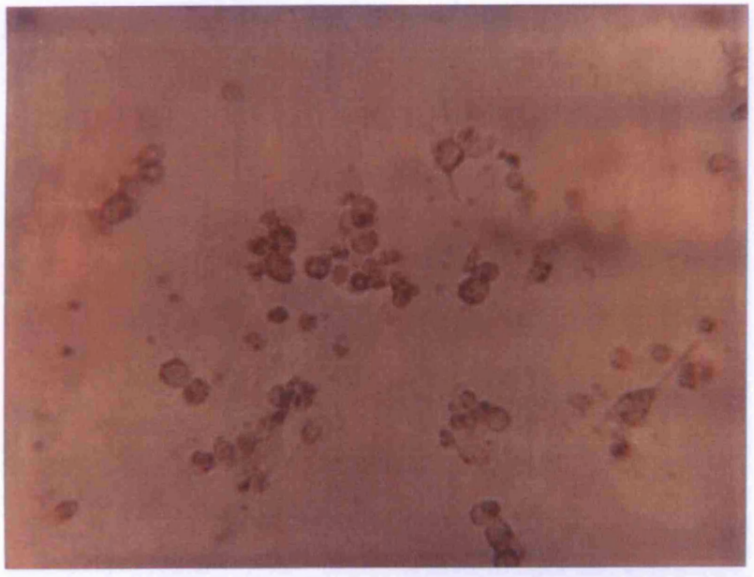

Figure 3.30 **Photomicrograph (x 40 magnification) of MADB106 cells co-cultured with 200 µL sodium nitroprusside in Ethiodol at concentration of 5 µg/µL for 2 days. Cells and culture pattern appeared non-viable, and trypan blue exclusion confirmed near total cell death (n=3/group; 3 experiments).**

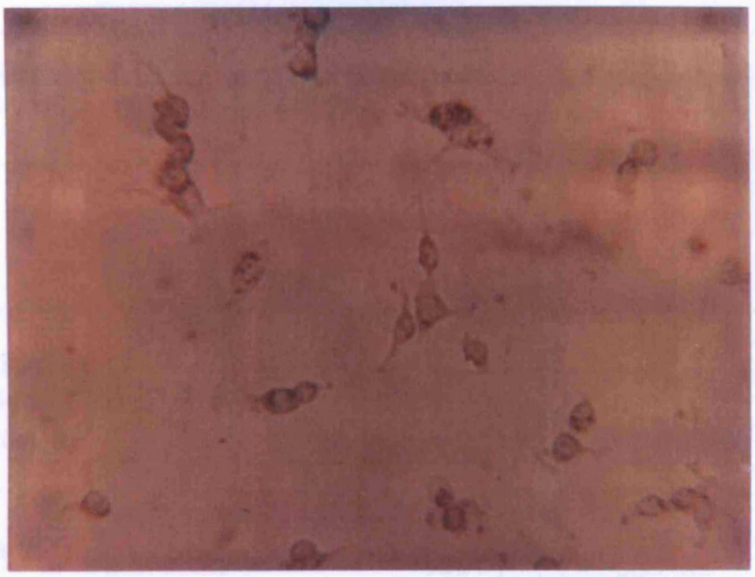

Figure 3.31 **Photomicrograph (x 40 magnification) of MADB106 cells co-cultured with 100 µL sodium nitroprusside in Ethiodol at concentration of 5 µg/µL for 2 days. Cells and culture pattern appeared mostly non-viable (trypan blue exclusion) (n=3/group; 3 experiments).**

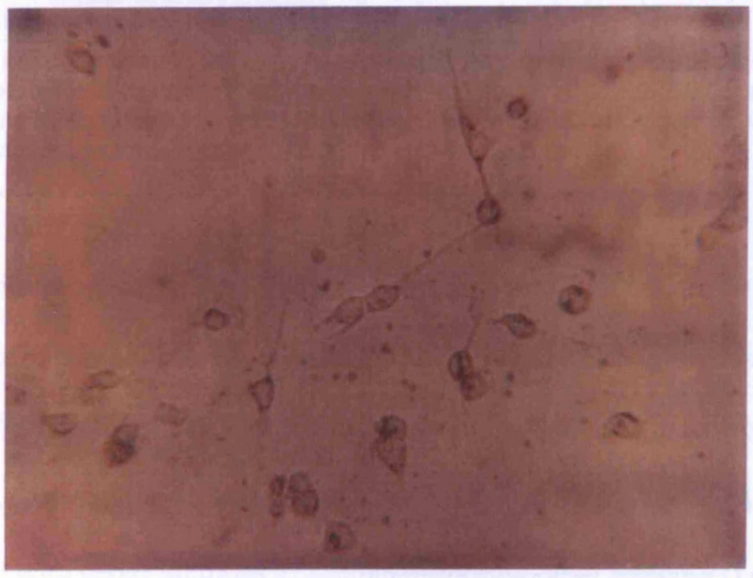

Figure 3.32 **Photomicrograph (x 40 magnification) of MADB106 cells co-cultured with 50 µL sodium nitroprusside in Ethiodol at concentration of 5 µg/µL for 2 days. Cells and culture pattern appeared mostly non-viable (trypan blue exclusion) (n=3/group; 3 experiments).**

Table 3.8

Culture appearance and cell viability with varied concentrations of sodium nitroprusside in Ethiodol

	Sodium nitroprusside					Ethiodol only
Volume sodium nitroprusside in Ethiodol (μL)	200	100	50	20	10	200
Appearance of culture	0	0	0	0	+	++
Number of viable cells	0	0	0	$1x10^4$	$4.7x10^4$	$13x10^4$
Number of dead cells	$4x10^4$	$3.8x10^4$	$4.2x10^4$	$0.5x10^4$	$1.2x10^4$	$0.7x10^4$

5×10^4 MADB106 cells co-cultured with varying volumes sodium nitroprusside in Ethiodol at 5 μg/μl concentration. Appearance of culture pattern and adherence noted at 48 hours. Cells then harvested and counted, and viability assessed with trypan blue exclusion. Appearance of culture pattern scored: 0 = non-viable, + = some viability, ++ = viable. (n=3/group; 3 experiments).

Sodium Nitroprusside also produces toxic cyanide ions. To determine whether antitumour effect of sodium nitroprusside in Ethiodol was due to NO or cyanide production, the NO scavenger PTIO and cyanide substrate thiosulfate were used to attempt cancellation of *in vitro* activity (see **Figures 3.33, 3.34, 3.35, 3.36, 3.37**). MADB106 cells were co-cultured with serial dilutions of sodium nitroprusside in Ethiodol (i.e. 500, 250, and 125 μg sodium nitroprusside in 100 μL Ethiodol), with or without 500 μg of PTIO in 100 μL Ethiodol, and also with or without 100 μL aqueous thiosulfate (125 mg/mL), for two days, and examined for culture appearance and adhesion, and cell viability (trypan blue exclusion).

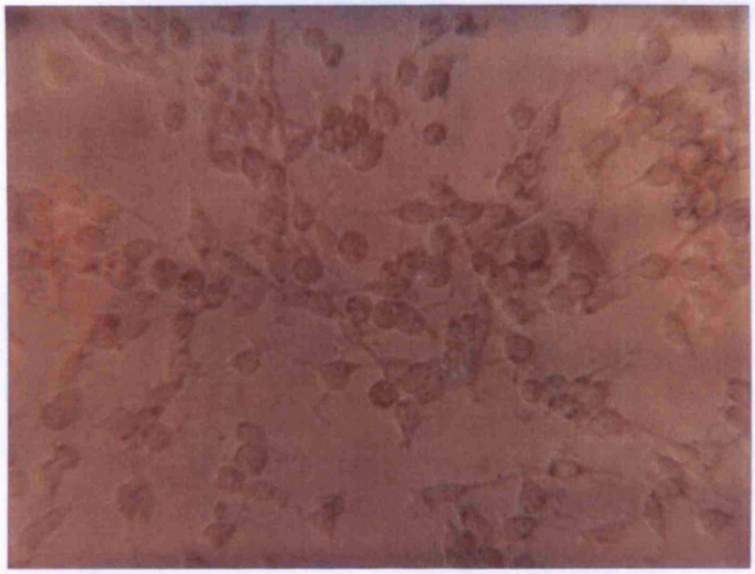

Figure 3.33 **Photomicrograph (x 40 magnification) of MADB106 cells co-cultured with 200 µL PTIO in Ethiodol at concentration of 5 µg/µL for 2 days. Cells and culture pattern appeared viable (trypan blue exclusion) (n=3/group; 3 experiments).**

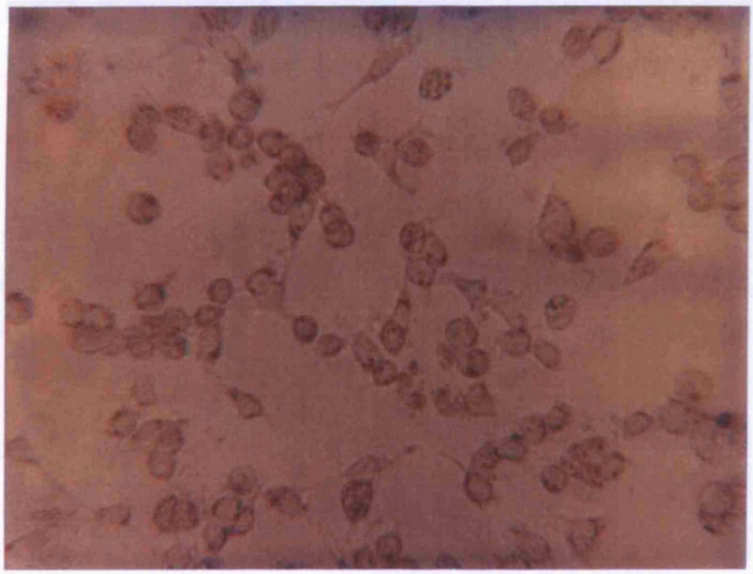

Figure 3.34 **Photomicrograph (x 40 magnification) of MADB106 cells co-cultured with 50 µL sodium nitroprusside in Ethiodol & 100 µl PTIO in Ethiodol, both at concentrations of 5 µg/µL for 2 days. Cells and culture pattern appeared moderately viable (trypan blue exclusion) (n=3/group; 3 experiments).**

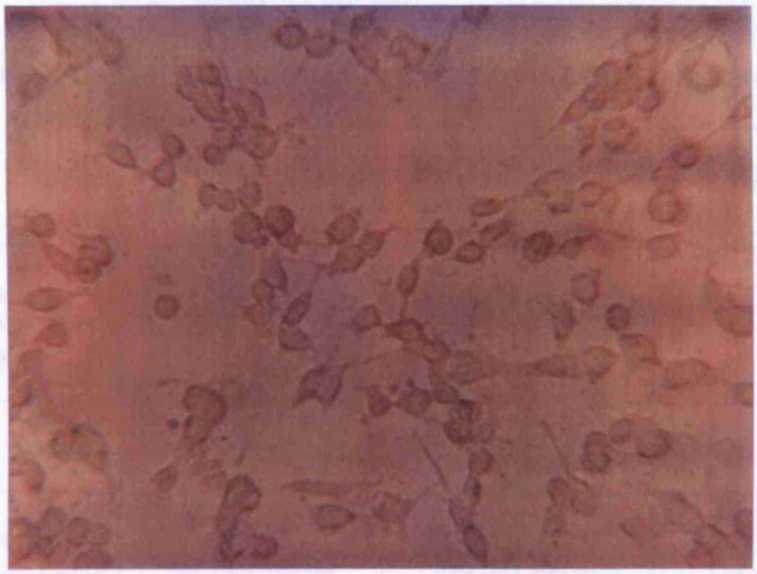

Figure 3.35 **Photomicrograph (x 40 magnification) of MADB106 cells co-cultured with 100 μL of aqueous thiosulfate at concentration of 125 mg/mL for 2 days. Cells and culture pattern appeared viable (trypan blue exclusion) (n=3/group; 3 experiments).**

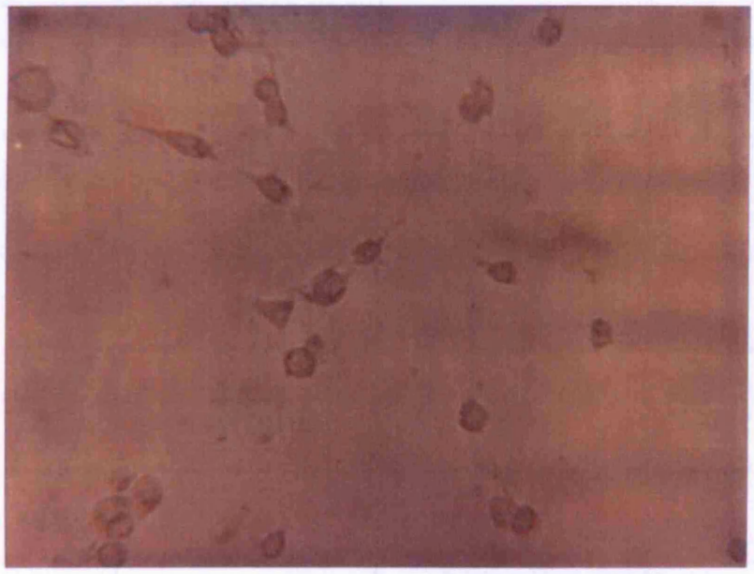

Figure 3.36 **Photomicrograph (x 40 magnification) of MADB106 cells co-cultured with 50 μL sodium nitroprusside in Ethiodol at concentration of 5 μg/μL and 100 μL of aqueous thiosulfate at concentration of 125 mg/mL for 2 days. Cells and culture pattern appeared minimally viable (trypan blue exclusion) (n=3/group; 3 experiments).**

Table 3.9

Cancellation of antitumour effect of sodium nitroprusside in Ethiodol

	Appearance of culture pattern and adherence for various chemical combinations		
Sodium Nitroprusside (μg given in columns to the right) in 100 μL Ethiodol only	**500 μg**	**250 μg**	**125 μg**
culture	**0**	**0/+**	**0/+**
Sodium Nitroprusside in 100 μL Ethiodol plus 500 μg PTIO in 100 μL Ethiodol	**500 μg / 500 μg**	**250 μg / 500 μg**	**125 μg /5 00 μg**
culture	**0**	+/++	+/++
Sodium Nitroprusside in 100 μL Ethiodol plus 12.5 mg/100 μL aqueous thiosulfate	**500 μg /12.5 mg**	**250 μg /12.5 mg**	**125 μg /12.5 mg**
culture	**0**	+	++
500 μg PTIO in 100 μL Ethiodol only	**500 μg CONTROL**		
culture	++		
12.5 mg/ 100 μL aqueous thiosulfate only	**12.5 mg CONTROL**		
culture	++		
100 μL Ethiodol only	**Ethiodol only CONTROL**		
culture	++		

5×10^4 MADB106 cells co-cultured with varying concentrations of reagents as given above. Appearance of culture pattern and adherence noted at 48 hours. Cells then harvested and viability assessed with trypan blue exclusion. Appearance of culture pattern scored: 0 = non-viable, + = some viability, ++ = viable. (n=3/group; 3 experiments).

