VASOACTIVE MANIPULATION TO ENHANCE THE DELIVERY AND UPTAKE OF REGIONAL INFUSION CHEMOTHERAPY IN THE TREATMENT OF COLORECTAL HEPATIC METASTASES

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Abstract

Hepatic arterial infusion chemotherapy for colorectal hepatic metastases increases tumour response rates and prolongs survival. Despite this response rates remain at approximately 50%. Vasoactive manipulation has been proposed as a strategy to increase the proportion of blood flow and consequently cytotoxic uptake to the tumour.

This study was designed to assess the changes in liver and tumour perfusion in response to a variety of vasoactive agents in a rat liver metastasis model. Laser Doppler flowmetry was used to assess the perfusion changes over a period of time. Vasopressin and angiotensin II led to a rapid onset hepatic vasoconstriction for a short period after which restoration of flow occurred despite continued infusion of the vasoconstrictor agent. Endothelin I had the most prolonged effect as a hepatic vasoconstrictor and at increasing the tumour to normal ratio. Nitric oxide inhibition led to a gradual onset and mild degree of vasoconstriction. However the tachyphylaxis seen in response to vasopressin could be prevented or reversed by the coadministration of a nitric oxide inhibitor.

In a separate experiment, blood flow and 5FU uptake ratios were measured at a single time point following a 30 minute infusion of a vasoactive agent. There was a close correlation between blood flow and 5FU uptake ratios, implying that strategies to enhance the proportion of blood flow to the tumour would also increase the uptake of 5FU. A range of vasoconstrictor and capillary permeability agents were assessed following a 30 minute infusion. Of the agents tested, angiotensin II and endothelin I were found to produce a significant increase in blood flow and 5FU uptake ratios by approximately two fold.

In conclusion, infusion of vasoconstrictor agents may be used to increase blood flow and 5FU uptake ratios. This effect varies between agents and varies throughout the duration of the infusion. Despite an increase in blood flow ratios, there was no absolute increase in tumour blood flow and tumours remained hypovascular in relation to surrounding liver parenchyma.
This thesis is dedicated to:

Flavia Olinda,

Raphael, Mateo and Sasha
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Statement of Originality

This thesis is based on an original idea proposed by Mr T G Allen-Mersh. The work described was performed entirely by the author in the perfusion laboratory at the Institute of Cancer Research at the Royal Marsden Hospital, with technical assistance as cited.

The Manuscript has been prepared and typed by the author using Word for Windows 2.0 on a Viglen 80386Sx personal computer.

No part of this work has been submitted in support of any other degree or qualification to this or any other University or Institution
1. **Introduction**

1.1. **The Clinical Problem**

Colorectal cancer is the second most common cause of cancer death in the United Kingdom, as in many other countries in the developed world. In the UK an estimated 27000 new cases occur each year, with a prevalence of 56000. In 1991, 3% of all deaths in the England and Wales were due to colorectal cancer with 17000 deaths (OPCS 1993). There has been little improvement in survival from colorectal cancer over recent years and the five year survival remains at only 37% (CRC 1990).

![Figure 1](image)

**Figure 1** Incidence of cancer related deaths in England and Wales 1991

A major difficulty in trying to improve survival is that 25-30% of patients have either locally advanced disease or distant metastases at presentation. In addition, a further 40% of patients can be expected to develop recurrent disease after primary tumour resection. Since 17,000 people develop advanced colorectal cancer each year in the United Kingdom, any small improvement in treatment will benefit a large number of people.
Strategies to enhance treatment may be aimed at;

- prevention or early diagnosis of colorectal polyps or tumours;
- optimal surgical excision with or without adjuvant therapies;
- improving the treatment of advanced disease.

It is important that all treatment strategies must originate from an understanding of the pathogenesis and spread of the disease.

The work contained within this thesis is aimed at enhancing the treatment of patients with advanced colorectal cancer and in particular the treatment of hepatic metastases which are present in over 50% of all patients dying from the disease (Pickren et al. 1982).

1.2. Pathogenesis of Advanced Colorectal Cancer

**Local Invasion**

Local invasion occurs when tumour originating in colonic mucosa penetrates the wall of the bowel and invades neighbouring structures. This is common in rectal cancer where the relative immobility of other pelvic organs makes them vulnerable to tumour invasion. Recognition of local involvement is important before resection of the primary tumour as failure to carry out complete resection leading to transgression of tumour planes and shedding of cancer cells leads to early local recurrence. Lateral extension and involvement of the pelvic side walls is also a common cause of tumour recurrence within the pelvis after rectal resection.

**Lymphatic spread**

Involvement of regional lymph nodes is an adverse prognostic factor and is associated with an increased risk of both local recurrence and distant metastasis. As involved lymph nodes that are not removed may be a source of local and regional recurrence the aim of primary tumour excision is to remove the local lymph nodes in continuity with the primary tumour. This in practice means removal of the lymphatic chain accompanying the main arterial pedicles to the tumour bearing area. In rectal carcinoma, the lymph nodes of the lower aortic, iliac and post rectal area must be excised although more extensive nodal stripping of the aortic nodes and the nodes in front of the vena cava does not lead to improved survival (Glass et al. 1985).

**Haematogenous spread**

More than 50% of patients dying from colorectal cancer will have liver involvement at the time of death and in 10-20% the liver is the only site of involvement (Pickren et al. 1982). It has been proposed that the initial hepatic metastasis develops from portal vein tumour emboli (Willis 1930). Dukes found evidence of venous spread in 17% of
operative rectal cancer specimens (Dukes 1957) and it is known that malignant cells may pass into the portal circulation at the time of tumour mobilisation (Fisher and Turnbull 1955). Having established themselves within the liver, metastatic cells invade the venous circulation of the liver and again metastasise to other organs such as lung, adrenal and bone. If this theory of stepwise metastasis is correct then this implies that there will be a group of patients in whom the disease has only spread to the liver. This is supported by the finding that there are some patients with liver metastases who can be cured by resection of primary tumour and liver metastases.

Studies extrapolating the growth rate of liver metastases on CT scan suggest that the average time course from the initial metastases to the death of the patient is four years (Finlay et al. 1988). A liver metastasis is not usually discovered until it has reached a diameter of greater than 2 cm, which usually requires three years of this four year growth period to have elapsed. Thus most of the liver metastases which appear after apparently curative primary tumour resection are present within the liver at the time of the initial surgery.

One approach to the management of these occult hepatic metastases currently under investigation is to treat all patients undergoing apparently curative primary resection with an infusion of 5-fluorouracil (5FU) via a catheter into the portal vein at and immediately after surgery for resection of the primary tumour (MRC Trials Protocol). Although two out of three patients receiving this therapy will not benefit (either because they are cured by primary tumour excision or because the disease is too advanced) it is hoped that the remaining patients who do have occult hepatic metastases will receive effective treatment for their early hepatic involvement. Initial studies of this approach suggest that there may be a survival benefit, particularly in the Dukes B group of patients (Taylor et al. 1985).

An alternative approach is to identify patients with clinically 'occult' liver metastases at an early stage using either intraoperative ultrasound (Boldrini et al. 1987) or CT portography (Soyer et al. 1992) which can detect metastases as small as 5 mm. However studies of the survival benefit of chemotherapy in patients identified in this way have not been done (Allen-Mersh 1991).

Despite adequate treatment of the primary tumour and possible adjuvant therapy, a significant group of patients will either have synchronous hepatic metastasis at the time of initial surgery or develop recurrent disease within the liver at a later stage. The vast majority of these patients will then be considered incurable. Until recently, few therapeutic options have been available to either prolong survival or improve their quality of life.
1.3. Options for the Treatment of Hepatic Metastases

1.3.1. Resection

Advances in anaesthetic and surgical techniques for liver surgery have made resection a much less hazardous undertaking and this is now associated with less than 10% operative mortality. Improved understanding of the segmental anatomy of the liver has meant that lesser resections are feasible and that bilobar metastases can be resected allowing sufficient healthy liver parenchyma to be preserved. The use of the ultrasonic dissector has helped to minimise operative blood loss which is a potent source of post-operative morbidity.

There are no randomised trials evaluating the benefits of liver metastasis resection. However, it is clear that for those patients with less than four liver metastases and no evidence of disease outside the liver, some long-term survivors (perhaps 25% of those operated upon) can be achieved (Adson and Van Heerden 1980). Patients with this degree of disease are unusual (only 3% of all patients with large bowel cancer) and the more controversial question of whether liver metastases resection should be undertaken for more advanced disease in order to sustain quality of life is unanswered.

Approximately 3% of patients with primary large bowel cancer are currently suitable for liver metastases resection, the number of patients actually offered resection is considerably less and it may be that these patients are being denied a treatment which could improve quality of life and offer a chance of cure. The majority of patients with liver metastases are diagnosed at an advanced stage when disease within the liver can be palpated abdominally. Thirty five percent of the liver is replaced by tumour on CT scan at this stage of diagnosis and it is unusual for metastases resection to be technically possible. The number of patients suitable for surgery may increase with improved techniques for identifying smaller liver metastases and intensive serum CEA monitoring after primary tumour resection. Thus for this approach to be applied more frequently, more intensive post-operative follow-up will be required for patients who have undergone primary large bowel cancer removal.

1.3.2. Radiotherapy

External beam radiotherapy in the treatment of hepatic metastases is of limited value due to the high radiosensitivity of the liver, although it may be of some value in symptom palliation (Prasad et al. 1977). One randomised study of 5FU with or without external beam radiotherapy failed to show any increase in response rates in those receiving additional radiotherapy (Wiley et al. 1989). An alternative method for delivery of radiotherapy to hepatic metastases is by radiolabelling regionally delivered microspheres or antibodies with an isotope such as 131-iodine. This may have a more selective action if delivered into the hepatic artery.
1.3.3. Physical Destruction Methods

A number of methods for the local destruction of liver metastases have been tried. Simple alcohol injection has been used while others have tried to necrose the tumours by heat (Masters et al. 1991) or cold (Ravikumar et al. 1987). More recently, focused ultrasound has been investigated in an experimental system to destroy intrahepatic tumours (Ter Haar et al. 1989).

While each of these methods is a potent method of tissue destruction, they are limited by the ability to treat a limited area within the liver. Such treatment is always likely to be palliative and of unproven survival or symptomatic benefit.

1.3.4. Systemic Chemotherapy

When colorectal cancer has become disseminated throughout the body, systemic therapy becomes the treatment of choice as it reaches all tumour bearing areas. Few cytotoxic agents are effective in colorectal carcinoma. The fluoropyrimidines; 5 fluorouracil (5FU) and fluorodeoxyuridine (FUdR) remain the most effective and widely used agents. Early studies of 5FU showed response rates of between 8-82% but were characterised by poorly defined criteria of response. When response criteria are more clearly defined, the response rates are between 15% - 20% with no clear survival benefit (Kemeny 1983, Wasserman et al. 1975).

Folinic acid is often used in conjunction with 5FU as a biochemical modulator. Its use is based on the rationale that an excess of reduced intracellular folates is necessary for the optimal inhibition of thymidylate synthetase thus potentiating the cytotoxic effect. Arbuck (1989) reported an overview of seven phase III trials comparing 5FU with 5FU and folinic acid in the treatment of advanced, previously untreated colorectal cancer. Five out of seven studies showed a higher response rate for the groups receiving folinic acid despite the overall 5FU dose being less. In addition a survival benefit was reported in two studies, increasing survival by 3 and 6 months.

One characteristic of virtually all systemic chemotherapy regimens is that further dose increases are limited by systemic toxicity, in particular affecting the gastrointestinal tract and bone marrow. Thus in order to increase dosage, techniques are required which will not be limited by systemic toxicity.
1.4. Regional Chemotherapy

1.4.1. Introduction

Klopp et al. (1950a 1950 b) and Bierman et al. (1950) independently first reported the use of nitrogen mustard administered intraarterially. Klopp's original discovery was based on the inadvertent injection of nitrogen mustard into the brachial artery instead of the antecubital vein. Initial treatment regimens involved intermittent boluses given through polythene cannulae placed into the artery supplying the tumour bearing region. The initial method was fraught with difficulties and in 1957 a method of intraarterial chemotherapy was introduced by Creech et al. (1957) using isolated limb perfusion in which the chemotherapy was administered to the tumour bearing region using a temporary oxygenated extracorporeal circulation between a local artery and vein.

There were several reports of intraarterial chemotherapy in the treatment of liver metastases in the 1960's (Clarkson et al. 1962, Sullivan et al. 1963, Watkins et al. 1970) although once again difficulties with catheter occlusion, cracking and displacement, were experienced. There was a resurgence of interest in the technique following the development and testing of a totally implantable system pump (Blackshear et al. 1972) which enabled continuous infusion chemotherapy to be carried out as an outpatient.

1.4.2. Rationale for Regional Treatment

1.4.2.1. Dose response relationship

The aim of hepatic artery infusion is to increase drug concentration in the liver while sparing the systemic circulation from excessive toxicity. While the relationship between dose and toxicity is clear, evidence for a dose relationship with tumour response is less obvious. In vitro such a relationship has been demonstrated but the multitude of trials with their variety of regimens and doses makes comparison between trials difficult. Hymiuk et al. have attempted to overcome this by using the concept of dose intensity (Hymiuk et al. 1987). This is the amount of drug received per unit time as mg/m²/week. It allows for time delays and dose reductions but assumes that scheduling is more important in reducing toxicity than in affecting tumour kill. From an analysis of published trials they demonstrated a steep dose response relationship for 5 FU in advanced colorectal cancer which has been confirmed by others (Frei and Canellos 1980).
1.4.2.2. The regional advantage

The benefit of regional delivery over systemic infusion is known as the regional advantage (Rd) and can be expressed as:

\[
Rd = 1 + \frac{CL_{tb}}{Qt(1-Et)}
\]

where CL\(_{tb}\) is the total body clearance, Qt is the blood flow through the perfused artery and Et is the extraction fraction of the perfused organ (Chen and Gross 1980, Ensminger and Gyves 1984).

This advantage will vary according to the individual pharmacokinetics of each agent. There is a greater regional advantage with drugs which have short plasma half lives and high clearance from the circulation such as 5 fluorouracil and fluorodeoxyuridine, while agents with low total body clearance such as methotrexate have virtually no regional advantage (Collins 1984). The first pass extraction ratios for 5FU and FUdR are around 50 and 90% respectively (Ensminger et al. 1978) and their plasma half lives only a few minutes. It is thus possible to achieve a regional advantage of several hundred fold using these drugs. Other agents have been used although the pharmacokinetics are rarely as well suited to regional treatment.

In addition to the regional advantage defined above, the regional advantage can also be increased by decreasing the blood flow (Qt) to the organ and this is the basis of treatment with arterial ligation or embolisation combined with regional cytotoxic therapy.

1.4.2.3. Infusion or bolus

Prolonged infusion appears to be the optimal method of drug administration. Most cytotoxic agents act only on the proliferating cell fraction and continuous exposure may induce other cells into the proliferating part of the cycle and increase tumour cell kill (Salmon 1979). Prolonged infusion may also decrease the drug toxicity as shown by Seiffert who randomised 70 patients to receive either bolus or infusion therapy and noted a reduction in myelotoxicity as well as an increased response in the infusion group (Seiffert 1975). A similar randomised study by Lokich reached the same conclusions (Lokich 1983).

1.4.2.4. Which route of administration

The route of administration of regional hepatic chemotherapy depends on the vascular pattern of hepatic metastases. This is more complex than at other sites due to the dual blood supply via the hepatic artery and portal vein. Established metastases within the liver are predominantly supplied by the hepatic artery rather than the portal vein (Breedis and Young 1954, Lien and Ackerman 1970, Ackerman 1974). It would therefore seem
logical to deliver chemotherapy for established hepatic metastases via the hepatic arterial rather than portal vein route. Daly et al. (1987) have shown superior response rates after hepatic artery compared with portal vein administration.

1.4.3. Results of Clinical Trials

Numerous reports in the literature have demonstrated response rates of 40-88% (Neiderhuber et al. 1984) (Schwartz et al. 1985) (Balch et al. 1983). Although response criteria vary, this is higher than that reached by systemic single agent chemotherapy and is also higher than response rates achieved when agents such as folinic acid are added (16-45%) (Arbuck 1989). However these have largely been series reports rather than randomised trials. It is therefore not possible to say from these studies if survival is prolonged and quality of life has rarely been measured.

There are few prospective controlled trials which assess whether hepatic artery infusion (HAI) chemotherapy is superior to systemic therapy. An early study by Grage et al. (1979) treated two groups with either a loading course of intraarterial (21 days) or systemic chemotherapy followed by maintenance bolus therapy. Following this both groups were given weekly systemic bolus therapy. Response criteria relied on abdominal palpation. This study showed an increase response rate in the HAI arm although survival was similar.

Kemeny et al. (1989) randomised 99 patients to receive HAI (48) or systemic (51) FUdR. All patients had a laparotomy and were given a 14 day infusion of FUdR alternating with normal saline. The dose administered in the intraarterial group was twice that of the systemic group due to the limitation of systemic toxicity. Patients in the HAI group had a higher response rate (50% v 20%). Survival analysis showed increased median survival in the HAI group (17 months versus 12 months) but this was not statistically significant and was difficult to interpret as many non responding patients were crossed over from the systemic into the HAI arm. A similar study by the Northern California Oncology group (Hohn et al. 1989) showed response rates of 42% v 10%.

Chang et al. (1987) performed a study with no crossover. They showed improved response rates of 62% v 17% in the HAI and systemic groups respectively but at 2 years survival was not significantly different: 22% and 15%. In this study patients randomised to HAI therapy had a staging laparotomy and in 9 out of 32 patients HAI therapy was not given due to extrahepatic disease. As analysis excluded these patients this meant that the HAI group was a more favourably selected group than the systemic group who did not receive a laparotomy.

Kirk Martin et al. (1990) randomised 74 patients to continuous HAI FUdR or weekly bolus 5FU and found increased response rate (48 v 21%) in the HAI arm with a slight but not significant increase in median survival (12.6 v 10.5 months)
Lokich stated in 1983 that one of the main reasons why hepatic artery chemotherapy was not the standard treatment for established hepatic metastases was the inability to demonstrate it prolongs survival more than systemic chemotherapy as well as a concern about cost (Lokich 1983). Until recently this has been true. Two recent randomised studies have shown prolonged survival as a result of intraarterial FUdR therapy compared with controls. Rougier et al (1992) carried out a multicentre trial of 166 patients randomly allocated to pump implantation and 14 days / month FUdR against a control arm in which patients received either symptomatic treatment alone or intermittent bolus 5FU. There was a significant (p<0.02) increase in median survival in the treated group (15 months) compared with the control group (11 months). There was however also a 1 year rate of sclerosing cholangitis of 25%. Treatment failure was commonly due to extrahepatic progression.

In a similarly randomised study Allen-Mersh et al. (1994) also found a significantly (p=0.03) improved survival for patients receiving intra-arterial FUdR (median survival 405 days) compared to the control arm (median survival 226 days). They also studied quality of life in both groups and concluded that intra-arterial FUdR treatment led to a prolongation of normal quality survival.

Thus it appears that the response rate after HAI is consistently higher within the liver than with other forms of chemotherapy. Survival is prolonged and this is associated with a prolonged period of normal quality of life. Where relapse occurs in patients receiving hepatic artery infusions it is frequently extrahepatic whereas intrahepatic progression leading to death is often the case with systemic chemotherapy.

One way around this dilemma may be to combine the systemic and regional approaches as attempted by Safi et al. (1989) (see table 1) infusing FUdR both systemically and regionally or Stagg et al. (1991) who combined shorter cycles of HAI FUdR with systemic 5FU. Although the aim was to reduce biliary toxicity they also noticed a lower incidence of extrahepatic failure.
<table>
<thead>
<tr>
<th>Study</th>
<th>Treatment Arm</th>
<th>No</th>
<th>Response</th>
<th>Median Survival (months)</th>
<th>Cross Over</th>
<th>Comment</th>
</tr>
</thead>
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<tr>
<td>Grage 1979 (COG)</td>
<td>IA 5FU loading then IV bolus IV bol.5FU</td>
<td>31</td>
<td>34%</td>
<td>13.5</td>
<td></td>
<td>response by palpation</td>
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<tr>
<td></td>
<td></td>
<td>30</td>
<td>23%</td>
<td>15.4</td>
<td></td>
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<tr>
<td>Chang 1987</td>
<td>IA FUdR</td>
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<td>62%</td>
<td>22</td>
<td>no</td>
<td>2 year survival</td>
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<tr>
<td></td>
<td>IV FUdR</td>
<td>29</td>
<td>17%</td>
<td>15</td>
<td></td>
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</tr>
<tr>
<td>Kemeny 1987</td>
<td>IA FUdR</td>
<td>48</td>
<td>50%</td>
<td>17</td>
<td>31/51</td>
<td>extrahepatic relapse</td>
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<tr>
<td></td>
<td>IV FUdR</td>
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<td>19.6%</td>
<td>12</td>
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</tr>
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<td></td>
<td></td>
<td>37%</td>
</tr>
<tr>
<td>Hohn 1989 (NCOG)</td>
<td>IA FUdR</td>
<td>50</td>
<td>42%</td>
<td>16.2</td>
<td>28/65</td>
<td>high biliary toxicity</td>
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<tr>
<td></td>
<td>IV FUdR</td>
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<td>10%</td>
<td>16.1</td>
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<td>Kirk-Martin 1990</td>
<td>IA FUdR</td>
<td>33</td>
<td>48%</td>
<td>12.6</td>
<td>no</td>
<td>extrahepatic relapse</td>
</tr>
<tr>
<td></td>
<td>IV bol.5FU</td>
<td>36</td>
<td>21%</td>
<td>10.5</td>
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<td>61%</td>
</tr>
<tr>
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<td></td>
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<td></td>
<td>33%</td>
</tr>
<tr>
<td>Safi 1989</td>
<td>IA FUdR</td>
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<td>52%</td>
<td>31</td>
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<td>extrahepatic relapse</td>
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<tr>
<td></td>
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<td>21</td>
<td>48%</td>
<td>16</td>
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<td>61%</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>33%</td>
</tr>
<tr>
<td>Rougier 1992</td>
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<td>43%</td>
<td>14</td>
<td>no</td>
<td>25% biliary sclerosis</td>
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<td></td>
<td>IV 5FU or symptomatic</td>
<td>82</td>
<td>9%</td>
<td>10</td>
<td></td>
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<tr>
<td>Allen-Mersh 1994</td>
<td>IA FUdR</td>
<td>51</td>
<td>40%</td>
<td>13.5</td>
<td>no</td>
<td>prolonged normal quality of life</td>
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<td>49</td>
<td>7.5</td>
<td>7.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Controlled trials of hepatic artery infusion chemotherapy
1.4.4. Problems with Regional Infusion