In vitro controls with Ethiodol, PTIO (**Figures 3.33**) and thiosulfate (**Figure 3.35**) only, all demonstrated good culture pattern and adherence while almost no cells survived any dilution of sodium nitroprusside in Ethiodol. Both PTIO and thiosulfate added independently to sodium nitroprusside in Ethiodol at 250 and 125 μg dilutions appeared to cancel cytotoxic activity and resulted in minimal to moderate culture pattern and adherence patterns (see **Figures 3.34 and 3.36, and Table 3.9**). Therefore, *in vitro* effects of sodium nitroprusside in Ethiodol were partially cancelled by the addition of PTIO and thiosulfate independently. This would imply that both antitumour mechanisms of NO and cyanide production contribute to the activity of sodium nitroprusside in Ethiodol.

D. Tumour admixture with NO donors in Ethiodol inhibits engraftment

BALB/C mice were inoculated with tumour admixed with 0.2 mL of Ethiodol with varying doses of experimental NO modulators, including the donors NOR1, NOR2, NOR3, NOR4 and SNAP, and the scavenger PTIO, with concentrations as given in **Table 3.7**. Engraftment of 2 x 10^5 TSA cells was prevented by NOR1 in Ethiodol (see **Figure 3.37**), and this effect was comparable to prevention of engraftment seen with hRIL2 in Ethiodol. Combination of NOR1, hRIL2 and Ethiodol had similar effect, whereas engraftment was not prevented by Hanks solution or Ethiodol only. Tumour engraftment was prevented by NOR1 in Ethiodol, combination of NOR1, hRIL-2 and Ethiodol, and hRIL2 in Ethiodol for as long as three weeks with 100% survival (see **Figure 3.38**). These results confirm the sustained-release characteristics of NOR1 in Ethiodol, and demonstrate that the preparation behaves similarly to an already established similar preparation, hRIL2 in Ethiodol. Statistical tests were not applied to engraftment models.

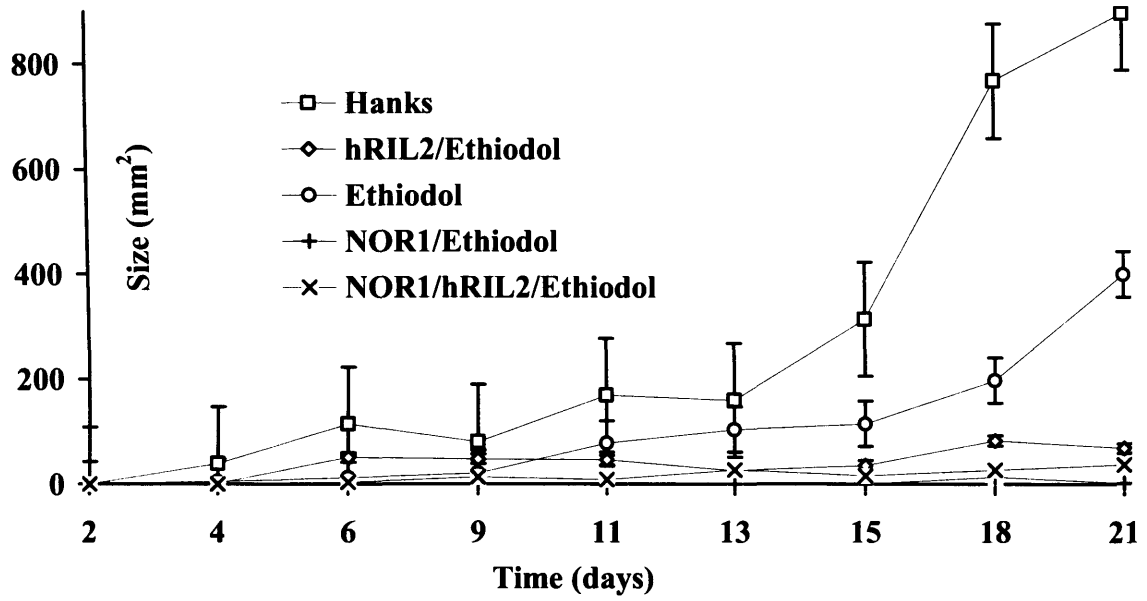

Figure 3.37 **NOR1, hRIL2 and NOR1/hRIL2 in Ethiodol, prevent tumour engraftment. 2 x 10^5 TSA tumour cells were injected subcutaneously into the flanks of BALB/C mice admixed with Ethiodol only, NOR1, hRIL2 or NOR1/hRIL2 in Ethiodol, or Hanks. Test groups administered doses as previously given in same volume (200 μL) (n=3/group; 3 experiments).**

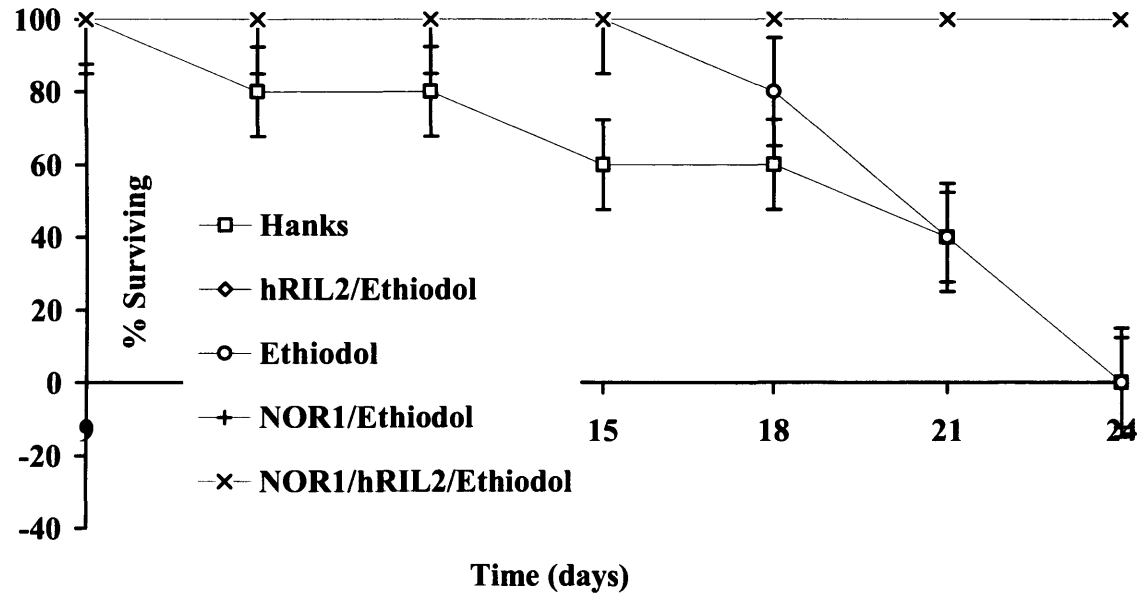

Figure 3.38 **NOR1, hRIL2 and NOR1/hRIL2 in Ethiodol, improve survival in an engraftment model. As above, 2 x 10^5 TSA tumour cells were injected into the flanks of BALB/C mice, admixed with Ethiodol only, NOR1, hRIL2 or NOR1/hRIL2 in Ethiodol, or Hanks. Test groups as above (n=3/group; 3 experiments).**

NOR3 in Ethiodol and SNAP in Ethiodol both demonstrated effects similar to NOR1 in Ethiodol, with prevention of tumour engraftment and 100% survival. However, NOR4 in Ethiodol was ineffective and displayed no antitumour effect, which was similar to Ethiodol only or the NO scavenger, PTIO in Ethiodol (see **Figures 3.39 and 3.40**).

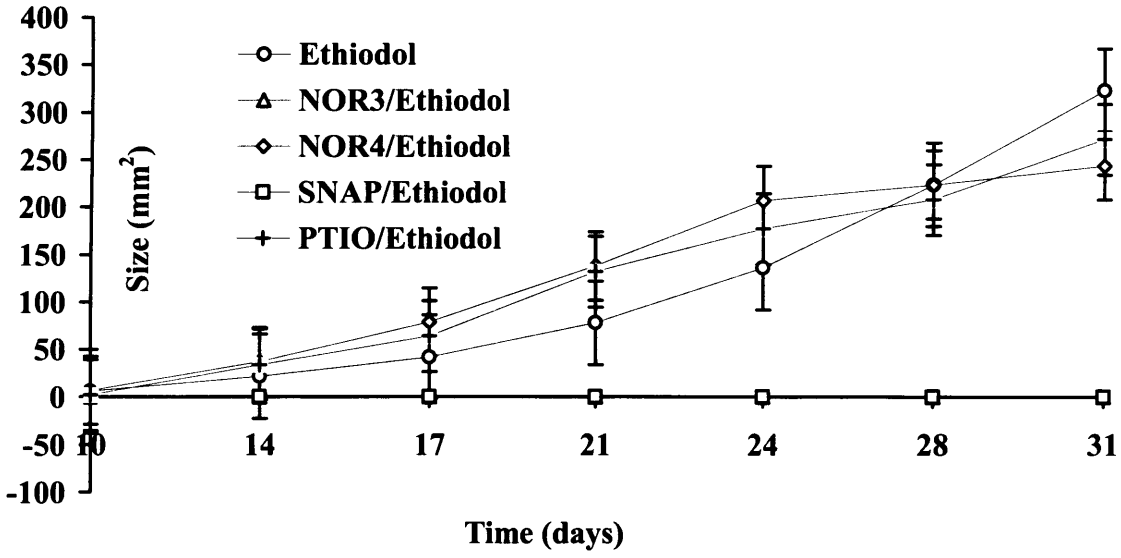

Figure 3.39 **NOR3 and SNAP in Ethiodol, prevent tumour engraftment. 2 x 10^5 TSA tumour cells were injected subcutaneously into the flanks of BALB/C mice admixed with Ethiodol only, or NOR3, NOR4, SNAP or PTIO in Ethiodol. Test groups administered doses as previously given in same volume (200 μL) (n=3/group; 3 experiments).**

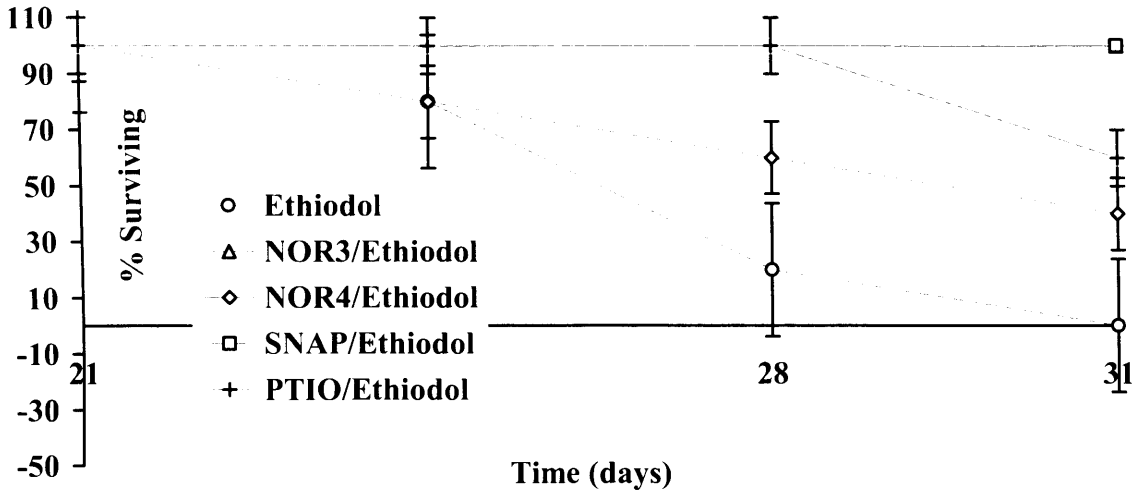

Figure 3.40 **NOR3 and SNAP in Ethiodol, improve survival in an engraftment model. As above, 2 x 10^5 TSA tumour cells were injected into the flanks of BALB/C mice admixed with Ethiodol only, or NOR3, NOR4, SNAP or PTIO in Ethiodol. Test groups administered doses as previously given in same volume (200 μL) (n=3/group; 3 experiments).**

NOR2 in Ethiodol also demonstrated effects similar to NOR1, NOR3 and SNAP in Ethiodol, with prevention of tumour engraftment and 100% survival (see **Figures 3.41 and 3.42**).

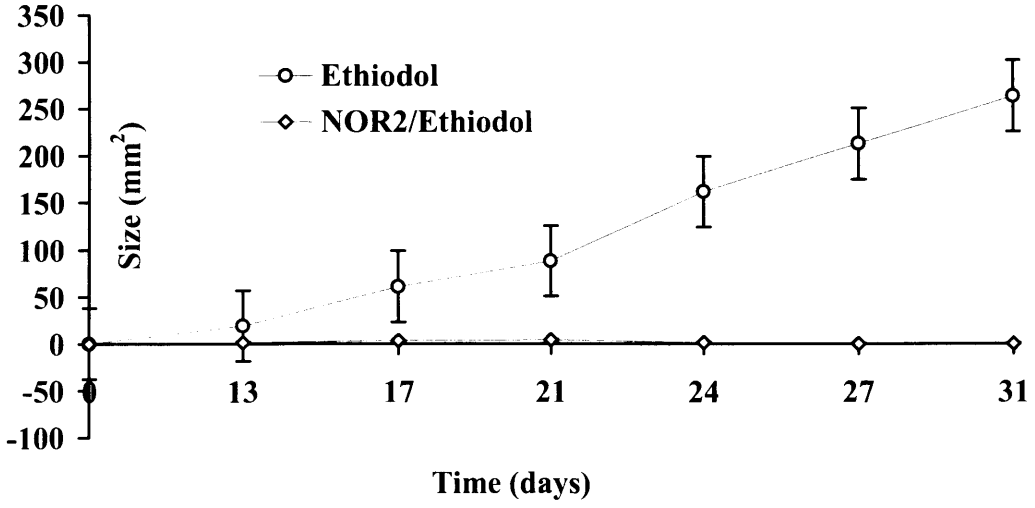

Figure 3.41 **NOR2 in Ethiodol prevents tumour engraftment. 2 x 10^5 TSA tumour cells were injected subcutaneously into the flanks of BALB/C mice admixed with Ethiodol only, or NOR2 in Ethiodol. Test groups as above (n=3/group; 3 experiments).**

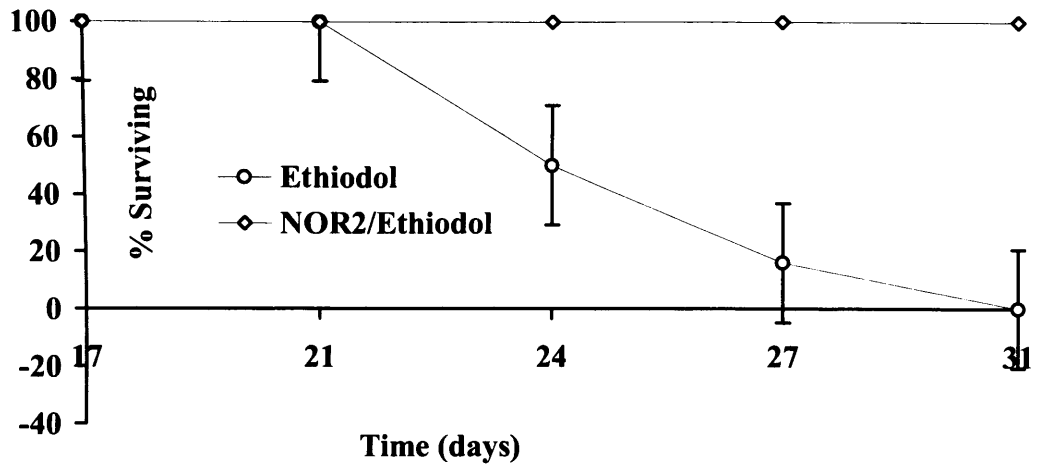

Figure 3.42 **NOR2 in Ethiodol improve survival in an engraftment model. As above, 2 x 10^5 TSA tumour cells were injected into the flanks of BALB/C mice admixed with Ethiodol only, or NOR2 in Ethiodol. Test groups administered doses as previously given in same volume (200 μL) (n=3/group; 3 experiments).**

Sodium nitroprusside in Ethiodol is a preparation made of clinically available pharmacologic agents. Sodium nitroprusside in Ethiodol demonstrated prevention of tumour engraftment when administered on the same side as the tumour inoculation (the cis position), but was ineffective when administered on the flank opposite to the tumour inoculation (or the trans position). Both PTIO and thiosulfate decreased the effectiveness of prevention of engraftment for sodium nitroprusside in Ethiodol almost equally. This supports the prior *in vitro* findings that NO and cyanide production may both be active antitumour agents released from sodium nitroprusside in Ethiodol (see **Figures 3.43**).

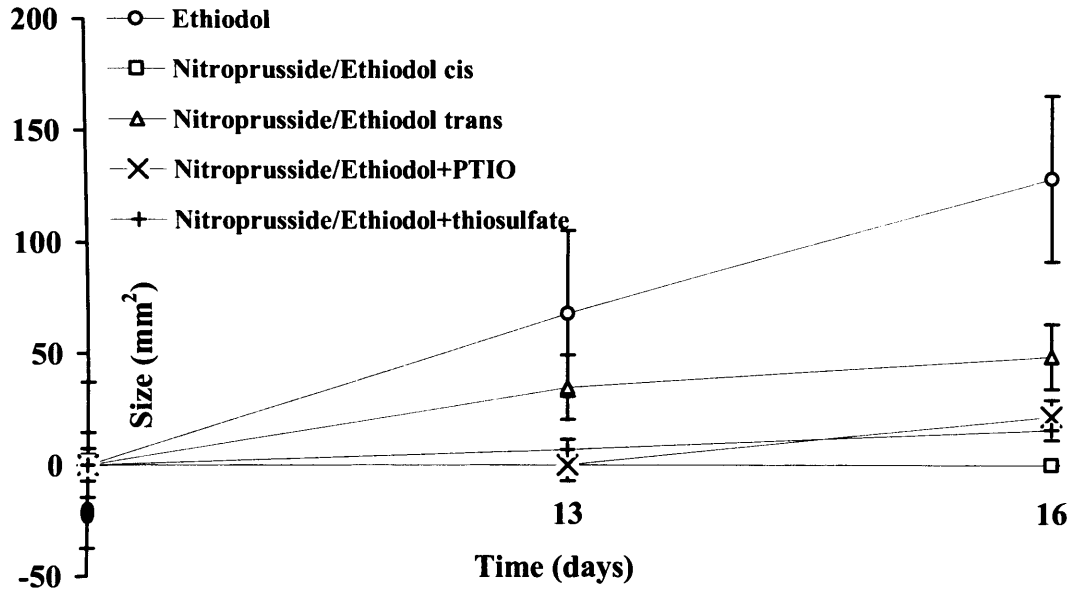

Figure 3.43 **Sodium nitroprusside in Ethiodol prevents tumour engraftment. 2 x 10^5 TSA tumour cells were injected subcutaneously into the flanks of BALB/C mice admixed with Ethiodol only, sodium nitroprusside in Ethiodol cis, sodium nitroprusside in Ethiodol trans, or sodium nitroprusside in Ethiodol with PTIO or thiosulfate. Test groups administered doses as previously given in same volume (200 μL) (n=3/group; 3 experiments).**

E. HAI infusion of NO donors in Ethiodol is superior to Ethiodol only

Rats bearing hepatic-metastatic adenocarcinoma were treated with HAI administration of NOR1 in Ethiodol, or Ethiodol only according to the methods outlined above. Briefly, F344 rats had 2 x 10^5 MADB106 breast adenocarcinoma cells inoculated intra-portally on day 0. They were then treated with 50 μL NOR1 in Ethiodol HAI on day 6 via the gastroduodenal artery. Control rats were treated with the same amount of Ethiodol only. Rats were sacrificed on day 16 and liver metastases were enumerated (see **Table 3.10**). Evaluation of livers ten days after therapy revealed marked differences in treatment groups. The number of liver metastases was significantly reduced among animals treated with HAI NOR1 in Ethiodol, compared to those treated with Ethiodol alone ($P < 0.01$).

Table 3.10

Comparison of numbers of liver metastases for rats treated with HAI NOR1 in Ethiodol versus Ethiodol only

Test Groups	Number of Metastases
Ethiodol only (n=6)	250, 155, 250, 250, 250, 250
NOR1 in Ethiodol (n=6)	90, 3, 13, 23, 3, 7
Wilcoxon Rank-Sum Test: $P = 0.0036$	

In addition, utilizing the same methodology, F344 rats were treated with 40, 60 and 80 µl sodium nitroprusside in Ethiodol as HAI therapy for hepatic-metastatic adenocarcinoma. Once again, evaluation of livers ten days after therapy revealed marked differences between treatment groups. The number of liver metastases was significantly reduced among animals treated with HAI sodium nitroprusside in Ethiodol at any dose, compared to those treated with Ethiodol alone ($P < 0.01$) (see **Table 3.11, Figures 3.44 and 3.45**).

As previously demonstrated, Ethiodol only treated animals consistently developed large numbers of sizable hepatic metastases by the time they were sacrificed. These control animals manifested hair loss, ruffled fur, abdominal distension, jaundice, or lethargy. In contrast, rats treated with NO donors in Ethiodol remained well throughout the period of observation.

Table 3.11

Comparison of numbers of liver metastases for rats treated with HAI sodium nitroprusside in Ethiodol versus Ethiodol only

	Ethiodol only	Sodium Nitroprusside in Ethiodol (5μg/μL)		
	60ul	**40ul**	**60ul**	**80ul**
No. of metastases	35	10	13	7
	250	3	39	37
	72	16	250	
	250	21	14	
	250	11	51	
	250	24	14	
	250			
	70			
Wilcoxon Rank-Sum Test: $P = 0.0012$				

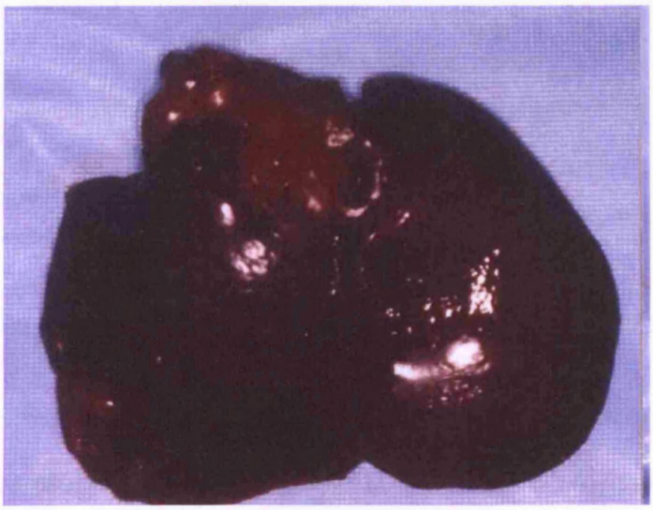

Figure 3.44 **Photograph of rat liver: HAI nitroprusside in Ethiodol treatment group.**

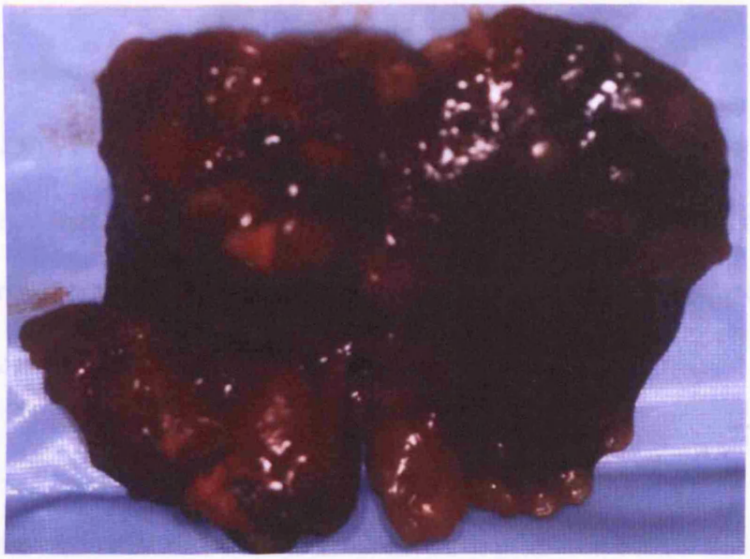

Figure 3.45 Photograph of rat liver: HAI Ethiodol only treatment group.

Review of the descriptive statistics for this experiment confirms the presence of an "outlier" value of 250 in the group treated with HAI sodium nitroprusside in Ethiodol (see **Figures 3.46)**. As demonstrated by the "box plot", the first quartile, media and third quartile values are 11.5, 15 and 33.5. This is a rare occurrence and is most likely due to an error in our biological experimentation system. The most likely explanation is an error in tumour seeding dose, which could be caused by tumour cells being clumped together prior to portal vein injection.

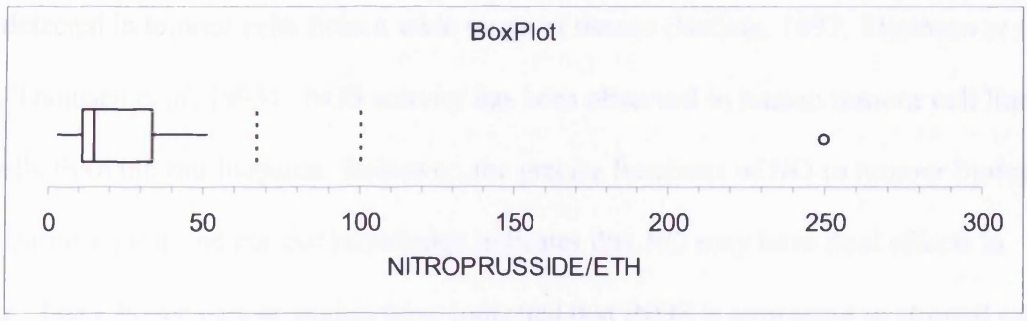

Figure 3.46 Box plot of "outlier" value in HAI sodium nitroprusside in Ethiodol treatment group.

3.34 Discussion

The role of NO in macrophage cytotoxicity was first described in 1987 (Hibbs Jr *et al*, 1987), and since that time numerous studies have shown that cytokine activated macrophages can generate large concentrations of NO by up-regulation of iNOS expression (Bosca *et al*, 2005; MacMicking *et al*, 1997).

Several direct and indirect mechanisms have been proposed for the antitumour properties of NO. These include direct damage of DNA, inhibition of DNA synthesis and inhibition of the rate-limiting enzyme ribonucleotide reductase. Reduced activity of cis-aconitase and loss of a large fraction of the iron pool, have also been suggested as possible mechanisms. NO generation can affect mitochondrial physiology leading to reduction of O_2 consumption and damage to mitochondrial electron transport chain complexes, and induction of apoptosis (Garbán & Bonavida, 1999; Hibbs Jr *et al*, 1987; Juang *et al*, 1998; MacMicking *et al*, 1997; Xie *et al*, 1995; Xu *et al*, 1998).