1.4.4.1. Anatomic variation

The hepatic arterial supply of the liver varies in 25% of persons. The major variations that commonly occur are when the left hepatic artery originates from the left gastric artery or when the right hepatic arises from the superior mesenteric artery. While the use of two separate catheters has been advocated, ligation of the aberrant vessel usually results in perfusion of both lobes by the remaining artery and this can be easily checked by the infusion of methylene blue into the catheter at the time of surgery (Burke et al. 1994).

1.4.4.2. Perioperative morbidity

Perioperative mortality from insertion of hepatic artery pumps is not high although safety of such procedures is related to the extent of hepatic disease. In 51 pump insertions, Allen-Mersh et al (1994) experienced two deaths due to liver failure, both in patients with over 45% hepatic replacement.

1.4.4.3. Toxicity

Unlike systemic therapy the toxic effects of FUdR infusion chemotherapy are almost entirely confined to the liver due to high local drug levels combined with a high first pass extraction. In this regard HAI of FUdR is similar to radiotherapy, as the dose limiting factor is of local tumour host tissue toxicity (Ensminger and Gyves 1984). In contrast, the limiting toxicity of regionally administered 5FU is frequently systemic due to its lower first pass uptake.

FUdR induced cholecystitis is prevented by cholecystectomy at the time of pump insertion (not possible when percutaneously placed catheters are used) and gastritis is rarely problematic if patients are placed on Hz antagonists. The most significant toxicity encountered is biliary sclerosis. Hohn et al. (1986) have pointed out that this can be reduced by careful enzyme monitoring and early dose reduction. Many trials have reported a high incidence of biliary toxicity possibly due to a lack of awareness of this approach. Most cases are reversible by early detection and dose reduction.

Corticosteroids may be of benefit in patients with hepatic toxicity or cholestatic jaundice and these may also be given through the infusion pump (Janis et al. 1988). There is also evidence that fluoropyrimidine metabolism is subject to a circadian rhythm with metabolism higher in the late afternoon and evening. In a randomised study using a time modified regimen of systemic FUdR those receiving the time modified regimen tolerated 50% more FUdR with less toxicity (Von Roemeling and Hrushesky1989).

However it is clear that there has been a switch of the dose limiting factor from systemic toxicity with systemic chemotherapy to hepatic toxicity with HAI chemotherapy.
1.4.4.4. Treatment Failure

Despite an improvement over conventional chemotherapy for the treatment of liver metastases, response rates are still only 50%. The question arises why half of patients treated derive no demonstrable benefit and what can be done to increase the response rates further.

Therefore there is a need to look for ways to potentiate the delivery and effect of HAI chemotherapy. Regional infusion gives a considerable dose advantage over systemic therapy in the treatment of liver metastases. However to further improve on this, delivery of the cytotoxic agent must be further targeted on the tumour itself while minimising that delivered to the surrounding liver parenchyma.

1.4.5. Alternative Strategies to Enhance Regional Chemotherapy

1.4.5.1. Regional antibody therapy

The concept of tumour associated antigens as a target for immunotherapy dates from the 1960's. Monoclonal antibodies are attractive because of their high specificity compared to conventional chemotherapy. Several problems remain including heterogeneity of antigen expression, development of high affinity antibodies and antigenic modulation. They may be used to induce host mediated toxicity or labelled with toxins or radio pharmaceuticals.

A number of phase I and phase II studies have been carried out such as that by Riva et al. (1991) in which a monoclonal directed against CEA or similar tumour antigens, was used and labelled with a therapeutic dose of $^{131}$I. Four responses out of 15 patients were achieved including two complete responses.

Animal studies suggest that compared to systemic administration, there may be some regional advantage for antibodies administered into the hepatic artery although this is only likely to be small. However the addition of histamine to the infusion caused a threefold increase in tumour antibody uptake ratios (Hennigan et al. 1991)

1.4.5.2. Ligation and embolisation

Hepatic artery ligation has been used in an attempt to decrease the growth of hepatic metastases. The potential benefit is reduced by revascularisation of the tumour from portal or collateral arterial sources. This has led some to develop methods of intermittent arterial occlusion in which the hepatic artery can be repeatedly occluded for short periods (Persson et al. 1990).

The combination of hepatic artery ligation and infusion chemotherapy into portal or arterial routes has been investigated. One randomised study of ligation and portal vein infusion in 24 patients did show a survival benefit although group numbers were small (Taylor 1978). Other studies have combined hepatic artery ligation with infusion through
the distal artery and some achieve high response rates (Didlokar et al. 1985) but once again therapeutic benefit is difficult to determine due to a lack of randomised studies.

More recently there has been interest in degradable starch microspheres. These commercially prepared spheres have a half life in the circulation of 20-30 minutes and provide a very effective way of reducing hepatic arterial flow. Administration of these spheres causes regional stasis in the tumour and this has been shown to increase the uptake of 5FU or FUdR experimentally (Flowerdew et al. 1987) and clinically (Thom et al. 1989) and to decrease the peak plasma drug levels. A number of phase II studies have been undertaken combining starch microspheres with a number of cytotoxic drugs and these report response rates of between 20-50%. One randomised controlled trial compared a combination of starch microspheres and 5FU with non reversible hepatic artery embolisation and a no treatment control arm (Hunt et al. 1990). This showed a slight, but non significant, increase in the median survival of the microsphere/5FU group.

An alternative approach is to use longer acting microparticles which have the cytotoxic agent encapsulated within (Kerr 1987). This approach can alter the pharmacokinetic profile of a drug so that its regional advantage can be enhanced and combined with the effect of short term ischaemia.

Another vehicle for selectively delivering treatment to tumours is to use lipiodol, an oily contrast medium which is retained within tumours. Lipiodol can be emulsified with several cytotoxic drugs or used to deliver Iodine-131 or Yttrium-90. It has been largely used in the treatment of hepatocellular carcinoma and less frequently with colorectal metastases although preliminary studies show reasonable response rates with low associated toxicity (Inoue et al. 1989).
1.4.6. Biological Response Modifiers

**tumour necrosis factor**

Experimental work with tumour necrosis factor (rTNF) has suggested a wide range of anti-tumour activity, both as a cellular cytotoxic agent and by an indirect effect on tumour vasculature. However, tumour response has been disappointing being associated with severe limiting hypotension, CNS dysfunction and cardiopulmonary toxicity (Kemeny et al. 1990). Mavligit carried out a phase I trial of hepatic artery infusion of rTNF (Mavligit et al. 1992) and found that by using a regional approach, doses of six times the intravenous dose could be tolerated. They demonstrated a partial response in some previously chemoresistant patients. Despite the improved regional delivery advantage, response rates were poor. An adequate therapeutic index may not be achieved and approaches such as isolated perfusion may be required if improved response rates are to be achieved.

**interleukin 2**

T cell growth factor IL-2 may have a synergistic effect with fluorouracil and may act by increasing LAK cells or tumour-infiltrating lymphocytes.

Mavligit et al. randomised 28 patients to receive IL-2 into hepatic or splenic artery with partial responses in only one in each group (7%) (Mavligit et al. 1990). Marked dose related toxicity was noted and 4 complications at the site of femoral puncture as well as 8 patients developing allergic reactions from contrast due to repeated percutaneous catheterisations.

There was no systemic treatment arm and it is therefore not possible to compare regional with systemic delivery although there was probably a lower incidence of cardiopulmonary toxicity when compared with other trials.

1.4.7. Biochemical Response Modifiers

Because of the low response of metastatic disease to systemic 5FU, strategies are being developed to enhance its anti-tumour effect through biological modulation

Folinic acid increases the stability of the FdUMP ternary complex necessary to inhibit thymidylate synthetase within the tumour cell and can increase the cytotoxicity of the fluoropyrimidines. Several randomised trials have been carried out of 5FU vs 5FU/Folinic acid given systemically. Most show an increased response rate and in two of seven studies a survival advantage was demonstrated (Erlichman et al. 1988, Poon et al. 1989).

Three regional infusion studies have included folinic acid in the regimen. Patt et al (1990) carried out a phase I trial of HAI FUdR and escalating doses of folinic acid. They had a partial response rate of 58% but had to terminate treatment in 35% of patients because of hepatobiliary toxicity which occurred more frequently than from FUdR alone.

27
Kemeny carried out a pilot study to evaluate administration of FUdR and folinic acid through the Infusaid pump at a variety of doses and showed an overall response rate of 72% with a median survival of greater than 27 months. However, once again, the combination of folinic acid and FUdR caused greater hepatic toxicity than with FUdR alone (Kemeny 1990).

Warren et al. (1994) carried out a phase II study in 31 patients using intra arterial 5FU and systemic high dose folinic acid. They found a response rate of 48% with a median predicted survival of 19 months. There were no cases of biliary sclerosis as FUdR was not used, although there was a fairly high rate of catheter related complications and occlusions. Due to the spillover effect toxicity was mainly systemic.

1.4.8. Vasoactive Manipulation

The benefits to hepatic métastasés patients of hepatic artery chemotherapy are now clear (Allen-Mersh et al. 1994) although up to 50% of patients are still not achieving a significant response. Dose intensification may lead to higher responses but further dose increases are limited by hepatic toxicity. Evidence suggests (deBrauw et al. 1991, Sigurdson et al. 1986) that the uptake of fluoropyrimidine into hepatic métastasés is less than half that of liver. Vasoactive manipulation may provide a means of increasing tumour perfusion and increasing the proportion of drug taken up by the liver.