All three isoforms of NOS, (iNOS, endothelial [eNOS], and neuronal [nNOS]), have been detected in tumour cells from a wide range of tissues (Nathan, 1997; Thomsen *et al*, 1994; Thomsen *et al*, 1995). NOS activity has been observed in human tumour cell lines and cells from tumour biopsies. However, the precise functions of NO in tumour biology is not entirely clear and current knowledge indicates that NO may have dual effects in cancer. Three breast cancer studies have indicated that iNOS is expressed in stromal cells, macrophages in tumour, and in tumour cells (Loibl *et al*, 2002; Reveneau *et al*, 1999; Vakkala *et al*, 2000a). iNOS activity has also been shown to be higher in less differentiated invasive breast carcinomas (Thomsen *et al*, 1995; Vakkala *et al*, 2000a).

eNOS expression and oestrogen receptor (ER) status have also demonstrated positive correlation (Martin *et al*, 2000; Vakkala *et al*, 2000b; Xu *et al*, 2002c). ER is known to bind to the p85α regulatory subunit of phosphatidylinositol-3-OH kinase (PI-3K), which leads to activation of protein kinase B/Akt (Haynes *et al*, 2000; Simoncini *et al*, 2000). The catalytic activity of eNOS is augmented by phosphorylation of a C-terminal serine residue (Ser-1177 of human eNOS) through the PI-3K/Akt pathway (Dimmeler *et al*, 1999; Fulton *et al*, 1999). It is therefore possible that activation of ER located on the surface of cell membranes, could indirectly activate the release of NO from membrane bound eNOS which may contribute to tumour cell survival under hypoxia and other stress conditions (Xu *et al*, 2002c).

iNOS overexpression has also been demonstrated in almost 60% of human adenomas and 20-25% of colon carcinomas, while expression was either low or absent in the surrounding normal tissue (Ambs *et al*, 1998a; Chhatwal *et al*, 1994). Human carcinoma cells transfected with a murine iNOS have demonstrated increased tumour growth (Jenkins *et al*, 1995). Growth of these NO generating tumours was accompanied by increased neovascularisation. Tumour growth studies on recombinant iNOS expressing human carcinoma cell lines containing mutant p53 demonstrated that NO mediated up-regulation of vascular endothelial growth factor (VEGF) corresponded with increased xenograft tumour vascularisation. NO generated by NOS (located either within the tumour or in the surrounding stroma) may therefore possibly promote new blood vessel formation by up-regulating VEGF with enhanced neovascularisation, invasiveness and metastatic ability (Ambs *et al*, 1998b; Xu *et al*, 2002b).

NO rapidly reacts intracellularly to form nitrite and nitrate, S-nitroso-thiols or peroxynitrate, and these metabolites can play key roles in mediating many of the NO associated genotoxic effects. These effects include DNA damage, which can be initiated by nitrosative deamination, DNA strand breakage or DNA modification (Wink *et al*, 1991).

NO mediated DNA damage may induce p53 accumulation with apoptosis. This is possibly a process by which NO may cause tumour cell death. An increase in NOS activity in tumour cells may cause the concentration of NO to be elevated such that it triggers p53-mediated growth arrest and apoptosis (Ambs *et al*, 1997; Forrester *et al*, 1996). Accumulation of p53 results eventually in down-regulation of iNOS expression by inhibition of iNOS promoter activity (Ambs *et al*, 1998c). Thus a negative feedback loop exists between NO generation and p53 accumulation, which may constitute part of a physiological mechanism that responds to endogenously produced DNA damage due to NO. This p53-mediated growth inhibition may be expected to provide a strong selection pressure for mutant p53 expression in tumour cells (Xu *et al*, 2002b).

NO has been shown to activate poly (ADP-ribose) polymerase (PARP) and it has been proposed that this activation is due to DNA damage (Zhang *et al*, 1994). This damage may take the form of DNA strand breaks or nitrosative deamination of DNA bases when NO is generated at high concentrations. Another important DNA repair enzyme, DNA-dependent protein kinase (DNA-PK), is also known to be essential for the maintenance of the structural integrity of the genome. NO mediated increase in active DNA-PK not only protects cells from the toxic effects of NO, but also provides cross-protection against clinically important DNA-damaging agents, such as radiation,

adriamycin, bleomycin and cisplatin (Xu *et al*, 2000). One of the major substrates of DNA-PK is p53, and DNA-PK is subject to ADP-ribosylation by PARP. It is therefore possible that NO mediated DNA damage and repair could play a significant role in tumour development (Woo *et al*, 1998).

Several investigators have demonstrated that NO releasing agents can also kill tumour cells, and as a consequence there have been attempts deliver NO to cells (Xu *et al*, 2002b). While NO-releasing drugs are being developed, an alternative method for delivery is transfer of NOS encoding cDNA sequences into cancer cells for gene therapy. Studies have shown that this approach may work. It was demonstrated that transfection of K-1735 melanoma cells with an iNOS cDNA suppressed tumourogenicity and abrogated metastasis (Xie *et al*, 1995). Transfection of human renal carcinoma cells with retroviral iNOS also showed similar results (Juang *et al*, 1998). A difficulty with current approaches is that constitutive expression of NOS can quickly result in death of the transfectant, shortening the time that NO can be generated, and potentially limiting the utility of the approach. NOS transfectants also often have to be cultured under conditions that reduce toxicity (for example in the presence of a NOS inhibitor), and transfection attempts may result in cells that are only capable of relatively low levels of NO generation (Ambs *et al*, 1998b). Paradoxically, this may result in low concentrations of NO that promote tumour growth rather than cell killing. NOS enzyme activity also requires several substrates and co-factors and these may be absent from the target cell type (Tzeng *et al*, 1996). Additionally, retroviral and adenoviral vector safety remains a major health concern (Lehrman, 1999).

A method to overcome the problems associated with gene therapy is use of a cell-based approach, which utilises the delivery of recombinant cells (rather than genes) to the

target site, with the advantage that the expression of the gene of interest can be optimised prior to delivery. Studies have shown the utility of two novel iNOS expressing human cell lines that can generate high concentrations of NO following treatment with analogues of either the insect hormone ecdysone or tetracycline (Xu *et al*, 2002a; Xu *et al*, 2000). In order to make the NO generating cells suitable for therapeutic delivery they have been encapsulated within a semipermeable alginate-poly-L-lysine membrane. Encapsulated cells are protected from environmental stresses encountered in the host (such as the host immune response) and can be delivered to tumour sites, with generation of high concentrations of NO and reactive nitrogen species when induced. Tumour model studies showed 100% killing of SKOV-3 tumours and 54% killing of DLD-1 tumours with this approach (Read *et al*, 2001; Xu *et al*, 2002a).

In summary, although initial findings suggested that immune-cell generated NO is cytostatic or cytotoxic for tumour cells, later findings have shown that NO can also possess apparently contradictory activity leading to increased tumour growth. NO can contribute to tumour angiogenesis by upregulating VEGF and modulating tumour DNA repair mechanisms by up-regulating p53, PARP and DNA-PK. High concentrations of NO, as generated by activated macrophages may mediate cancer cell apoptosis and the inhibition of cancer growth. Relatively low concentrations of NO, seen in many different types of clinical cancer samples, promote tumour growth and proliferation.

Furthermore, hypoxic upregulation in tumour cells with increased invasiveness has been linked to reduced nitric oxide signalling (Postovit *et al*, 2002). It was also demonstrated that NO mediates chemosensitivity in tumour cells, and hypoxia-induced drug resistance appears to result, in part, from downstream suppression of endogenous NO

production (Matthews *et al*, 2001). It has therefore been suggested that these results raise the possibility that administration of small doses of NO mimetics could be used as an adjuvant in chemotherapy. The regulation of tumour growth by NO therefore represents an important new area in cancer research.

Data from prior studies therefore generally support the hypothesis of this study that depot or sustained-release NO modulators, which donate relatively high levels of NO, may be effective locoregional antitumour agents.

This study demonstrates that Ethiodol can be used as a sustained-release vehicle for several biologically active NO donors. NO donor in Ethiodol preparations in a dose-dependant manner, inhibit both *in vitro* tumour growth, and also tumour engraftment when delivered subcutaneously *in vivo*, admixed with tumour inoculum in animals. It is important to note that sodium nitroprusside in Ethiodol, a preparation made of clinically available pharmacologic agents, demonstrated prevention of tumour engraftment when given at the same site as the tumour inoculation, but was ineffective when given at a site opposite tumour inoculation. This demonstrates that the antitumour effect was not systemic in nature but locoregional with sustained-release. The sustained-release of NO from donors in Ethiodol within tumours would potentially generate high concentrations NO locally, without concomitant systemic toxicity. Both NO and cyanide generation appear to be central to the observed antitumour effect of sodium nitroprusside in Ethiodol. HAI of Ethiodol containing sodium nitroprusside was associated with marked antitumour activity against established hepatic-metastatic adenocarcinoma. In addition, MADB106 used in the hepatic-metastatic model are not iNOS producing tumour cells (Dr. Alarcon,

University of Pittsburgh, personal communication), and hence would not produce endogenous NO to modulate the therapeutic antitumour effect.

As discussed previously (see section 3.24), the clinical applicability of infusional approaches is limited by the need for operative placement of vascular catheters and cumbersome infusions for variable periods of time. In addition, the regional drug advantage over systemic infusion is often limited by unfavourable first pass pharmacokinetics. Results of this study suggest that Ethiodol may serve as a vehicle for delivering biologically active NO donors selectively to hepatic-metastatic and other tumours by several possible routes. The demonstrated ability of Ethiodol to target tumours after bolus HAI, may obviate the need for indwelling arterial catheters. The sustained-release of NO donors from Ethiodol within tumours would generate high concentrations locally, without concomitant systemic toxicity. Repeated doses could easily be administered through temporary arteriography catheters positioned transfemorally. The ability of Ethiodol to deliver and sustain high concentrations of NO donors at sites of subcutaneous inoculation also suggests an alternative to gene or cell therapy. Ethiodol based delivery of NO donors may be equivalent to such therapy and may actually obviate the need for costly, complex and time consuming methodology. Finally with regard to transition of this research from laboratory to clinical evaluation, patents have been applied for formulations and methods regarding use of nitric oxide mimetics against malignant cell phenotype, which incorporate experimental details reported in this body of work (Adams *et al*, 2005).

4.0 CONCLUSIONS

This research demonstrates that several novel anticancer agents are soluble in Ethiodol, and exhibit depot or sustained-release characteristics. These preparations are effective *in vitro* and *in vivo* and eradicate hepatic-metastatic disease in an experimental animal model, when administered as locoregional HAI therapy.

A reproducible experimental syngeneic animal hepatic-metastatic model was established using F344 rats, which allowed evaluation of Ethiodol based HAI therapeutic preparations.

The topoisomerase-1 inhibitor 9AC, in Ethiodol inhibited tumour engraftment after subcutaneous inoculation. Bolus HAI therapy was found to be well tolerated and was significantly superior in reducing the number of hepatic metastases with decreased toxicity, when compared to the aqueous colloidal dispersion preparation administered in the same manner, or by continuous intraperitoneal systemic therapy.

The cytokine hRIL2, in Ethiodol inhibited tumour engraftment after subcutaneous inoculation in a dose dependent manner. Macrophages appeared to be central to this event. Bolus HAI therapy was well tolerated and was significantly superior in reducing the number of hepatic metastases compared to systemic hRIL2.

The NO donors NOR1, NOR2, NOR3, SNAP and sodium nitroprusside, when dissolved in Ethiodol, all inhibited tumour engraftment after subcutaneous inoculation. NOR1 and sodium nitroprusside in Ethiodol as bolus HAI therapy, were well tolerated and

significantly superior in reducing the number of hepatic metastases compared to controls. For sodium nitroprusside in Ethiodol, both NO and cyanide ion production appeared to contribute to the antitumour effect.

The hypothesis of this body of work was that anticancer agents soluble in Ethiodol will (1) demonstrate depot or sustained-release characteristics; (2) demonstrate effective antitumour activity; and (3) eradicate hepatic-metastatic disease when administered as regional HAI therapy.

The result of this body of work largely supports the hypothesis. In addition, the result validates the principle of depot or sustained-release regional HAI therapy, as an effective method to eradicate hepatic-metastatic disease

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6.0 APPENDIX

6.1 Raw data

RAW DATA FOR FIGURE 3.13

	n=3			mean values				
TIME (hours)	1	3	5	7	24	48	73	96	120
IL2	1220	674	572	327	1208	829	994	701	336

RAW DATA FOR FIGURE 3.14

	n=3		mean values		
	333	166.5	33.3	3.33	0.333
hRIL2	60	55	45	29	0.8
hRIL2/Ethiodol	28	25	2.1	0.4	0.2

RAW DATA FOR FIGURE 3.15

	n=3		mean values	
	333	33.3	3.33	0.333
hRIL2	39	35	24.9	6
hRIL2/Ethiodol	30	14	4.3	1.9

RAW DATA FOR FIGURE 3.16

n=3 in each group	%surviving							
TIME (days)	13	15	17	20	23	26	29	32
Ethiodol	100	100	80	0				
hRIL2/Ethiodol	100	100	100	100	100	100	100	100
PEG/hRIL2	100	100	100	75	25	0		
Incomplete Freunds	100	100	100	100	60	60	40	40

RAW DATA FOR FIGURE 3.17

	n=3 in each group		mean values		
TIME (days)	14	17	19	21	24
Carrageenan	14.1	45	69.25	112.25	164.25
Untreated	0	0	0	0	0

RAW DATA FOR TABLE 3.5

Number of metastases counted in each group

n=14	n=14	n=12	n=4
9AC/E	**CD/9AC/HIA**	**CONTROLS**	**IP/CD/9AC**
19	73	250	
12	250	250	
2	145	180	
28	183	250	
13	235		
3	250	105	
8	139	102	
27	85	250	
32	250	250	
19	174	233	149
13	189	247	162
17	181	250	128
24	223	250	86
42	250		

Wilcoxon - Mann/Whitney Test

n	sum of ranks	
14	105	9AC/E
14	301	CD/9AC/HIA
28	406	total
	203.00	expected value
	21.73	standard deviation
	-4.49	z, corrected for ties
	0.00000721405663006713	p-value (two-tailed)

Wilcoxon - Mann/Whitney Test

n	sum of ranks	
14	105	9AC/E
12	246	CONTROLS
26	351	total
	189.00	expected value
	19.25	standard deviation
	-4.34	z, corrected for ties
	0.00001437843933405070	p-value (two-tailed)

Wilcoxon - Mann/Whitney Test

n	sum of ranks	
14	105	9AC/E
4	66	IP/CD/9AC
18	171	total

133.00	expected value
9.41	standard deviation
-2.92	z, corrected for ties
.0035	p-value (two-tailed)

Wilcoxon - Mann/Whitney Test

n	sum of ranks	
14	161	CD/9AC/HIA
12	190	CONTROLS
26	351	total

189.00	expected value
18.70	standard deviation
-1.47	z, corrected for ties
.1413	p-value (two-tailed)

Wilcoxon - Mann/Whitney Test

n	sum of ranks	
14	149	CD/9AC/HIA
4	22	IP/CD/9AC
18	171	total

133.00	expected value
9.37	standard deviation
1.65	z, corrected for ties
.0980	p-value (two-tailed)

Wilcoxon - Mann/Whitney Test

n	sum of ranks	
12	120	CONTROLS
4	16	IP/CD/9AC
16	136	total

102.00	expected value
7.90	standard deviation
2.22	z, corrected for ties
.0267	p-value (two-tailed)

Descriptive statistics

	CONTROLS
count	12
mean	218.08
sample variance	3,263.90
sample standard deviation	57.13
minimum	102
maximum	250
range	148
1st quartile	219.75
median	250.00
3rd quartile	250.00
interquartile range	30.25
mode	250.00
low extremes	2
low outliers	0
high outliers	0
high extremes	0

Descriptive statistics

	9AC/E
count	14
mean	18.50
sample variance	127.35
sample standard deviation	11.28
minimum	2
maximum	42
range	40
1st quartile	12.25
median	18.00
3rd quartile	26.25
interquartile range	14.00
mode	19.00
low extremes	0
low outliers	0
high outliers	0
high extremes	0

Descriptive statistics

	CD/9AC/HIA
count	14
mean	187.64
sample variance	3,627.94
sample standard deviation	60.23
minimum	73
maximum	250
range	177
1st quartile	152.25
median	186.00
3rd quartile	246.25
interquartile range	94.00
mode	250.00
low extremes	0
low outliers	0
high outliers	0
high extremes	0

Descriptive statistics

	# 1
count	4
mean	131.25
sample variance	1,106.25
sample standard deviation	33.26
minimum	86
maximum	162
range	76
1st quartile	117.50
median	138.50
3rd quartile	152.25
interquartile range	34.75
mode	#N/A
low extremes	0
low outliers	0
high outliers	0
high extremes	0

RAW DATA FOR TABLE 3.6

Number of metastases counted in each group

n=14	n=12	n=8
hRIL2/E	**ETH only**	**hRIL2 only**
81	250	
85	250	
25	250	
102	250	
17	250	67
120	250	250
35	180	53
10	250	105
24		
27	105	115
18	102	63
15	250	108
32	250	250
12		

Wilcoxon - Mann/Whitney Test

n	sum of ranks	
14	107.5	hRIL2/E
12	243.5	ETH only
26	351	total

189.00	expected value
19.04	standard deviation
-4.26	z, corrected for ties
0.00002089256089643500	p-value (two-tailed)

Wilcoxon - Mann/Whitney Test

n	sum of ranks	
14	120	hRIL2/E
8	133	hRIL2 only
22	253	total

161.00	expected value
14.65	standard deviation
-2.77	z, corrected for ties
.0057	p-value (two-tailed)

Wilcoxon - Mann/Whitney Test

n	sum of ranks	
12	153.5	ETH only
8	56.5	hRIL2 only
20	210	total

126.00	expected value
11.84	standard deviation
2.28	z, corrected for ties
.0225	p-value (two-tailed)

Descriptive statistics

	hRIL2/E
count	14
mean	43.07
sample variance	1,375.30
sample standard deviation	37.09
minimum	10
maximum	120
range	110
1st quartile	17.25
median	26.00
3rd quartile	69.50
interquartile range	52.25
mode	#N/A
low extremes	0
low outliers	0
high outliers	0
high extremes	0

Descriptive statistics

	ETH only
count	12
mean	219.75
sample variance	3,349.84
sample standard deviation	57.88
minimum	102
maximum	250
range	148
1st quartile	232.50
median	250.00
3rd quartile	250.00
interquartile range	17.50
mode	250.00
low extremes	2
low outliers	1
high outliers	0
high extremes	0

Descriptive statistics

	hRIL2 only
count	8
mean	126.38
sample variance	6,345.13
sample standard deviation	79.66
minimum	53
maximum	250
range	197
1st quartile	66.00
median	106.50
3rd quartile	148.75
interquartile range	82.75
mode	250.00
low extremes	0
low outliers	0
high outliers	0
high extremes	0

RAW DATA FOR FIGURE 3.7

n=3 in each group **mean values**

	TIME/DAYS				
TREATMENT	10	15	20	25	40
1 mg 9-AC	59	48	44	22	5
0.5 mg 9-AC	84	71	57	46	17
0.25 mg 9-AC	69	68	63	32	13
Ethiodol only	153	431	791		

RAW DATA FOR FIGURE 3.21

n=2 for each sample **n=3 for standard controls**

Nitrite concentration values calculated from mean absorbence values

Days	**NOR1/Ethiodol**	**NOR2/Ethiodol**	**NOR3/Ethiodol**	**SNAP/Ethiodol**
0	0	0	0	0
1	17148.5	18715.5	14178.5	24838
2	9246.5	10999	8763	14986
3	3670	6767	4681.5	10745

RAW DATA FOR FIGURE 3.22

Days	Sodium nitroprusside concentrations with corresponding nitrite concentrations over time					
	200µL	100µL	50µL	20µL	10µL	Ethiodol
0	0	0	0	0	0	0
1	283	117	77	97	36	0
2	167	103	94	147	89	0
3	214	195	213	165	11	0
4	342	315	299	154	87	0
5	489	361	327	129	75	0
6	588	377	392	131	60	0
7	842	573	434	126	75	0
8	867	503	380	109	32	0
9	692	424	302	109	108	0
11	665	331	208	85	31	1

RAW DATA FOR FIGURE 3.27

n=3 in each group **mean values**

	2	4	6	9	11	13	15	18	21
Hanks	0	39	115	81	170	160	315	770	900
hRIL2/Ethiodol	0	1	51	48	46	26	36	83	69
Ethiodol	0	5	12	21	78	104	115	198	402
NOR1/Ethiodol	0	0	0	0	0	0	0	14	3
NOR1/hRIL2/Ethiodol	0	0	4	14	9	27	16	27	38

RAW DATA FOR FIGURE 3.28

n=3 in each group

	9	11	13	15	18	21	24
Hanks	100	80	80	60	60	40	0
hRIL2/Ethiodol	100	100	100	100	100	100	100
Ethiodol	100	100	100	100	80	40	0
NOR1/Ethiodol	100	100	100	100	100	100	100
NOR1/hRIL2/Ethiodol	100	100	100	100	100	100	100

RAW DATA FOR FIGURE 3.39

n=3 in each group **mean values**

	10	14	17	21	24	28	31
Ethiodol	6	22	42	78	136	224	323
NOR3/Ethiodol	0	0	0	0	0	0	0
NOR4/Ethiodol	7	37	79	138	207	224	244
SNAP/Ethiodol	0	0	0	0	0	0	0
PTIO/Ethiodol	2	34	64	132	177	208	272

RAW DATA FOR FIGURE 3.40

n=3 in each group

	21	24	28	31
Ethiodol	100	80	20	0
NOR3/Ethiodol	100	100	100	100
NOR4/Ethiodol	100	80	60	40
SNAP/Ethiodol	100	100	100	100
PTIO/Ethiodol	100	100	100	60

RAW DATA FOR FIGURE 3.41

n=3 in each group **mean values**

	Ethiodol	NOR2/Ethiodol
0	0	0
13	19	1
17	61	3
21	88	4
24	162	1
27	213	0.17
31	264	0.17

RAW DATA FOR FIGURE 3.42

n=3 in each group

	Ethiodol	NOR2/Ethiodol
17	100	100
21	100	100
24	50	100
27	16	100
31	0	100

RAW DATA FOR FIGURE 3.43

n=3 in each group **mean values**

	Ethiodol	Nit/Eth/cis	Nit/Eth/trans	Nit//Eth/PTIO	Nit/Eth/thio
0	0	0	0	0	0
13	68	0.25	35	0.25	7
16	129	0.125	49	22	16

RAW DATA FOR TABLE 3.9

HAI NOR1/E	ETHIODOL
90	250
3	155
13	250
23	250
3	250
7	250

Wilcoxon - Mann/Whitney Test

n	sum of ranks	
6	21	Group 1
6	57	Group 2
12	78	total

39.00	expected value
6.01	standard deviation
-2.91	z, corrected for ties
.0036	p-value (two-tailed)

Descriptive statistics

	# 1
count	6
mean	23.17
sample variance	1,128.97
sample standard deviation	33.60
minimum	3
maximum	90
range	87
1st quartile	4.00
median	10.00
3rd quartile	20.50
interquartile range	16.50
mode	3.00
low extremes	0
low outliers	0
high outliers	0
high extremes	1

Descriptive statistics

	# 1
count	6
mean	234.17
sample variance	1,504.17
sample standard deviation	38.78
minimum	155
maximum	250
range	95
1st quartile	250.00
median	250.00
3rd quartile	250.00
interquartile range	0.00
mode	250.00
low extremes	0
low outliers	0
high outliers	0
high extremes	0

RAW DATA FOR TABLE 3.10

NITROPRUSSIDE/ETH	ETHIODOL
7	35
37	250
13	72
39	250
250	250
14	250
51	250
14	70
10	
3	
16	
21	
11	
24	

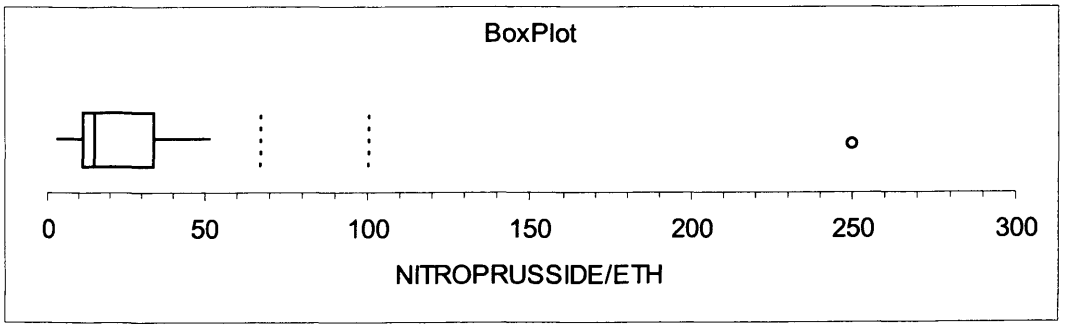
Wilcoxon - Mann/Whitney Test

n	sum of ranks	
14	113.5	NITROPRUSSIDE/ETH
8	139.5	ETHIODOL
22	253	total

161.00	expected value
14.50	standard deviation
-3.24	z, corrected for ties
.0012	p-value (two-tailed)

Descriptive statistics

	NITROPRUSSIDE/ETH
count	14
mean	36.43
sample variance	3,963.49
sample standard deviation	62.96
minimum	3
maximum	250
range	247
1st quartile	11.50
median	15.00
3rd quartile	33.75
interquartile range	22.25
mode	14.00
low extremes	0
low outliers	0
high outliers	0
high extremes	1



Descriptive statistics

	ETHIODOL
count	8
mean	178.38
sample variance	9,895.41
sample standard deviation	99.48
minimum	35
maximum	250
range	215
1st quartile	71.50
median	250.00
3rd quartile	250.00
interquartile range	178.50
mode	250.00
low extremes	0
low outliers	0
high outliers	0
high extremes	0

6.2 Sources of laboratory agents

AGENT	SOURCE
3,3'-diaminobenzidine	Sigma Labs, St. Louis, MO
[^{3}H]thymidine	NEN/Dupont, Boston, MA
9AC	Pharmacia, Columbus, OH
10% foetal calf serum	GIBCO, Grand Island, NY
30 gauge PE-10 Intramedic polyethylene tubing	Clay Adams, Parsippany, NJ
Acid: HCL, phosphoric	Sigma Labs, St. Louis, MO
AIM V	GIBCO, Grand Island, NY
Anti-CD3, CD4, CD8 antibodies	Pharmingen, San Diego, CA
Avidin-biotin-peroxidase complex	Vector Labs, Burlingame, CA
Anti-asialo GM1	Wako Bioproducts, Richmond, VA
Athymic (nu/nu) mice	Harlan Sprague Dawley, Inc, Indianapolis, IN
BALB/C mice	Jackson Laboratory, Bar Harbor, ME
C57BL/6 mice	Jackson Laboratory, Bar Harbor, ME
Carrageenan	Sigma Labs, St. Louis, MO
Cautery device	Aaron Medical Inc., St. Petersburg, FL
Cell-culture inserts	Becton Dickinson, Franklin Lakes, NJ
Colloidal dispersion (CD) 9AC	Pharmacia, Columbus, OH
Cryogenic vials	Corning Glassware, Corning, NY
Daudi cells	ATTC, Rockville, MD
Dexon 4-0	Davis & Geck, Manati, PR
DMSO	Sigma Labs, St. Louis, MO
Ethanol 70%	Fisher Scientific, Pittsburgh, PA
Ethiodol	Savage Labs., Melville, NY
Fisher (F344) rats	Taconic Farms, Germantown, NY
Glutamine	GIBCO, Grand Island, NY
Goat serum	GIBCO, Grand Island, NY