Despite the effectiveness of many cytotoxic agents in vitro, their use in the treatment of solid tumours is disappointing. Physical properties inherent within the tumour structure make treatment of solid tumours much less successful than their petri dish counterparts. To have an effect on a cancer cell, an agent delivered through the blood stream must pass into vessels supplying the tumour, pass out across the vessel wall, across the interstitium and be taken up within a cell. Several barriers to this process exist because of the structure and form of solid tumours (Jain 1994). One major problem in the delivery of cytotoxic agents to tumours is the marked heterogeneity of the tumour vasculature. Without a suitable vascular network cytotoxic drugs will not even pass close to the centre of a tumour. Raised tumour interstitial pressure limits the efflux of molecules by convection although the effect on small molecules transported predominantly by diffusion is likely to be less. Tumour vessels, where they exist, demonstrate marked differences from normal tissues in structure as well as distribution. Initial tumour growth incorporates the presence of existing tissue vessels but as the tumour increases in size, these vessels are thought to collapse and new sinusoidal leaky channels develop, which lack smooth muscle lining and adrenergic vasoconstrictor tone. They are said to exist in a state of maximal dilatation and lack the ability to constrict. Thus the use of vasoactive agents to create a differential effect between tumour and normal tissue vessels, such that the delivery of cytotoxic agents to the former can be increased while minimising it to the latter, might be possible.
Vasoactive manipulation depends on differences between normal and tumour vasculature to enhance the proportion of drug delivered to tumour while minimising that reaching normal tissues. Its use depends on an understanding of the tumour vascular system and the effects of vasoactive compounds on both tumour and normal vessels. Tumour vasculature will be described first and a review of the results of previous studies of vasoactive manipulation is discussed in more detail below.

1.5. Tumour Vasculature

1.5.1. Tumour Vessel Morphology

The efficacy of most non surgical approaches to the treatment of liver metastases is ultimately dependent on the tumour microcirculation. Initially tumours were thought to be hypervascular when compared with surrounding tissues (Ribbert 1904) and this was generally accepted. More recently however, studies have indicated that this is rarely the case.

Tumour vasculature develops as a result of tumour angiogenesis (Folkman 1985). A solid tumour is dependent on such angiogenesis for development beyond a certain critical size (Folkman 1972). Angiogenic factors are produced which induce the development of capillary buds and these develop into the tumour neovasculature. In addition to new vessel formation the tumour incorporates existing vessels in host tissue. Lindgren (1945) described the tumour vasculature as 'low differentiated and of a foetal type... the wall lacking elastic lamellae.' He observed a similarity between the angioarchitecture of the primary tumour and the metastasis.

The features of tumour microvasculature are mainly based on observations in experimental tumours. Characteristically the small vessels are irregular, often sinusoidal with a large lumen and an irregular covering of endothelium (Warren 1970). There is frequently a network of giant capillaries around the periphery of the tumour composed of endothelial cells and some fibrous supporting tissue (Warren and Chauvin 1977). Arterioles may be incorporated from normal host tissues into tumours (Intaglietta et al. 1977) but show a considerable resistance to tumour invasion (Willis 1973). These arterioles do have some smooth muscle component in their walls although do not necessarily communicate with the smaller tumour vessels and may act as shunts through the tumour. Tumour arteriovenous shunts may account for a considerable proportion of blood flow, as Weiss et al. (1979) observed in a rat mammary tumour model in which up to 40% of blood passed through such shunts. Shunting of blood has been described as having a stealing effect from the tumour centre (Muller Klieser and Vaupel 1984), which frequently shows signs of necrosis.
Adrenergic innervation is generally absent in tumours (Krylova 1968, Mattsson et al. 1977, Hafstrom et al. 1980) although this does not preclude an effect by adrenergic agents acting directly on smooth muscle receptors.

1.5.2. Intratumour Blood Flow Distribution

Early qualitative studies of intratumour perfusion were carried out by Goldacre and Sylven (1959) and by Owen (1960) using lissamine green.

Tumours possess both macroscopic and microscopic heterogeneity (Jirtle 1988). Chaplin et al. (1987) used a fluorescent dye combined with microspheres and demonstrated that tumour blood flow may be transient and perfusion absent for periods of time up to 20 minutes rendering areas of tumour hypoxic with a consequent decrease in tumour pH (Vaupel 1977).

1.5.3. Effect of Blood Pressure

Tumour blood flow has been shown to have a linear relationship with arterial blood pressure with no evidence of autoregulation of flow (Vaupel 1975). The idea that tumours are passive vascular beds was investigated by Suzuki et al. (1981) who elevated the systemic blood pressure of rats with a subcutaneous tumour, by the infusion of angiotensin II. Using a thermoelectric method for the measurement of tumour blood flow they found a 5.7 fold selective increase in tumour blood flow and went on to demonstrate an increased response to mitomycin C when administered with angiotensin induced hypertension. Such an approach was extended to the treatment of patients suffering from advanced cancers (Wakui and Suzuki 1983).

1.5.4. Effect of Tumour Size

Continuous structural and physiological changes take place as tumours expand. Gullino and Grantham concluded from their work that tumours tended towards a uniform blood flow irrespective of tumour size. In a study on Guerin carcinoma in rats measuring Rubidium-86 ($^{86}$Rb) uptake, Takacs et al. (1975) found no significant difference between blood flow in small versus large tumours. In a study to specifically investigate this relationship, Cataland et al. (1962) studied the blood flow to subcutaneous transplants of mammary carcinoma 755 and sarcoma 180 using $^{86}$Rb. Unlike Gullino and Grantham they found an inverse relationship between tumour size and perfusion. Such studies are influenced by both tumour type and the method used for flow measurement. Peterson (1979) concludes that the flow in non necrotic parts of the tumour probably remains fairly stable despite increasing tumour size.
1.5.5. Effect of Anaesthesia

The vast majority of studies concerning tumour blood flow have been performed in anaesthetised animal models. Investigators have frequently reached widely differing conclusions, particularly in assessing the response to vasoactive agents. However little attention has been paid to the possible effect of the anaesthetic agent used and whether this could affect tumour vasculature in a different way to that of the normal vasculature. Zanelli et al. (1975) investigated the effects of sodium pentobarbitone and urethane in five types of transplanted mouse tumour and normal organs. They concluded that these anaesthetics led to a decrease in the total blood volume of the tumour while at the same time increasing the relative flow i.e. as a fraction of cardiac output, by up to 2 fold. Such studies show the way in which tumour blood flow measurements can vary and may in part explain some of the contradictory results in the literature.

1.5.6. Tumour Permeability and Drug Transport

Tumour vasculature is highly heterogeneous. Vessels are dilated, saccular and tortuous. As tumours grow in size their vascular area decreases leading to a reduction in the transvascular exchange of molecules and cells. Tumour vessels have wide interendothelial junctions a large number of fenestrae and a discontinuous basal lamina (Jain 1989). This suggests that tumour vessels should be leakier than normal vessels although extravasation of macromolecules is decreased. Two factors contributing to this are the decrease in vascular pressure and the increased tumour interstitial pressure which were demonstrated in tumours grown in transparent chambers (Jain 1988). The increased tumour interstitial pressure (Young et al. 1950) rises with tumour size, presumably due to absence of lymphatic drainage (Gullino 1975). This correlates with a decrease in tumour blood flow and the development of necrosis with tumour growth (Jain 1987). Tumour interstitial pressures are higher towards the centre of the tumour and zero at the periphery. Interstitial pressures have been measured in human melanoma (Boucher et al. 1991) and showed increased values in larger tumours.

In order for drugs to reach a tumour cell they must be transported through the vascular endothelium lining the tumour vessels and through the interstitial space. Microvascular or interstitial solute transport is facilitated by two transport processes:

1. Convective flow is dependent on bulk fluid movement through tissues. Such flow is proportional to the vascular area and is driven by the difference between the hydrostatic and oncotic pressures in plasma and interstitial fluids which is known as the hydraulic conductivity (Jain 1989).

2. Diffusion occurs down a concentration gradient and is dependent on the surface area for exchange. The constant that relates to the amount diffusing is called the diffusion coefficient and is measured in cm$^2$/sec.
As well as these factors, molecules may bind to proteins or other tissue components, be metabolised or undergo active uptake into a cell.

In tumours, convection is likely to be reduced because of the decreased pressure gradient and this will impair the movement of large molecules such as antibodies. Transport of small molecules such as 5FU (m.w. 130) may occur fairly rapidly due to the concentration gradient facilitating diffusion. Measures to enhance the blood flow or pressure gradient across tumours may increase transport out of the circulation. However the limiting factor in the uptake of small molecules, such as 5FU, is likely to be due to flow rather than the uptake of the drug out of the circulation. Thus measures which enhance flow would be predicted to increase drug uptake into tumour
1.6. **The Blood Supply of Hepatic Tumours**

The blood supply of hepatic tumours is more complex than in other parts of the body because of the presence of a dual blood supply from both hepatic artery and portal vein. Breedis and Young (1954) demonstrated in both live rabbits and human livers at necropsy that the blood supply to the tumour was virtually exclusively from the hepatic artery. Ackerman (1974) studied the vascularity of solitary rat hepatic Walker Carcinosarcoma tumours by the injection of coloured silicone rubber solutions. Tumours of less than 1mm in diameter had no evidence of a newly developed circulation. Tumours between 1-2mm developed a plexus of vessels which encircled the tumour and were derived from either portal or arterial systems. Larger tumours developed more extensive and complex vascular patterns although the arterial system predominated. These vessels were irregular and tortuous and different to the vasculature of the host and again mainly supplied by the hepatic arterial system (Lien and Ackerman 1970). Larger tumours still developed avascular areas and more varied patterns of vascularity. Honjo and Matsumura (1965) noted a portal component to the supply of the rims of large tumours which they believed may indicate a nutritive role for the portal blood at the tumour rim. However Ackerman interpreted the appearance of the tumour at the edge of the encircling plexus as a compression phenomenon in which the vessels were displaced during tumour growth. Rogers et al. (1967) found that the centres of tumours did not stain with lissamine green whereas smaller ones stained uniformly and that larger tumours had lower flow per unit volume. In vivo studies of the vasculature of human tumours have been performed using angiography although these tend to show the encircling vessels around the tumour and less about the intratumour vasculature (Bragg 1976).