Hank's balanced salt solution (HBSS)	GIBCO, Grand Island, NY
hRIL2	Chiron Corporation, Emeryville, CA
Incomplete Freund's adjuvant	GIBCO, Grand Island, NY
K562	ATTC, Rockville, MD
M 2-mercaptoethanol	Sigma Labs., St. Louis, MO
MADB106 cells	W. Chambers, Univ. Pittsburgh, PA
Methoxyflurane	Pitman-Moore, Mundelein, IL
Mayer's haematoxylin	Sigma Labs., St. Louis, MO
MC38 cells	ATTC, Rockville, MD
Microtiter plates	Becton-Dickinson & Co., Franklin Lakes, NJ
Mini-osmotic pump	Alzet Corp., Palo Alto, Ca
Needles 30 gauge	Becton-Dickinson & Co., Franklin Lakes, NJ
Nembutal	Abbott Laboratories, North Chicago, IL
Nitropress (Sodium Nitroprusside)	Abbott Laboratories, North Chicago, IL
NO modulators: SNAP, NOR1, NOR2, NOR3, NOR4, PTIO	Alexis Biochemicals, San Diego, CA
Nonessential amino acids	GIBCO, Grand Island, NY
Nytex	Lawshe Industrial Co. Inc., Bethesda, MD
OCT	Tissue Tek, Elkhart, IN
PEG IL2	Chiron Corporation, Emeryville, CA
Penicillin	GIBCO, Grand Island, NY
PHA	Sigma Labs, St. Louis, MO
Polyinosinic: polycytidylic acid	Sigma Labs, St. Louis, MO
Rabbit serum	Wako Bioproducts, Richmond, VA
RPMI 1640	GIBCO, Grand Island, NY
Saline 0.9%, TBS, PBS	Sigma Labs, St. Louis, MO
Scintillation counter	Pharmacia, Gaitherburg, MD
Silk suture 7-0	Ethicon Inc., Somerville, NJ
Skatron Titertek System	Skatron AS, Lierbyen, Norway
Sodium ^{51}Cr-chromate	NEN/Dupont, Boston, MA
Sodium pyruvate	GIBCO, Grand Island, NY
Streptomycin	GIBCO, Grand Island, NY
Thiosulfate	Sigma Labs, St. Louis, MO

Triton-X	Sigma Labs, St. Louis, MO
Trypsin/EDTA	GIBCO, Grand Island, NY
TSA cells	ATTC, Rockville, MD
Type 4 collagenase	Sigma Labs, St. Louis, MO
YAC1 cells	ATTC, Rockville, MD

6.3 Details of standard laboratory techniques

Cell Tissue Culture:

In this procedure, a primary culture is grown to confluency in tissue culture medium. Cells are dispersed by trypsin treatment and then reseeded into secondary cultures. The process of removing cells from the primary culture and transferring them to secondary cultures constitutes a passage, or subculture. All culture incubations should be performed in a humidified 37°C, 5% CO2 incubator unless otherwise specified.

Materials:

- Primary cultures of cells
- HBSS
- Trypsin/EDTA solution
- Complete medium
- Sterile Pasteur pipets
- 37°C incubator
- Tissue culture plastic ware or glassware including pipets and flasks

Protocol:

- Remove all medium from primary culture with a sterile Pasteur pipette. Wash adhering cell monolayer once or twice with a small volume of 37°C HBSS to remove any residual FBS that may inhibit the action of trypsin
- Add enough 37°C trypsin/EDTA solution to culture to cover adhering cell layer

- Place plate on a 37°C warming tray 1 to 2 min. Tap bottom of plate on the countertop to dislodge cells. Check culture with an inverted microscope to be sure that cells are rounded up and detached from the surface
- Add complete medium at 37°C. Draw cell suspension into a Pasteur pipette and rinse cell layer two or three times to dissociate cells and to dislodge any remaining adherent cells. As soon as cells are detached, add serum or medium containing serum to inhibit further trypsin activity that might damage cells.
- Add an equal volume of cell suspension to fresh plates or flasks that have been appropriately labelled
- Add fresh medium to each new culture. Incubate in a humidified 37°C, 5% CO_2 incubator
- If necessary, feed subconfluent cultures after 3 or 4 days by removing old medium and adding new 37°C medium
- Passage secondary culture when it becomes confluent

Cell Freezing:

In this procedure, cultured cells are frozen. To preserve cells, avoid senescence, reduce the risk of contamination, and minimize effects of genetic drift, cell lines may be frozen for long-term storage. Without the use of a cryoprotective agent freezing would be lethal to the cells in most cases. A cryoprotective substance such as dimethylsulfoxide (DMSO) is used in conjunction with complete medium for preserving cells at –70°C or lower. DMSO acts to reduce the freezing point and allows a slower cooling rate. Gradual freezing reduces the risk of ice crystal formation and cell damage.

Materials:

- Monolayer culture of cells
- Complete medium
- Freezing medium: complete medium supplemented with 10% to 20% (v/v) FBS and 5% to 10% (v/v) DMSO, 4°C
- Bench top clinical centrifuge

Protocol:

- Prepare a single-cell suspension of target cells in complete medium
- Trypsinize cells
- Transfer cell suspension to a sterile centrifuge tube and add 2 mL complete medium with serum. Centrifuge 5 min at 300 to 350 × *g* (~1500 rpm), room temperature
- Remove supernatant and add 1 mL of 4°C freezing medium. Resuspend pellet
- Add 4 mL of 4°C freezing medium, mix cells thoroughly, and place on wet ice
- Count cells using a haemocytometer. Dilute with more freezing medium as necessary to get a final cell concentration of 10^6 or 10^7 cells/mL
- Pipette 1-mL aliquots of cell suspension into labelled 2-mL cryovials. Tighten caps on vials
- Place vials 1 hr to overnight in a –70°C freezer, then transfer to liquid nitrogen storage freezer

Chromium-Release Assay for Measuring CTL Activity:

In this procedure, target cells are briefly labelled with ^{51}Cr, washed, then mixed with effector CTL at appropriate effector-to-target (E:T) ratios for varying periods of time. The amount of ^{51}Cr released into the supernatant by killed target cells is quantitated. By comparison with ^{51}Cr release of controls, the corrected percent lysis is calculated for each concentration of effector cells.

Materials:

- Target cells
- Control target cells
- Complete medium
- Sensitization medium
- Mitogen solution
- ~1 mCi/ml $Na_2{}^{51}CrO_4$ in isotonic medium, sterile and pyrogen-free (200 to 500 μCi/μg; DuPont NEN or Amersham)
- Fetal bovine serum (FBS), heat-inactivated 1 hr at 56°C
- Effector cells
- Control effector cells
- 2% (v/v) Triton X-100 in H_2O
- 25-cm^2 tissue culture flasks
- 24-well flat-bottom microtiter plates, 2-ml capacity, with lids
- Nylon filtration fabric, 112-μm mesh (optional)
- 15-ml disposable polystyrene conical tubes with screw caps
- Sorvall H-1000B rotor (or equivalent) and microtiter plate carrier

- Multiwick supernatant harvesting system (Skatron)
- 96-well round-bottom microtiter plates with lids to fit supernatant harvesting system
- Multichannel pipettor (50- to 200-µl) with disposable tips
- ^{51}Cr counting tubes (Skatron)

Protocol:

- Prepare a single-cell suspension of target cells in complete medium
- Transfer cells to 15-mL conical tube and wash cells once in 14 mL complete medium by centrifuging 5 min in ~200 × *g* (1000 rpm in Sorvall H-1000B rotor), room temperature. Discard supernatant
- Resuspend cells in 5 mL complete medium. Allow cell aggregates to settle by gravity for several minutes or pass cell suspension through a single layer of 112-µm-mesh nylon filtration fabric resting on the open top of a 15-mL conical tube (press a pipette tip into the nylon fabric to form a small funnel)
- Determine viable cell count of unsettled or filtered cells by trypan blue exclusion
- Centrifuge $\leq 5 \times 10^7$ cells 5 min at 200 × *g*, room temperature, in a 15-mL conical tube. Discard most of supernatant, but leave ~0.1 mL medium on the pellet
- Gently resuspend cells in remaining complete medium. Add 0.2 mL of 1 mCi/mL ^{51}Cr solution and 20 µL FBS. Mix gently and incubate in a loosely capped 15-mL conical tube ~45 min for lymphocytes or 1 to 2 hr for tumour cells at 37°C, 5% CO2
- Prepare effector cells by repeating prior steps and resuspend in complete medium at 10^7 cells/mL. Prepare controls that differ from the test effector cells only in antigen

specificity (no sensitized cells or cells sensitized against irrelevant antigens). Prepare a series of tubes with 3-fold serial dilutions in complete medium for each source of effector cells

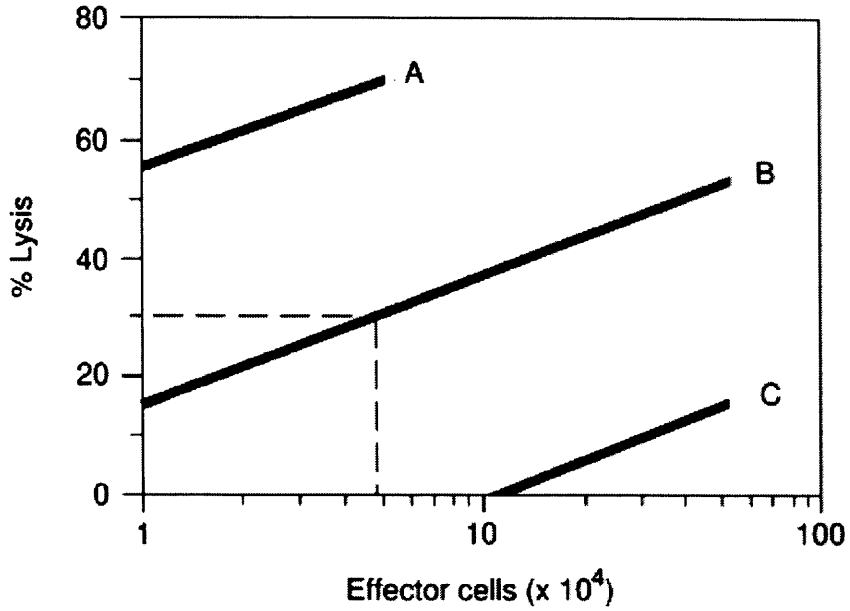
- Dispense 0.1 mL effector cells (or controls) to the wells of 96-well microtiter plates, with replicates of three or four wells for each effector cell concentration
- Wash ^{51}Cr-labeled target cells 2 to 3 times with 14 mL complete medium as before, aspirating down to the cell pellet between washes and collecting the supernatant in a radioactive waste container (tube should be capped in the centrifuge). Resuspend labelled target cells in complete medium to 104–106 cells/mL
- Add 0.1 ml ^{51}Cr-labeled target cells to replicate wells containing one of the following, for a final volume of 0.2 mL/well:
    - 0.1 mL effector cells
    - 0.1 mL control lymphocytes
    - 0.1 mL medium (to measure spontaneous 51Cr release)
- Centrifuge plates 30 sec at 200 × *g* in a Sorvall H-1000B rotor with microtiter plate carrier to promote contact between effector and target cells. Incubate plates 3 to 6 hr in a humidified 37°C, 5% CO2 incubator
- Centrifuge plates 5 min at 200 × *g*. Add 0.1 mL of 2% Triton X-100 (lysing agent) to measure maximum releasable ^{51}Cr to a replicate set of empty wells with 0.1 mL target cells and mix by pipetting. Harvest ~0.1 mL of each supernatant to ^{51}Cr counting tubes using a multichannel pipettor or all of the supernatants with the supernatant harvester system
- Count ^{51}Cr in γ-scintillation counter, 1 to 2 min/sample
- Calculate corrected percent lysis for each concentration of effector cells, using the mean cpm for each replicate of wells, where test refers to effector cells with CTL

activity and control refers to nonlytic cells ("control effector cells") or cell-free medium. (Release of ^{51}Cr from target cells incubated in medium alone is often referred to as "spontaneous release"):

$$\text{corrected \% lysis} = 100 \times \frac{\text{test } ^{51}\text{Cr released} - \text{control } ^{51}\text{Cr released}}{\text{maximum } ^{51}\text{Cr} - \text{control } ^{51}\text{Cr released}}$$

- Present CTL data in lytic units, graphs, or lysis values

  [Lytic units: A lytic unit (LU) is arbitrarily defined as the number of lymphocytes required to yield the selected lysis value (e.g., 30%). It is determined in a manner similar to calculating antibody titer as follows. Plot corrected % lysis values versus the log of the effector cell number for each effector cell preparation. Select a lysis value (e.g., 30%) through which most of the declining titration curves pass. Ideally, titration plots for each of the different sources of effector cells should be straight and parallel in this region. With this value, determine the number of LU per 106 effector cells or per culture (if the effector cells were generated *in vitro*) or per organ (if the effector cells were freshly explanted). For example if 5×10^4 cells from a particular sample produces 30% lysis then the sample has 20 LU/10^6 cells. If the titration curve of an effector-cell preparation fails to reach the selected lysis value (e.g., 30%), refer to activity as greater or less than x LU, where "x" is the calculated maximum or minimum level. Figure below illustrates sample results of a lytic unit plot.]



Sample figure: Lytic units (LU). Population A has >20 LU/106 cells, and C has <20 LU/106 cells.

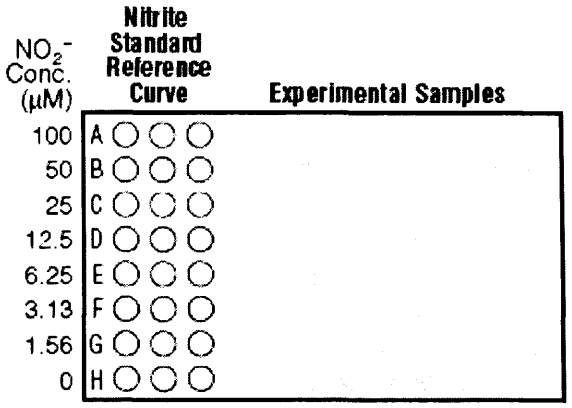
Greiss Assay:

In this procedure, the Griess reaction system measures a surrogate for estimation NO formation. Greiss assay relies on a diazotization reaction, and measures nitrite production (NO_2^-), which is one of the two primary, stable and non-volatile breakdown products of NO.