In a study using radioiodinated human serum albumin (Ackerman et al. 1972), major haemodynamic changes were found when flow in either portal or arterial systems was interrupted. When the portal vein was ligated, the tumour to liver ratio of an agent injected into the hepatic artery fell. This suggests the presence of arterioporal shunts allowing blood from the hepatic artery to reach the capillaries in the distribution of the portal vein and also that the portal vein must be intact to maintain a high tumour to liver ratio of regionally delivered agent.
1.7. Vasoactive Manipulation of Tumour Blood Flow

1.7.1. Rationale for Vasoactive Manipulation

In the 19th century the French Physician Jean Poiseuille described the relationship between blood flow, the pressure differential across the capillary bed, the viscosity of the blood and the geometric parameters of the vessels:

\[ Q = \pi D^4 \Delta P / 128 \eta L \]

where \( Q \) is blood flow, \( D \) the vessel diameter, \( \Delta P \) the pressure difference, \( \eta \) the viscosity and \( L \) the vessel length.

Tumour vessels originate as sinusoidal outgrowths of normal vessels (Folkman 1986) and therefore lie in parallel to normal vessels. Thus changing \( \Delta P \) alone is unlikely to cause a selective difference between tumour and normal blood flow and both are likely to be influenced by a similar amount. As tumour vessel length is fixed the only two variables likely to lead to a selective difference are the diameter of the vessel and the blood viscosity. As blood flow is proportional to \( D^4 \), a small change in vessel diameter will effect a major flow change. Therefore drugs that may differentially affect the diameter of normal and tumour vessels will lead to a considerable blood flow difference.

The vasculature has sometimes been illustrated by an electrical circuit diagram to show the sites of vascular resistance within the circulation (see figure 2). In the figure, \( R_1 \) represents the variable resistance of the major feeding arteries, \( R_2 - R_6 \) represent the precapillary, capillary and post capillary vessels of tumour and liver vascular beds. Different vasoactive agents may then be postulated to act at different sites. Agents acting at \( R_1 \) will in theory affect blood in liver and tumour equally. The vascular beds of liver and tumour differ in both their resting state and the proportion of smooth muscle arterioles within them so that a drug increasing the vascular resistance at \( R_5 \) or \( R_6 \) is likely to have a proportionately greater effect than on \( R_2 \) or \( R_3 \). It has also been suggested that by increasing the vascular resistance in the liver bed it may be possible to cause an absolute increase in tumour flow by shunting blood from the liver bed through the tumour bed.
Figure 2 A circuit diagram representing blood flow through normal (I_N) and tumour (I_T) vascular beds. R1 represents the variable resistors present in the large feeding arteries. R2 and R5 represent the resistances in host arteries in normal tissue or incorporated into tumour. R3 and R6 represent the resistance in the normal arterioles, pretumour arterioles and arterioles incorporated into the tumour bed. R4 and R7 represent the fixed vascular resistances in the normal and tumour capillary beds. (after Jirtle 1988)

1.7.2. Pharmacoangiography

Early attempts to manipulate tumour blood flow were described by radiologists who administered vasoconstrictor agents to enhance the vascular blush of tumours seen at angiography. Abrams presented the first results on the demonstration of a canine renal tumour using adrenaline (Abrams 1962) and in further clinical studies reported improved imaging in renal tumours (Abrams 1964). Other vasoconstrictors used for pharmacoangiography were angiotensin (Ekelund and Lunderquist 1974) and vasopressin (Carlsson and Erikson 1970). Vasodilators were also used although less widely as it was felt that the enhanced tumour visualisation was due to the vasoconstriction of normal arterioles while the tumour vessels were unable to react.
1.7.3. Pharmacology of Vasoactive Agents

1.7.3.1. Vasopressin

Vasopressin is an oligopeptide (Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly) and acts via specific receptors of which 2 species are recognised with one type (V1) predominantly mediating the pressor effects while the V2 receptors mediate via the adenylate cyclase system to mediate the antidiuretic effects. Vasopressin given systemically increases blood pressure and reduces cardiac output. It acts directly to constrict smooth muscle arterioles and is clinically administered in the case of variceal bleeding to cause splanchnic vasoconstriction. It is rapidly inactivated in the liver and kidney with a circulating half life of 18 minutes.

1.7.3.2. Angiotensin II

Angiotensin II is an octapeptide (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) with pressor and vasoconstrictor effects. It is synthesised in the kidney from angiotensinogen via angiotensin 1 catalysed by renin and angiotensin 1 converting enzyme. It is a potent pressor agent and is 40 times more potent than noradrenaline. Its plasma half life is only 15 to 60 seconds. It binds to vascular smooth muscle receptors particularly in precapillary sphincter arterioles as well as stimulating constriction through the sympathetic nervous system. As there are fewer arterioles incorporated into tumour than the surrounding normal tissue, angiotensin II may decrease tumour blood flow by a proportionately lesser amount than the surrounding tissue and as a result of the associated hypertension, blood flow may even rise.

1.7.3.3. Endothelin I

Endothelin I is a potent vasoconstrictor polypeptide produced by vascular endothelial cells. It is the most potent vasoconstrictor on a molar basis and is 5 times as potent as angiotensin. It consists of 21 amino acids (molecular weight 2492 Daltons) and is structurally related to the venom of the Israeli burrowing asp (*Atractaspis engaddenesis*). The endothelins originate from a large prepropeptide called big endothelin and consists of 38 amino acids and is broken down to form endothelins I, II and III. The physiological effects include bronchoconstriction (Turner et al. 1989) and it has positive inotropic effects on the heart (Ishikawa et al. 1988). Its complete physiological role is not fully understood but it appears to have a role in the maintenance of vascular smooth muscle tone and the regulation of systemic blood pressure (Brain et al. 1988, Davenport et al. 1990, Hamblin 1990). When infused into the human brachial artery endothelin initially increased forearm flow and then subsequently decreased it. At higher doses, there was a toxic reaction of sweating and vomiting (Dahlof et al. 1990).
1.7.3.4. Histamine

Histamine is well known for its role in inflammation. It combines with specific receptors of which two species are recognised. The H\textsubscript{1} receptor is mediated by cAMP and increases capillary permeability and blood flow (Bhargava et al. 1977) while the H\textsubscript{2} is mediated by cGMP and increases gastric acid secretion. Histamine exerts a predominantly small vessel vasodilator effect on the circulation lowering peripheral resistance and blood pressure but may also have a direct vasoconstrictor response in larger vessels. Capillary permeability is increased by histamine and the effect can be blocked by H\textsubscript{1} antagonists as in the lung where the increase is blocked by terfenadine but not by the H\textsubscript{2} blocking agent cimetidine (Chan et al. 1987). Agents such as diphenhydramine which have an antihistamine property reduce capillary permeability in tumours (Ackerman and Jacobs 1988). However, there is a H\textsubscript{2}-mediated component of the effect on permeability (Braude et al. 1984).

1.7.3.5. Leukotriene C\textsubscript{4}

Leukotriene C\textsubscript{4} is a vasoconstrictor metabolite of arachidonic acid (Samuelson 1983) produced from the 5-lipoxygenase pathway. 'Slow reacting substance of anaphylaxis' (SRS-A) has been recognised for some time (Feldberg and Kellaway 1938) but has been broken down and identified as three specific leukotrienes - C\textsubscript{4}, D\textsubscript{4} and E\textsubscript{4} (Lewis et al. 1990). The biological effects of leukotrienes suggests that they play a role in inflammatory and allergic disease (Samuelson 1983). They influence the microvasculature to increase the margination of leucocytes, increase permeability of the post-capillary venule and stimulate leucocyte diapedesis (Lewis and Granger 1988, Lewis et al. 1990). Increased vascular permeability produced by the contraction of adjacent endothelial cells can occur in response to leukotriene C\textsubscript{4}, D\textsubscript{4} or E\textsubscript{4} produced by adherent leucocytes. Leukotriene C\textsubscript{4} is approximately 100-fold more potent than histamine (Hedqvist et al. 1980) in its ability to increase pulmonary epithelial permeability. Leukotriene B\textsubscript{4} increases mucus secretion in the bronchi, produces bronchoconstriction and is approximately 1,000 times more potent than histamine on a molar basis (Dahlen et al. 1980). Leukotriene C\textsubscript{4} is a potent vasoconstrictor (Badr et al. 1987, Frolich and Yoshiza 1987) and has been identified as a potent cause of vasoconstriction in subarachnoid haemorrhage (Rodriguez et al. 1987). The microvascular effects of the leukotrienes include the increase in capillary permeability seen with extravasation of Evans blue and fluorescein labelled dextran and in this test were 5,000 times more potent than histamine (Dahlen et al. 1980).
1.7.3.6. Calcitonin Gene Related Peptide (CGRP)

CGRP is a 37 amino acid peptide discovered in 1982 by isolation of messenger RNA from a rat calcitonin cancer similar to human medullary thyroid cancer. It is found in all tissues, particularly the nervous system. It is considered an important neurotransmitter in the human cardiovascular system acting as a powerful endogenous vasodilator. It is a positive inotrope and chronotrope and causes general vasodilation throughout the peripheral vasculature (Ledda et al. 1993). CGRP has been shown to be expressed in cultures of liver cells along with specific receptors (Bracq et al. 1994). It acts to oppose the vasoconstrictor effects of endothelin I (Lopez-Belmonte and Whittle 1993) and in the mesenteric bed acts to release endothelially derived nitric oxide (Ledda et al. 1993).

1.7.3.7. Nitric oxide / N-nitro-L- arginine methyl ester (L-NAME)

Furchgott and Zawadzki (1980) characterised an endothelially derived substance necessary for the vascular relaxation of smooth muscle which later became known as endothelium dependant relaxation factor (EDRF). This was later characterised as nitric oxide (NO) synthesised from L-arginine by NO synthetase and acting by stimulation of the soluble guanylate cyclase leading to a continuous vasodilator tone and opposed by the vasoconstrictor tone of the smooth muscle and the action of endothelin (Moncada et al. 1991). Inhibitors of NO include L-NG mono methyl arginine (L-NMMA) and N-nitro-L-arginine methyl ester (L-NAME) which act as competitive inhibitors of NO synthetase. It has been shown that NO is the mediator of ATP induced vasodilation in the hepatic arterial bed (Mathie et al. 1991) and may be involved with endothelin I in the regulation of basal sinusoidal tone within the liver (Kawada et al. 1993).
1.7.4. Vasoactive Manipulation in Extrahepatic Tumour Models

1.7.4.1. Vasoconstrictors

Cater recognised the importance of blood flow manipulation in the treatment of malignant tumours and studied the effect of both bolus and slow systemic infusion of vasoactive drugs on the oxygen tension of subcutaneous rat hepatomas (Cater et al. 1962, Cater et al. 1966). He found that both adrenaline and noradrenaline led to a fall in tumour oxygen tension, although when the former was given by infusion there "did appear to be the possibility of raising the oxygen tension". Angiotensin also did not produce an increase in oxygen tension. Isoprenaline given for its vasodilator action also caused a fall in tumour oxygen tension which was attributed to associated hypertension, while acetyl choline led to a small rise in tumour oxygen tension. In this study an indirect method of blood flow measurement is used, the vasoactive agents are given systemically and the tumour model is a subcutaneous hepatoma. Nonetheless, the study is of interest due to a differential response between the effects of adrenaline when given as a bolus and as a 15 minute infusion.