Materials:

- 1% sulfanilamide solution in 5% phosphoric acid
- 0.1% N-1-napthylethylenediamine dihydrochloride
- Nitrite standard (0.1M sodium nitrite in water)
- Reagent reservoirs and multichannel pipettor
- 96-well flat-bottom enzymatic assay plate
- Plate reader with 520-550nm filter

Protocol:



Preparation of nitrite standard reference curve; methods reference sample 96-well plate as illustrated above:

- Prepare 1ml of a 100μM nitrite solution by diluting the provided 0.1M Nitrite Standard 1:1,000 in the matrix or buffer used for the experimental samples
- Designate 3 columns (24 wells) in the 96-well plate for the Nitrite Standard reference curve. Dispense 50μl of the appropriate matrix or buffer into the wells in rows B-H
- Add 100μl of the 100μM nitrite solution to the remaining 3 wells in row A
- Immediately perform 6 serial twofold dilutions (50μl/well) in triplicate down the plate to generate the Nitrite Standard reference curve (100, 50, 25, 12.5, 6.25, 3.13 and 1.56μM), discarding 50μl from the 1.56μM set of wells. Do not add any nitrite solution to the last set of wells (0μM). The final volume in each well is 50μl, and the nitrite concentration range is 0.100μM

Nitrite measurement (Griess reaction):

- Allow the Sulfanilamide Solution and NED Solution to equilibrate to room temperature (15-30 min)
- Add 50μl of each experimental sample to wells in duplicate or triplicate

- Using a multichannel pipettor, dispense 50µl of the sulfanilamide solution to all experimental samples and wells containing the dilution series for the nitrite standard reference curve
- Incubate 5-10 min at room temperature, protected from light
- Using a multichannel pipettor, dispense 50µl of the NED Solution to all wells
- Incubate 5-10 minutes at room temperature, protected from light. A purple/magenta colour will begin to form immediately
- Measure absorbance within 30 min in a plate reader with a filter between 520.550nm
- Measure absorbance within 30 min. Colour may fade after this time

Determination of nitrite concentrations in experimental samples:

- To generate a nitrite standard reference curve, plot the average absorbance value of each concentration of the nitrite standard as a function of "Y" with nitrite concentration as a function of "X"
- Determine average absorbance value of each experimental sample
- Determine its concentration by comparison to the Nitrite Standard reference curve. [Sulfanilamide and NED compete for nitrite in the Griess reaction; thus greater sensitivity is achieved when the two components are added sequentially. Add the Sulfanilamide Solution to the sample first, incubate for 5.10 minutes, then add the NED Solution. To ensure accurate nitrite quantitation, prepare a reference curve with the nitrite standard for each assay, using the same matrix or buffer used for experimental samples. Due to substances that interfere with the Griess reaction, different levels of sensitivity may be achieved in different buffers or matrices. Absorbance spectrum of the coloured azo compound and a sample series of

representative reference curves for the nitrite standard in various matrices are given below.]

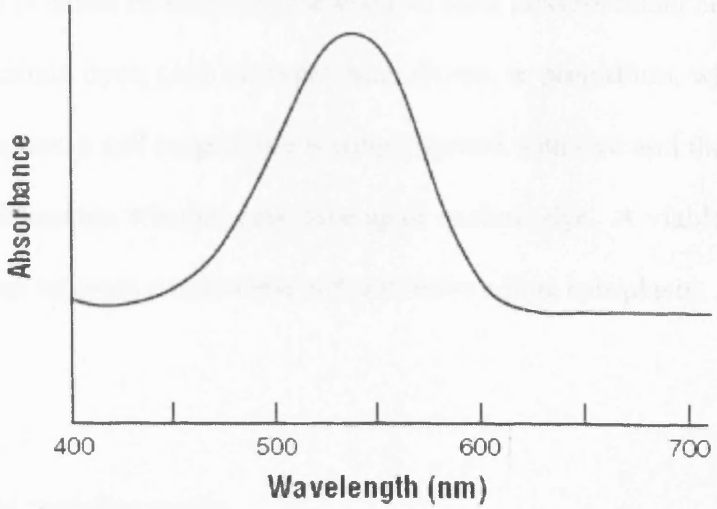
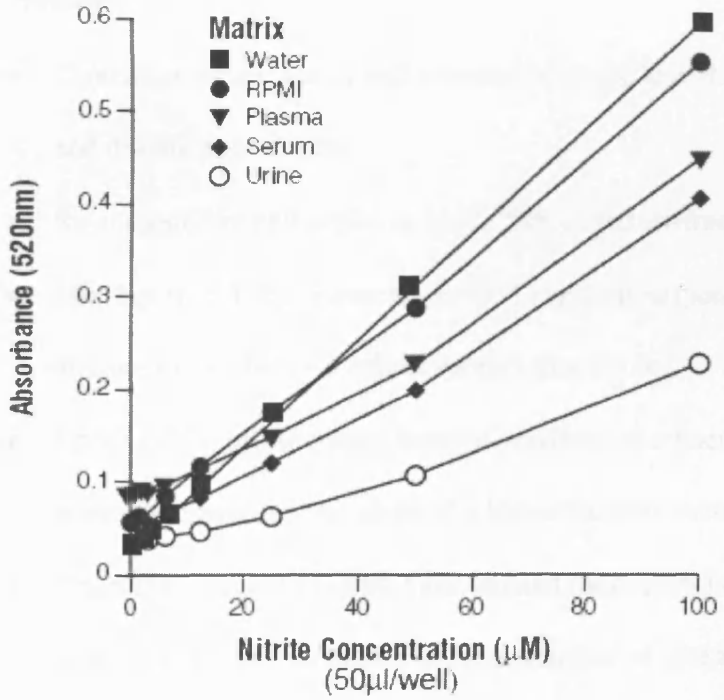


Illustration (above) of the absorbance spectrum of the coloured azo compound.



Sample representative nitrite standard reference curves (above) in various matrices. Assays were performed as described as given in the methods using the nitrite standard in the following undiluted matrices: water, RPMI1640 containing 15% serum and 5.3mg/L phenol red, bovine plasma, bovine calf serum and human urine.

Trypan Blue Exclusion Test of Cell Viability:

The dye exclusion test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, Eosin, or propidium, whereas dead cells do not. In this test, a cell suspension is simply mixed with dye and then visually examined to determine whether cells take up or exclude dye. A viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm.

Materials:

- PBS
- Serum free complete media
- 0.4% trypan blue

Protocol:

- Centrifuge an aliquot of cell suspension being tested for viability 5 min at 100 × *g* and discard supernatant
- Re-suspend the cell pellet in 1 mL PBS or serum-free complete medium
- Mix 1 part of 0.4% trypan blue and 1 part cell suspension (dilution of cells). Allow mixture to incubate ~3 min at room temperature
- Apply a drop of the trypan blue/cell mixture to a haemocytometer. Place the haemocytometer on the stage of a binocular microscope and focus on the cells
- Count the unstained (viable) and stained (nonviable) cells separately in the haemocytometer. To obtain the total number of viable cells per mL of aliquot, multiply the total number of viable cells by 2 (the dilution factor for trypan blue). To obtain the total number of cells per mL of aliquot, add up the total number of viable and nonviable cells and multiply by 2

- Calculate the percentage of viable cells as follows:

$$\text{viable cells (\%)} = \frac{\text{total number of viable cells per ml of aliquot}}{\text{total number of cells per ml of aliquot}} \times 100$$

7.0 PUBLICATIONS

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A Comparison of Locoregional Depot and Systemic Preparations of 9-Aminocamptothecin for Treatment of Liver Metastases in a Rat Tumor Model: Superior Antitumor Activity of Sustained-Release Preparation

Keith A. Dookeran, MBBS FRCS (Ed), Ann Brumfield, BS, and Joshua T. Rubin, MD

Background: Locoregional therapy of hepatic-metastatic disease may overcome the limitations of systemic therapy by allowing tumor drug dose intensification without increasing systemic toxicity. This may result in improved efficacy. We have demonstrated that the novel topoisomerase I inhibitor 9-Aminocamptothecin (9-AC) when dissolved in Ethiodol acts as a sustained release preparation, with good antitumor activity. Its benefit as a depot hepatic intraarterial (IA) therapy for hepatic metastases was compared to systemic therapy with an aqueous colloidal dispersion (CD) preparation of 9-AC in a rat model, since a lack of demonstrable benefit of the locoregional therapy, would argue against further clinical evaluation.

Methods: Fisher rats underwent direct intraportal injection of 2×10^5 MADB106 adenocarcinoma cells and were treated 6 to 7 days later with: (a) bolus IA 9-AC (60 μg) in 60 μl of Ethiodol; (b) bolus IA CD/9-AC (60 μg) in 200 μl water; (c) bolus IA Ethiodol only (60 μl), or (d) CD/9-AC (60 μg) in 200 μl water, via a mini-osmotic pump pumping at 1 μl/hr for 7 days intraperitoneally (IP). Livers were harvested 10 to 12 days later, and the number of metastases on the surface were counted blindly.

Results: Bolus hepatic IA 9-AC/Ethiodol was found to be significantly superior in reducing the number of hepatic metastases, when compared to the aqueous CD preparation administered in the same manner, or by continuos infusion via mini-osmotic pump IP ($p < 0.01$). Systemic therapy was also associated with substantial toxicity.

Conclusions: These results suggest that locoregional therapy of hepatic neoplasms with 9-AC/Ethiodol would be associated with clinical efficacy far exceeding that associated with its systemic administration.

Key Words: 9-Aminocamptothecin—Sustained-release preparation—Locoregional treatment—Hepatic metastases.

We remain in need of effective therapies for treatment of hepatic-metastatic disease (1). It would be beneficial if new therapies were inexpensive and relatively simple to administer. Locoregional therapy for patients with hepatic-metastatic disease is attractive because it allows for tumor-directed dose intensification without associated increase in systemic toxicity (2). This may translate to improved efficacy. The use of sustained-release or depot preparations may obviate the need for continuous infusion via implantable pumps. 9-Aminocamptothecin (9-AC) is a water-insoluble topoisomerase-1 inhibitor that has been shown to be superior to 5-fluorouracil (5-FU) for treatment of liver metastases in mouse animal models, with significant prolongation of survival (3). We have demonstrated that the lymphangiographic oil Ethiodol, which is selectively retained in liver

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tumors after hepatic intraarterial (IA) infusion (4), acts as a delivery vehicle for 9-AC. We have also shown that 9-AC is released from Ethiodol (solution of 5 mg/ml) in a sustained fashion beyond 5 days, reaching concentrations of 10 μg/ml in plasma, of which approximately 50% of the drug [detected by high-performance liquid chromatography (5)] is the active lactone form. The released drug also shows both *in vitro* and *in vivo* antitumor activity (6,7).

It is important to compare the antitumor activity of sustained release 9-AC/Ethiodol and systemic aqueous 9-AC, as treatment for isolated liver metastases in a preclinical model, since a lack of demonstrable advantage in efficacy for the locoregional treatment would argue against its implementation in phase I-II clinical trials. This study sought to determine whether locoregional 9-AC/Ethiodol was a more effective antitumor preparation than systemically administered aqueous 9-AC, as treatment for isolated liver metastases in an experimental rat tumor model.

MATERIALS AND METHODS

Rats

Fisher (F344) rats (Taconic Farms, Germantown, NY, USA) were housed and fed in accordance with University of Pittsburgh research guidelines. They were allowed to acclimatize for at least 2 weeks prior to use in these studies. They were exposed to alternating 12-h light and dark cycles. Animals used in these experiments weighed approximately 350 g.

Tumor Cells Lines

MC38 is a colonic adenocarcinoma sygenic to C57BL/6 mice. MADB-106 (8) (W. Chambers, Pittsburgh, PA, USA) is a cultured breast adenocarcinoma cell line syngenic to F344 rats. All tumor cell lines were cultured in complete medium consisting of RPMI 1640 supplemented with 10% fetal calf serum (Gibco, Grand Island, NY, USA), 100 mg/ml streptomycin (Gibco), 100 U/ml penicillin (Gibco), 3% glutamine (Gibco), 1 mM sodium pyruvate (Gibco), 0.1 mM nonessential amino acids (Gibco), and 5×10^{-5} M 2-mercaptoethanol (Sigma Labs., St. Louis, MO, USA).

In Vitro Studies

The sustained release antitumor activity of 9-AC (Pharmacia, Columbus, OH, USA) in Ethiodol (Savage Labs., Melville, NY, USA) was confirmed using a previously detailed method (6,7) prior to commencing *in vivo* studies. Briefly, MC38 and MADB-106 cell lines were cultured on cell culture inserts in 6-well plates at a seeding dose of 5×10^4 cells. Once growth was established, 200 μg 9-AC in 40 μl Ethiodol was inserted into the test wells. The same amount of Ethiodol only was inserted into control wells. Cell death in the test wells was indicative of release of active 9-AC (as determined by trypan blue exclusion). The cell culture insert was then replaced with an insert with fleshly cultured cells. Half the media in the wells was also replaced with fresh medium daily. This was done for a period of 10 days which was equal to the planned period of drug exposure for *in vivo* experiments.

The effectiveness of the mini-osmotic pump (Alzet Corp., Palo Alto, CA, USA) in releasing-water soluble, active colloidal dispersion (CD) 9-AC was tested by inserting the pump containing 60 μg CD/9-AC in 200 μl water, into a 50-cm^2 culture flask with 5×10^4 MADB-106 cells established in culture. Cell death was indicative of release of active drug. The mini-osmotic pump was then transferred to a flask with freshly cultured cells and new media. This was also done for a period of 10 days.

In Vivo Studies

Titration of 9-AC/Ethiodol Drug-Efficacy in an Engraftment Model

1×10^6 MADB-106 tumor cells were admixed with 200 μl Ethiodol only, or plus 9-AC in doses of 1000, 500, or 250 μg and injected into the abdominal flank of F344 rats. Tumor growth was measured with calipers over a 6-month period. Animals with tumors larger than 2×2 cm in size were sacrificed.

Generation of Hepatic Metastases

Detailed methods of tumor cell preparation and inoculation are described elsewhere (9). Briefly, F344 rats underwent laparotomy and direct intraportal injections with 2×10^5 MADB-106 tumor cells in 150–200 μl RPMI 1640 under general anesthesia.