Kligerman and Henel (1961), using an Algire chamber technique concluded that vasoactive compounds modified the tumour blood supply in a similar way as normal tissues and concluded that although tumour vessels might be unable to react, they were subject to the control of the arteriolar blood supply. Intravenous adrenaline led to a fall in tumour blood flow (Gullino and Grantham 1961). Rankin and Phernetton (1976) found the vasculature of the rabbit VX2 carcinoma very sensitive to noradrenaline. Wickersham et al. (1977) studied the tumour circulation in mouse mammary tumours by direct inspection of the most prominent arteriole. He found that as the tumour enlarged there was a loss of response to the vasoactive agent which was most marked after topical administration rather than intravenous.

Mattsson et al. (1978) used a rubidium-86 uptake technique to study the flow in a rat intramuscular tumour. Noradrenaline led to a fall in isotope uptake in both muscle and tumour which was blocked by pretreatment with phenoxybenzamine, while ischaemia led to a slight rise in both. In order to try to establish whether the fall in tumour blood flow was occurring within tumour vessels or within arterioles supplying the tumour Mattsson et al. (1980) used a local isotope washout technique to measure the response to noradrenaline. However in this study both isotope and noradrenaline were mixed together and given by direct injection into the tumour. Once again there appeared to be a reduction in tumour flow implying a direct response by the intratumour vasculature.
1.7.4.2. Vasodilators

Vasodilators have been used to lead to a selective reduction in tumour blood flow. Hydralazine acts by direct relaxation of vascular smooth muscle. As the tumour vasculature is in a state of greater vasodilation than normal tissue, hydralazine decreases the blood flow through tumour by a steal phenomenon. Vorhees and Babbs (1982) have demonstrated such a reduction in tumour to normal ratio in dogs bearing intramuscular tumours. Similar studies have been produced using calcium channel blockers causing a selective decrease in the tumour to normal ratio (Knapp et al. 1985). Such reductions in the tumour to normal blood flow ratio are of benefit in conjunction with treatment modalities such as radiotherapy and hyperthermia but are the opposite of that required to enhance the uptake of cytotoxic agents.

1.7.5. Vasoactive Manipulation in Hepatic Tumour Models

1.7.5.1. Catecholamines

In a study of the effect of intravenous adrenaline and noradrenaline on rats with an intrahepatic hepatoma or adenocarcinoma, Hafstrom et al. (1980) measured the tumour to liver blood flow ratios before and after the onset of vasoactive manipulation with a dual microsphere technique. They found an increase in the tumour to liver blood flow ratios for noradrenaline but not for adrenaline, although the absolute tumour flow changes were not given. However others (Burton and Gray 1987) have found a fall in tumour to liver ratio with noradrenaline but an increase when given with propanolol. Young et al. (1979) also found a fall in hepatic tumour blood flow with noradrenaline.

Ackerman carried out a series of studies of the effects of vasoactive agents in a rat intrahepatic Walker carcinosarcoma. Ackerman et al. (1980a) examined tumour vasculature by perfusion with silicone rubber solution and found that capillary like vessels appeared to open in the central area of the tumour in response to intra-arterial or intraportal adrenaline. He put forwards several possible explanations for this. Firstly the effect could be on the arterioles supplying the tumour leading to increased pressure and flow into both encircling plexus and central tumour areas. Secondly, there may be a decrease in arterioporal shunting around the tumour leading to an increased internal flow and finally that the effect may be on the venous outflow leading to a congestion in the central areas of blood flow. This was later confirmed using a different technique of fluorescent dye injection (Ackerman et al. 1989). Ackerman also studied the effect of adrenaline using laser Doppler (Ackerman and Jacobs 1985). In this study rats were given bolus doses of adrenaline into the portal system. Tumour blood flow was seen to rise initially for a period of up to 1 minute and was followed in some groups by a secondary fall in perfusion which was sustained over several minutes before returning to baseline. This study was important in appreciating that the effects of vasoactive manipulation may vary with time. Ackerman postulated that the variable results obtained
previously (Gullino and Grantham, 1961, Edlich et al. 1966, Hafstrom et al. 1980) could be explained on the basis of a delay after injection of adrenaline before measurements were made which meant that the acute redistribution of blood to the tumours would be missed. In a further study (Bloom et al. 1987) in which doxorubicin was given with or without intraportal adrenaline, tumours showed greater response in the group given adrenaline. In one of the few previous studies to measure tissue cytotoxic levels when regionally administered with vasoactive manipulation, Iwaki et al. (1978) showed a higher tumour to liver mitomycin C ratio when given with intra arterial adrenaline. Although group sizes were small they found that the tumour drug uptake was little affected by the adrenaline while levels in normal liver were reduced.

1.7.5.2. Angiotensin II

Tvete et al. (1981) demonstrated an increased tumour/renal flow ratio while Suzuki et al. (1981) reported a six fold increase in tumour to liver ratios. Burton et al. (1985) investigated the potential of angiotensin to enhance the tumour to liver ratio for the delivery of 90-Yttrium microspheres which had previously been postulated as a treatment for hepatic metastases (Stribley et al. 1983). They found a 3 fold increase in the tumour to liver ratio and greater penetration into the centre of the tumours. Hemingway et al. (1991a) investigated the uptake of a low weight molecular marker and found a 4 fold increase in marker retention in tumours where injection was preceded 30 seconds earlier by intra-arterial angiotensin. Carter et al. (1992) showed that this effect was additive with the effect of degradable starch microspheres. Studies in humans are limited but angiotensin II was shown to cause a 3 fold increase in the tumour to liver ratio for the uptake into open biopsies of dual labelled albumin microspheres injected into the hepatic artery at laparotomy (Goldberg et al. 1991). Hemingway et al. (1992) showed an increase in absolute tumour flow using laser Doppler flowmetry at laparotomy in 8 out of 10 patients given a 90 second regional angiotensin infusion, although did not measure the corresponding liver perfusion changes. Furthermore the maximal increase in tumour blood flow was not until after the end of the angiotensin infusion.

In all of the preceding studies angiotensin has been given either as a bolus or over a period of less than 5 minutes. Sasaki et al. (1985) studied the effect of regional angiotensin II infusion in patients suffering from hepatocellular carcinoma (n=9) and metastatic colorectal carcinoma (n=2). 81-Krypton a short lived gamma emitting isotope ($t_{1/2}$ 13 seconds), was continuously infused into the hepatic artery. Angiotensin II was administered into the hepatic artery or peripheral vein for 3-4 minutes. Planar measurements were made using a gamma camera and tumour to liver ratios calculated by comparing regions of interest. There was a 3 fold increase in tumour to liver ratio although the increase was maximal at around 100 seconds after which the ratios tended to decrease. Intravenous infusion produced lesser rises although the administered dose of
angiotensin was less. This study was important as the only one where the beneficial effect of angiotensin infusion was studied with a declared aim of use with continuous intra-arterial infusion. Despite this infusions of only 3-4 minutes were given.

1.7.5.3. Vasopressin

Vasopressin has also been shown to lead to the preferential delivery of blood to hepatic tumour when administered as a 10 minute intravenous infusion (Hemingway et al. 1991b) although previous work (Jenkins et al. 1984) showed that whether flow is increased or decreased in tumours is likely to depend on dose administered.

1.7.6. Manipulation of Tumour Permeability

Ackerman and Hechmer (1978) investigated the capillary permeability of an experimental rat hepatic tumour using an Evans blue technique and found initially greater permeability in liver which then became greater in tumour from 2 hours onwards. Vasoactive manipulation may influence the transport of molecules by increasing tumour blood flow, by increasing tumour vascular pressure or decreasing tumour interstitial pressure.

Vascular permeability agents have been investigated as potential agents to enhance the uptake of therapeutic cytotoxic agents. Papadimitriou and Woods (1975) found that histamine increased the leakage of trypan blue into subcutaneous sarcomas while Underwood and Carr (1972) however found that histamine and serotonin did not increase the escape of Evans blue but increased the escape of saccharated iron oxide and colloidal carbon. Ackerman et al. (1980a) found that liver but not tumour had an increased permeability to Evans blue in response to regional histamine, bradykinin and serotonin. Hennigan et al. (1991) demonstrated increased uptake of an anti CEA antibody in a rat xenograft model of up to 3 fold using histamine, and also found lesser increases with Leukotriene C4 and interleukin-2.

The variable results may arise from the differences in how permeability has been quantified and it is unclear if the same increase might be seen with smaller molecules than antibodies. As a result, the potential role of permeability agents in therapy is unclear. Furthermore it is often difficult to separate the effects on permeability from those as a vasoconstrictor or vasodilator agent.
1.7.7. Choice of Vasoactive Agent

A variety of vasoactive agents were used in these studies. Agents were selected on the basis of previous studies suggesting a potential benefit or on the basis of their mechanism of action which suggested that they may be of value. Vasopressin and angiotensin II are both peptide hormones which act by binding to smooth muscle receptors. Angiotensin has a shorter half life than vasopressin and may thus have a greater regional advantage and binds more specifically to receptors in the precapillary arteriolar region. Both agents have been suggested for use to increase the tumour to normal ratio. Endothelin is also a polypeptide with predominantly vasoconstrictor properties. It has been shown in a pilot study to have the potential to produced sustained hepatic vasoconstriction (Hennigan and Allen-Mersh 1994).

Nitric oxide has recently been identified as an important endogenous endothelial vasodilator which provides a continuous vasodilator tone to small vessels (Moncada et al. 1991). The vascular effects of nitric oxide and nitric oxide inhibition as a method to enhance anti tumour therapy have not been previously investigated.

In addition to these agents it was decided to investigate the effects of the potent vasodilator Calcitonin Gene Related Peptide (CGRP). Its has an action on the liver sinusoids and leads to a general vasodilation and leads to the release of nitric oxide.