Liver Metastases Treatment Groups

All groups were treated 6–7 days after inoculation as follows. Group A received bolus hepatic IA 9-AC (60 μg) in 60 μl of Ethiodol. Group B received bolus hepatic IA aqueous colloidal dispersion (CD) 9-AC (60 μg) in 200 μl water. Group C received bolus hepatic IA Ethiodol only (60 μl). Group D received CD/9-AC (60 μg) in 200 μl water, via mini-osmotic

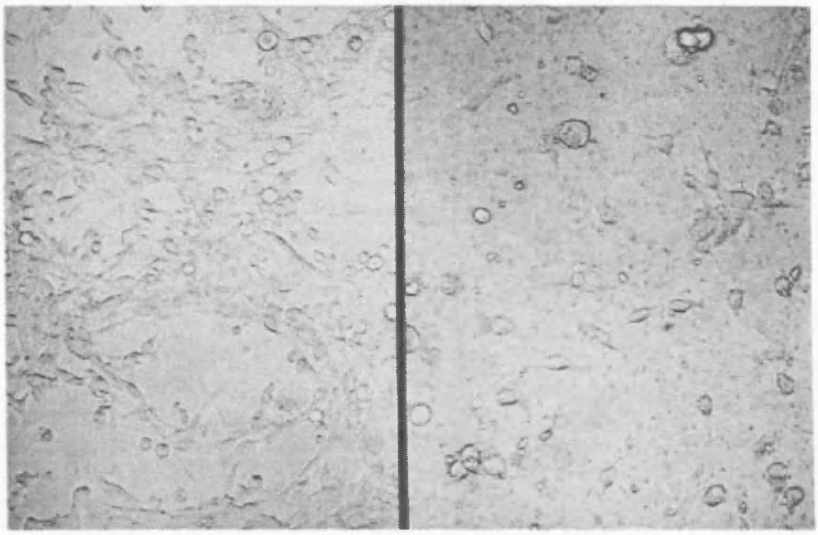

FIG. 1. Cytotoxicity and growth inhibition of MC38 culture. Left photograph shows normal culture growth, right photograph shows extensive cell death.

pump pumping at 1 μl/hr for 7 days intraperitoneally (IP).

Detailed methods of hepatic IA treatment are described elsewhere (9). Briefly rats underwent laparotomy under general anesthesia and cannulation of the gastroduodenal artery (GDA) with a 30-gauge cannula. The common hepatic artery was clamped and the GDA ligated distally to prevent retrograde flow. The hepatic IA treatment was administered and the cannula was flushed with 100 μl of 0.9% saline (Sigma) to ensure that the entire treatment volume was given.

Tumor Evaluation

Rats were sacrificed and livers were harvested 10–12 days after drug treatment. The number of metastases on the liver surface was counted blindly. Based on our previous observations, this method was determined to be the most sensitive and reliable for evaluation of tumor progression in this model. No other method of tumor evaluation was therefore employed.

Statistical Analysis

Statistical analysis between groups was performed using Kruskal-Wallis nonparametric analysis of variance and Wilcoxon rank sum test.

RESULTS

9-AC/Ethiodol Demonstrates Effective Sustained Antitumor Activity In Vitro

9-AC in Ethiodol was effective in causing cell death (Fig. 1). Fresh cells in established culture on inserts were replaced at 3-day intervals over a 10-day period. All cultures experienced almost total cell death at 2 days after insertion into test wells. This confirmed sustained release of active drug over a 10-day test period.

Mini-Osmotic Pump Continuously Releases CD/9-AC with Effective Antitumor Activity In Vitro

Aqueous CD/9-AC released from the mini-osmotic pump was also effective in causing cell death. Fresh cells in an established culture in a 50-cm^2 flask were exposed at 3-day intervals to the primed pump. Cultures experienced almost total cell death at 2 days after pump insertion. This confirmed continuos release of active drug over a 10-day test period.

9-AC/Ethiodol is Effective in Preventing Tumor Engraftment

Tumor engraftment occurred in rats treated with Ethidol only. Engraftment was effectively prevented in all test doses of 9-AC (Fig. 2).

Treatment of Liver Metastases: IA 9-AC/Ethiodol is Superior to Systemic CD/9-AC

There were no significant differences in the mean number of metastases between groups treated with hepatic IA CD/9-AC and those treated with Ethiodol only (Table 1). There was significant reduction in the mean number of metastases for both the hepatic IA 9-AC/Ethidol and systemic IP CD/9-AC groups when compared to the IA CD/9-AC and Ethiodol groups

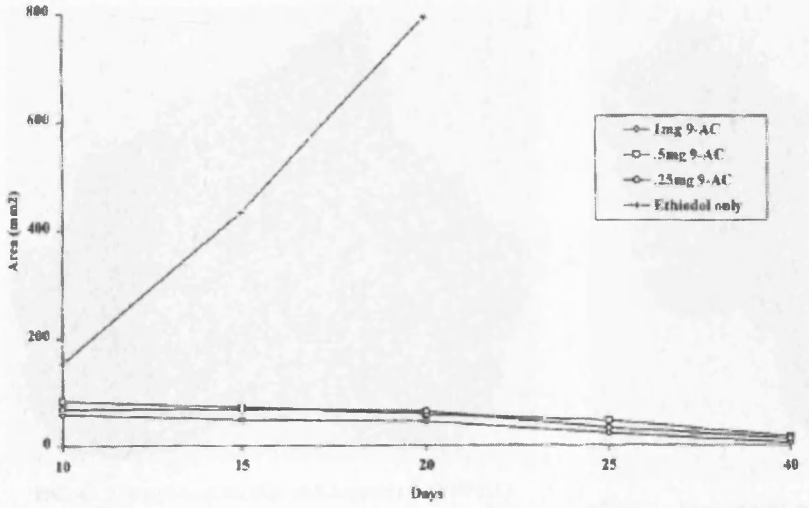

FIG. 2. 9-AC in Ethiodol prevents MADB-106 tumor engraftment in F344 rats.

(Fig. 3–6). However, the reduction in the mean number of metastases for the hepatic IA 9-AC/Ethidol group was noted to be greater than in the IP CD/9-AC group. This difference was statistically significant ($p < 0.01$). 9-AC/Ethiodol hepatic IA therapy was well tolerated. In contrast, systemic continuous infusion of aqueous CD/9-AC via mini-osmotic pump IP was associated with anorexia, vomiting, weight loss, and ruffled fur with hair loss.

DISCUSSION

The iodinated poppyseed oil Ethiodol, is selectively retained within hepatic tumors for weeks to months (4), in contrast to normal liver parenchyma where it is cleared in days (10). Ethiodol is thought to have no antitumor activity itself, and although it is not truly an embolic agent, it is possible that it may cause temporary microembolic phenomenon that could potentiate drug efficacy (10). It is more plausible, however, that Ethiodol is simply a vehicle for drug delivery, with unique sustained release characteristics that may be related to its density, and the selective retention in tumors probably occurring because of poor tumor venous and lymphatic drainage (11–14).

Sustained release or depot preparations allow for a change in approach to therapy for patients with hepatic-metastatic disease. This may obviate the need for the expensive Infusaid pump with implantation. Same-day surgery treatments are performed in

TABLE 1. *Mean number of liver metastases per treatment group*

Treatment group	Liver metastases (mean n)		
	Experiment 1	Experiment 2	Experiment 3
A. IA 9-AC/Eth	15 (5)[a]	18 (4)[a]	23 (5)[a,c]
B. IA CD/9-AC	178 (5)	181 (4)	204 (5)
C. IA Eth only	233 (4)	177 (4)	245 (4)
D. IP CD/9-AC	—	—	132 (4)[b]

IA, hepatic intraarterial; Eth, Ethiodol; 9-AC, 9-Aminocamptothecin; CD, colloidal dispersion; IP, intraperitoneal. Numbers in parentheses are rats per group.

[a] Group A vs B: $p < 0.05$; group A vs C: $p < 0.05$.

[b] Group D vs B: $p < 0.05$; group D vs C: $p < 0.05$.

[c] Group A vs D: $p < 0.01$.

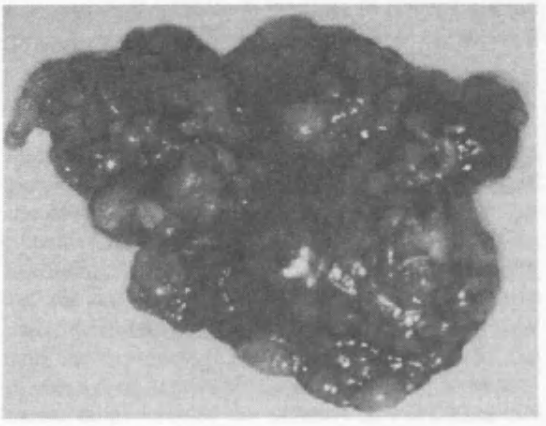

FIG. 3. Liver of rat treated with hepatic IA Ethiodol only. Tumors appear as white coalescent nodules.

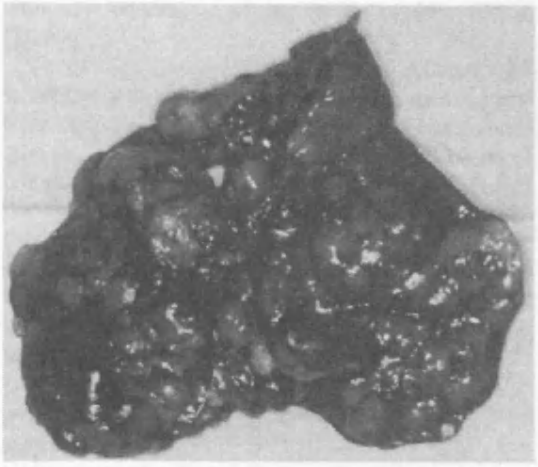

FIG. 4. Liver of a rat treated with hepatic IA CD/9-AC.

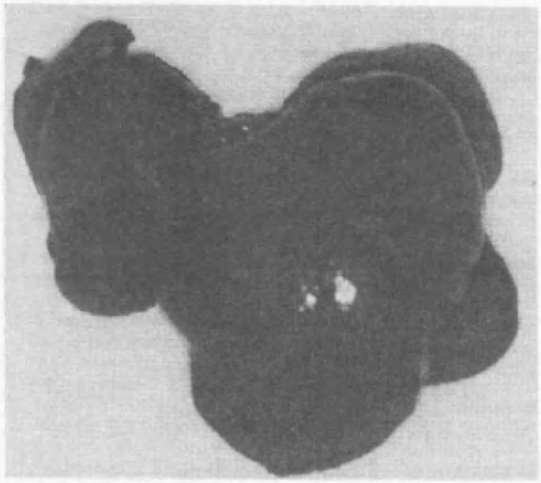

FIG. 5. Liver of a rat treated with hepatic IA 9-AC/Ethiodol.

the radiology suite with 1–2 days of patient hospitalization. This treatment may even be applied to patients who would not traditionally be eligible for hepatic IA infusional therapy because of lack of fitness for operation. New agents that are effective in patients previously treated with 5-FU regimens are needed. It has been shown that colon carcinomas possess higher levels of topoisomerase I than normal intestinal mucosa (15,16). This feature may be taken advantage of with topoisomerase I inhibitors.

Topoisomerase I is an essential nuclear enzyme that relaxes supercoiled duplex DNA enabling replication and transcription. A cleavable complex is formed between DNA and topoisomerase I, with single strand nicks occurring in the phosphodiesterase backbone, allowing swiveling at the nicks and passage of the intact strand through the nicks. Camptothecins are topoisomerase I inhibitors that bind to the cleavable complex and prevent religation of the single strand DNA breaks (17). Topoisomerase I inhibitors are S-phase-specific drugs that result in inhibition of RNA synthesis. This effect is rapidly reversible following drug removal, suggesting that prolonged exposure is important for efficacy (17–21). 9-AC has been shown to be more potent that the mother compound 20-*S*-Camptothecin and superior to 5-FU for treatment of liver metastases in murine animal models, with significant prolongation of survival (3,22–24).

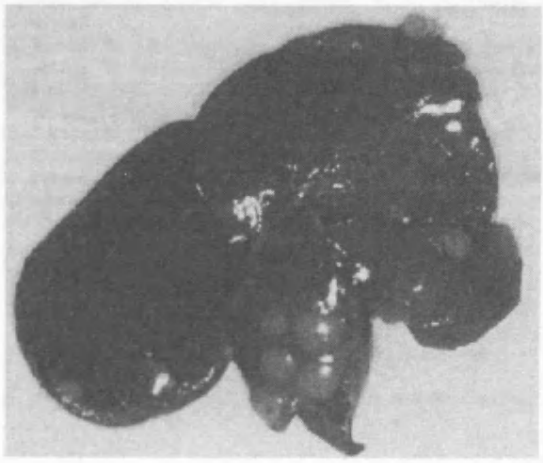

FIG. 6. Liver of a rat treated with systemic CD/9-AC via mini-osmotic pump.

We have demonstrated that bolus IA CD/9-AC given in identical fashion to 9-AC/Ethiodol has no appreciable antitumor effect, and this may be due in part to rapid conversion to the inactive acid form of the drug at physiological pH (25) and relatively low hepatic extraction (26). *In vitro* studies confirm the antitumor activity of both 9-AC/Ethiodol and CD/9-AC via mini-osmotic pump for periods of at least 10 days, demonstrating that both preparations deliver drug as intended. This period was equal to the planned drug exposure time *in vivo*. Titration experiments demonstrated the potency of 9-AC/Ethiodol in preventing engraftment of 1×10^6 cells in doses as small as 250 μg in rats observed for up to 6 months.

This experiment guided the choice of dose for IA therapy.

To advance to clinical testing, 9-AC/Ethiodol would have to be more effective as a locoregional treatment for hepatic metastases than a comparable systemic treatment, since IA therapy will be more complex and expensive to institute than systemic therapy. To address this problem, we used the mini-osmotic pump for systemic continuous infusion of the same dose of aqueous CD/9-AC. The results indicate that although comparable systemic treatment results in a reduction in the number of liver metastases (mean: 132) when compared to controls treated with Ethiodol only (mean: 245) this reduction is not as great as that for 9-AC/Ethiodol (mean: 23), which is significantly better ($p < 0.01$). Systemic therapy was also associated with substantial clinical systemic toxicity, which has been a problem generally with the camptothecins, and is one reason for the long delay in their clinical evolution (25). The significant reduction in hepatic metastases in the 9-AC/Ethiodol group was not associated with toxicity and the treatment was well tolerated.

These results suggest that locoregional therapy of hepatic neoplasms with sustained release 9-AC/Ethiodol would be associated with clinical efficacy far exceeding that associated with its systemic administration. Phase I clinical trials are therefore being planned.

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