Capillary permeability agents may have a potential to increase the uptake of molecules into tumours. Histamine has been shown to increase the uptake of isotope antibody conjugates by three fold (Hennigan et al. 1991). While the barriers to the uptake of smaller molecules such as 5FU are likely to be different from antibodies, there may also be a beneficial effect on the uptake of smaller molecules. In particular, most capillary permeability agents also have some vasoactive effects and these may contribute to the changes in molecular uptake. Two capillary permeability agents are investigated, histamine and leukotriene C4, the former associated with vasodilator effects and the latter vasoconstrictor.
1.8. Fluoropyrimidine Pharmacology

5 Fluorouracil (5FU) was introduced over 30 years ago and has gained widespread usage as an anticancer drug with activity against gastrointestinal, breast and ovarian tumours. It is an analogue of the naturally occurring uracil pyrimidine molecule required for nucleic acid synthesis which was synthesised by Duschinsky et al. (1957). The 5FU molecule itself is not cytotoxic and requires in vivo metabolism. Two main metabolic pathways exist in which the 5FU either undergoes catabolism accompanied by elimination and inactivation or anabolism to nucleotides, the cytotoxic metabolite.

Entry into the cell was initially thought to be by simple diffusion but is now thought to be via a saturable carrier mediated mechanism. However at physiological concentrations, entry into the cell is by first order kinetics and thus flow and not transport is likely to be the rate limiting mechanism. Therefore an increase in tumour blood flow could be expected to increase tumour uptake of a fluorinated pyrimidine.

Most studies of 5FU metabolism have focused on anabolite production although the availability of 5FU for anabolism may be dependent on the rate of catabolism (Heggie et al. 1987). There are at least three anabolic pathways in which 5FU may form cytotoxic nucleotides. The mechanism by which cell death comes about is unclear. Inhibition of thymidylate synthetase by FdUMP has been shown to relate to tumour chemosensitivity in some tumour models although the 5FU derived nucleotides are incorporated into both RNA and DNA.

Fluorodeoxyuridine (FUdR) is a 5FU analogue with a plasma half life of about 10 minutes which is rapidly hydrolysed into 5FU. Membrane transport is extremely rapid and the one step phosphorylation to the active anabolite FdUMP by thymidylate synthetase would appear to be an advantage. However toxicity patterns differ considerably from 5FU and is mainly used in association with regional chemotherapy.

Studies of fluoropyrimidine pharmacokinetics have largely been following intravenous bolus administration rather than protracted continuous infusion. The plasma half life after an intravenous bolus of 5 FU is between 8-22 minutes although its metabolites are present for longer. (Heggie et al. 1987). When given by continuous infusion there appears to be marked diurnal variation in the level of 5 fluorouracil (Petit et al. 1988) and FUdR (von Roemeling and Hrushesky 1989).

FUdR and 5FU infused into the hepatic arterial system have extraction fractions of 94-99% and 19-51% respectively (Ensminger et al. 1978).
1.9. How Can Tumour Blood Flow be Measured?

The combination of low tumour blood flow and marked vascular heterogeneity make the accurate measurement of tumour blood flow difficult to achieve. As a result, the study of tumour blood flow has been hampered by methodological difficulties. A wide variety of techniques have been described, many of which reflect one aspect of tumour perfusion and not the overall tumour flow. Initial studies utilised indirect methods such as the oxygen tension (Cater et al. 1962) or dye distribution (Owen 1960). A variety of techniques were used which each appeared to measure one aspect of tumour perfusion. Bierman et al. (1951, 1952), studied human tumours by angiography and venous oxygen content and concluded that tumours were highly perfused.

Flow measurements may give absolute flow (ml/min/g) or may be in relative terms, often as a proportion of cardiac output. Absolute measurements are more desirable but not always obtainable. It must be borne in mind that where flow is measured in relative terms, there may be a change in cardiac output over the course of the experiment in particular where vasoactive manipulation is used.

1.9.1. Direct Flow Measurement

1.9.1.1. Collection of venous effluent

Measurement of tissue blood flow can be achieved by the placement of an electromagnetic flow probe directly on the supplying artery. No such method is possible in the measurement of tumour blood flow as the blood supply that directly and exclusively supplies the tumour is rarely identifiable. The closest such direct measurement was that of Gullino and Grantham (1961) who devised a method by which they transplanted tumour tissue to a kidney or ovary with total isolation from the host other than by preservation of the vascular pedicle. Once the tumour had completely taken over the organ the venous effluent was cannulated and collected by means of a peristaltic pump circuit. This was the first direct measurement of tumour flow although the effects of tumour growth isolated from surrounding tissues is not known. They found that where a tumour was implanted into an ovary, above 4g there was no evidence of residual ovarian tissue, unlike in kidney where some residual renal tissue remained. The average blood flow for a hepatoma implanted into the kidney was 0.14ml/hr/mg; 20 times less than that of normal liver flow.

1.9.1.2. Plethysmography

This method can be used to measure the blood flow of tumours implanted into the paws of animals (Kjartansson et al. 1976). The paws were placed in a water filled cylinder at a controlled temperature. A venous occlusion cuff was rapidly inflated and the change in paw volume measured by the change of pressure within the cylinder. The tumour blood flow was calculated from the difference in volume change between the tumour and non
tumour bearing paws. Such a method is however complex and does not exclude from measurement normal tissue adjacent to the tumour.

1.9.2. Radiotracer Methods

Radioactive Microspheres Methods

Use of radioactive microspheres for the measurement of organ blood flow is now well established. The sphere chosen is designed to be trapped in the arterioles and capillaries of the first vascular bed. Spheres are prepared by cleaning and resuspending in glucose solution and are injected into the left ventricle of the heart. The spheres are then distributed to the various organs of the body according to the proportion of cardiac output. Absolute flow values can be calculated using the artificial reference organ method (Bartrum et al. 1974) in which a reference sample is collected by withdrawal of blood at a constant rate through a cannulated artery such as the femoral artery over the period of injection of the microspheres.

Multiple measurements can be made using microspheres with different radioactive labels and up to four serial measurements have been made (Voorhees and Babbs 1982).

Because part of the liver inflow is from a portal system, measurement of liver flow using microspheres will only measure the flow arriving through the hepatic artery. The portal contribution can be calculated indirectly by summation of the blood flow from the organs that drain into the portal vein. Groszmann et al. (1982) described a similar organ summation method for the measurement of portal flow and also measured portasystemic shunting by the direct injection of microspheres into the spleen.

Microspheres may also be used to measure tumour flow. There are however certain potential pitfalls of which to be aware (Buckberg et al. 1971). It is of importance to ensure that the spheres are adequately mixed. Streaming or clumping of microspheres will lead to invalid results. Many tumours have very low blood flow levels and it is difficult to ensure an adequate number of microspheres present for adequate statistical precision. Increasing the number of systemically injected microspheres may help although above a certain level the microspheres will have cardiovascular effects.

1.9.2.1. Isotope Distribution Method According to Sapirstein (1958)

This method is based on the fractional distribution of an intravenous injection of 86-Rb or radiolabelled iodoantipyrine. The injected isotope mixes in the heart and is initially distributed according to cardiac output. Although uptake is not 100% the remainder recirculates and in approximately 10-15 seconds, virtually all is taken out of the circulation. The animal is killed before the isotope redistributes throughout the body by diffusion, and the organs are counted. A dose sample is required to measure the total injected radioactivity and the results are expressed as the percentage of injected dose taken up or as a percentage of cardiac output.
The disadvantages for measurement of liver blood flow are that because of the portal inflow to the liver, the concentration of isotope delivered portally will be uncertain and may vary. Because of the differing proportions of blood supply of normal and tumour tissue in the liver this may bias the findings. The second disadvantage is that it is necessary to kill the animal within a short space of time after injection and therefore only one measurement can be made.

1.9.2.2. Isotope Uptake

According to the method of Kety(1960)

In this method iodoantipyrine is given into the arterial supply of the area of interest and the animal is killed within seconds of the completion of the injection to prevent redistribution of the iodoantipyrine. Tissue counting can either be counted in scintillation vials or autoradiographs can be prepared and quantified by quantitative microdensitometry.

133-xenon uptake

In this method an intra-arterial injection is given into the blood supply of an organ and the residual activity in the tissue measured with a carefully shielded collimator. This method can be used to measure tumour flow (Oikawa et al. 1975) and can be repeated at intervals but is subject to the inaccuracies of using an external collimator such as the detection of radioactivity from tissue adjacent to the tissue of interest. In addition, while isotope partition coefficients are generally available for most normal tissues, the tissue blood partition coefficient for tumour is variable and not easily measured. As a result, the tumour partition coefficient in most studies is usually assumed to be similar to that of the host tissue thus incorporating an unquantifiable error.

1.9.2.3. Local Tissue Clearance Methods

This technique involves the local injection of 133-Xe or 85-Kr into the tumour. The isotope equilibrates with the venous blood at that site and the residual activity in the tumour as it is washed out is measured with a collimated detector. The flow rate can be calculated from the time taken for the activity to halve. Although fairly widely used for tumour blood flow measurement, there are several potential sources of error which detract from the accuracy of the results, including those referred to above concerning tissue partition coefficients and external collimators. Furthermore, tumour heterogeneity means that any local measurements from one part of the tumour may not reflect the situation throughout the tumour as a whole. In addition the effect of an injection of isotope into the tumour substance creates an artefact both in isotope leakage and the effects of the isotope on the immediate vascular perfusion. These potential sources of error detract from the accuracy of the technique, although its relative simplicity makes it a useful technique in some circumstances.
1.9.2.4. High uptake / High retention tracers

More recently a number of radiotracers have been developed which have high tissue retention but unlike iodoantipyrine and 133-Xe have high tissue retention due to a chemical reaction within the cells. This combination allows for the regional or systemic administration of the radiotracer which will then be distributed according to blood flow or cardiac output but which will not then redistribute by diffusion. Two such tracers are $^{99m}$Tc-hexamethyl propyleneamineoxime ($^{99m}$Tc-HMPAO) and Copper (II)-pyruvaldehyde bis(N-4 methyl thiosemicarbazone) (Cu-PTSM). These have both been used in the studies in this thesis and their properties are described in more detail in sections 3.3.4 and 3.3.5.

1.9.3. Quantitative Perfusion Fluorometry

This is an alternative method of blood flow measurement (Silverman et al. 1981) which has the advantage of avoiding the use of radioisotopes. Sodium fluorescein diffuses across the capillary wall to stain the extracellular fluid. When exposed to blue or ultraviolet light, it emits yellow-green fluorescence. Dye uptake correlates with vascularity and can be quantified by a perfusion fluorometer. Injected into the regional arterial supply it has been used to measure the relative tumour and liver perfusion in association with the injection of degradable starch microspheres (Thom et al. 1988).

1.9.4. Thermodynamic Methods

These methods involve heating or cooling of the tissue under examination. Thermal flux is measured a short distance away from a heat sink maintained at a constant temperature (Johnson 1976). The technique allows multiple measurements but is mainly limited to measurements at superficial sites and is likely to influence flow itself during the process of heating or cooling. Furthermore the technique assumes a linear relationship between blood flow and heat transfer over the range of interest. Alternative techniques include thermal washout measurement but are subject to the same errors and limitations (Samulski et al. 1987).
1.9.5. Laser Doppler Flowmetry

1.9.5.1. Development of laser Doppler flowmetry

1.9.5.1.1. The Doppler principle

Johann Christian Doppler (born Salzburg Austria, 1803 - died Venice Italy 1853) worked as a mathematical assistant in Salzburg writing on mathematics and electricity before being appointed Professor of Elementary Mathematics and Practical Geometry at the State Technical Academy in Prague. In May 1842 he presented his paper "On the coloured light of double stars and some other heavenly bodies" (White 1982) in which he derived the formula for the motion of source or observer along a line between them. He mentioned the application of this principle to both light and sound. The first experimental validation was conducted two years later by Buys Ballot. In this experiment he borrowed a locomotive and a flatcar. A trumpeter rode on the flatcar towards a second musician at the station who confirmed that as the train approached the trumpet note fell by one half tone. Initial application of this finding was confined to astronomy and little use was made for other purposes until the advent of the laser.

Charles Townes and Arthur Schawlow (1958) developed the idea in which stimulated molecules encounter an electromagnetic wave and give up their energy to the wave thus strengthening it and as this process repeats itself the strength of the wave quickly multiplies. The first laser was developed with the first patent awarded in 1960.

The properties of laser light which are of use in laser Doppler flowmetry are the narrow range of wavelengths emitted, their coherence and the fact that the waves all have the same frequency, amplitude and direction.
1.9.5.1.2. Laser Doppler Velocimetry

With the advent of laser light, a technique known as light beating spectroscopy was further developed. The output of two independent lasers simultaneously illuminating a single photomultiplier led to a beat note at the difference in the frequency of the two laser wavelengths. By mixing scattered and unscattered light, Yeh and Cummings (1964) measured the flow velocities in different regions of a fluid undergoing lamellar flow. Laser Doppler Velocimetry then gained widespread use in industrial research.

Figure 3 Portrait of Johann Christian Doppler (1803 - 1853)

1.9.5.1.3. Laser Doppler flowmetry

Riva et al. (1972) made the first measurements of blood cell velocity in individual vessels of the retina of rabbits. The first measurements of perfusion were made by Stern from his finger tip (Stern 1975). Having devised an algorithm to quantify the flow from the root
mean square of the bandwidth, he compared flow readings in skin to those made by the xenon washout technique (Stern et al. 1978). Early commercial instruments were produced by Medpacific and Perimed using Helium / Neon laser light.

1.9.5.2. Principles of laser Doppler flowmetry

The tissue is illuminated under laser light which is scattered in both static structures as well as red blood cells in the capillary bed. Photons scattered by moving red blood cells undergo a frequency shift according to the Doppler effect. Conversely light photons scattered by static structures do not undergo a frequency shift. Some of the scattered light reaches a sensitive photodetector and the photocurrent signal contains components from light which has been frequency shifted and that which has not. This signal is processed and the output is proportional to both red blood cell concentration and velocity.

The average depth and subsequent volume of the tissue samples increases with the separation of the laser and photodetector fibres. However because the intensity of the detected light falls off rapidly one is restricted in practice to a separation of not more than 1-2mm.

Figure 4 Illustration of the principles of laser Doppler flowmetry showing how laser light is scattered and undergoes a frequency shift when it strikes a blood cell
1.9.5.3. Application of laser Doppler flowmetry

1.9.5.3.1. General

Laser Doppler flowmetry has developed over the past 15 years into a widely used technique for the monitoring of tissue perfusion in a wide variety of applications (Belcaro et al. 1994). Initial studies of the effects of pharmacological agents and hyperthermia were mainly carried out in skin although studies in animals have been carried out in muscle, brain, spinal cord, teeth, gut, kidney and many other tissues. Laser Doppler flowmetry has particular clinical applications in the monitoring of free tissue transfers in plastic surgery, in the assessment of intestinal ischaemia and the effects of microvascular perfusion in peripheral occlusive vascular disease, Raynauds disease and diabetic microangiography. A proliferation of probe types now enables probes to be passed along endoscope working channels and future variations are likely to include remote monitoring of probes implanted within the body.

1.9.5.3.2. Liver

Previous studies (Shepherd et al. 1987, Almond and Wheatley 1992) have shown that laser Doppler can be used within the liver substance and produce a linear response to changes in blood flow. However the simultaneous application of the probe to both liver and tumour in a small rodent has not previously been reported. Arvidsson et al. (1988) investigated whether laser Doppler flowmeter readings from the surface of the liver reflected total hepatic flow in the pig and found that they did although the technique was found to be more sensitive to flow changes arising in the hepatic artery rather than portal vein.

1.9.5.3.3. Tumour

Acker et al. (1990) has measured interstitial tumour blood flow during hyperthermia treatment and has evaluated the amount of tissue disruption as a result of interstitial insertion of the laser Doppler probe. He found that the disrupted tissue was limited to within 0.12 mm of the probe tip.
2. Hypothesis and Aims

It can be seen from the background section that tumour and liver blood flow might be influenced by vasoactive agents. This has been previously demonstrated when such agents are administered as a bolus or short infusion. It has been assumed that this effect will increase the tumour to normal ratio for the uptake of cytotoxic agents although this has been little tested and not for prolonged infusions. By administering these agents into the hepatic arterial system for a more prolonged infusion period, it was hoped to understand more of the early vasoactive changes and to establish which agents produced the most beneficial effects.

The hypothesis which has been addressed in this thesis is:

Intra-arterial vasoactive infusion can increase the blood flow ratios to intrahepatic tumours and consequently increase the uptake ratios of a cytotoxic drug.

Aims

In order to investigate this hypothesis the following points have been addressed:

The effect of specific vasoactive agents on parenchymal and tumour blood flow - specifically the duration and magnitude of effect.

The relationship between vasoactive-induced change in parenchymal and tumour blood flow and the effect on the tumour to normal ratio.

The relationship between tumour blood flow and 5FU uptake.

The magnitude of increase in 5FU uptake which can be achieved with vasoactive manipulation.

The development of the methods required to achieve the above aims using a tumour bearing rat model.
3. Materials and Methods

3.1. Tumour Model

3.1.1. Animal Care

All animal work was carried out in a designated laboratory in the Institute of Cancer Research at the Royal Marsden Hospital (Sutton, Surrey). All procedures carried out were covered by a Home Office personal licence (PIL/7009596) under a project licence (DPL/90/00005).

Male Chester Beatty Hooded (CBH/cbi) rats (weight 275-375g) were used for all experiments. Animals were housed in cages (RC1) containing graded sawdust with up to six animals per cage. They were watered and fed ad libitum (total maintenance diet pellets SDS No.1).

3.1.2. Tumour Cell Culture

The tumour used in all experiments was the HSN sarcoma which is a syngeneic sarcoma line originally induced with 10mg 3,4 dibenzpyrene and is passaged and stored as frozen cell suspensions at -80°C. It has similar vascular characteristics to human colorectal hepatic metastases (Hemmingway et al. 1991b) both being hypovascular in relation to the surrounding hepatic parenchyma. Intraportal injection of this tumour as a cell suspension produces 1-8 intrahepatic tumours in about 75% of animals, becoming established between 22 to 28 days from implantation.

Cells were cultured in Dulbecco's modified Eagles medium (Sigma) enriched with 10% foetal calf serum in an incubator. Prior to injection into the animals the cells were trypsinized and resuspended in saline at the required concentration and kept cold by placing the container on ice. For the laser Doppler experiments a single tumour and an area of normal liver parenchyma were required. For these studies, the standard HSN tissue culture (HSN/tc) cells were used in which 1x10^6 cells were injected. For the experiments in which a larger number of tumours were required, a subculture of the HSN/tc cells was used in which cells from a liver tumour had been sub cultured (HSN/lv). With this cell line a smaller number of cells were required to produce a larger number of tumours and between 300-800 cells were injected into each rat according to the cell passage number. However in both cases the original cell line was the same as was the growth rate and there were no apparent differences in the macroscopic or microscopic characteristics of the individual tumours and thus no likelihood of a difference in vascularity.
Figure 5  HSN tumours in the liver of a CBH rat

Figure 6  Photomicrograph of HSN tumour (x300  H and E stain)
3.1.3. Tumour Implantation

Animals were anaesthetised with halothane and a short lower midline incision was performed. A loop of bowel was withdrawn from the abdomen in readiness for cell injection. Prior to injecting the cell suspension, the cells were gently mixed by agitating the container and by drawing the cells into the syringe and emptying them several times. When the cells were judged to be adequately mixed, tumour cells were injected into a portal vein tributary such as a mesenteric vein. Care was taken to ensure clean venepuncture with a 23G needle and subsequent injection of a 0.2ml suspension. Inadequate tumour cell injection led to large mesenteric tumours and precluded the animal from being studied. Following injection the needle was withdrawn and light pressure immediately applied to the area for not less than one minute to ensure haemostasis.

Figure 7 HSN cells being injected into a mesenteric vein
Following this the abdominal contents were replaced within the abdomen. The abdomen was closed with 4/0 chromic catgut (Ethicon) and Michel clips for skin. Animals were returned to their cage and allowed to recover.

This led to the development of intrahepatic tumours (figure 5) suitable for use between 21-26 days after injection. While tumour take was high there were a number of animals which did not develop tumours. The proportion in which there was no tumour take and the number of tumours was found to vary between different batches of injections. This may have related to the technique of cell preparation, in particular the period of cell trypsinisation.

3.1.4. Anaesthesia

Induction of anaesthesia was initially facilitated using a rising concentration of CO₂, but this was abandoned in favour of induction by halothane inhalation (4%) (May and Baker) because of concern that the carbon dioxide may induce vascular changes which might persist throughout the experiment. Maintenance of anaesthesia was with inhaled halothane (1-2%).

Injectable agents such as thiopentone were avoided because of their more marked hypotensive effect. In addition agents such as Saffan which did not result in so marked hypotension were not suitable because their gradually diminishing effect meant that in the laser Doppler experiment conditions were not maintained throughout. Although halothane is recognised to have vasodilator properties, it did produce a stable and sustainable level of anaesthesia. Blood pressure was better maintained with halothane than the barbiturate agents and it was therefore selected for use. During all experiments, animals were placed on a warming pad to maintain body temperature.

3.1.5. Hepatic Artery Infusion

An upper midline laparotomy was performed. A fine haemostat was placed on the lower aspect of the first part of the duodenum and retracted downwards to expose the lesser omentum. The gastroduodenal artery, which leads into the common hepatic artery was dissected free with microvascular forceps and bipolar diathermy for haemostasis. This was then cannulated with polythene tubing (Portex Ltd. 0.28 x 0.61mm, i.d. x o.d.) with the aid of an operating microscope. The cannula was held in place with two 6/0 silk ligatures and the haemostat removed from the duodenal border to ensure no traction on the hepatic vessels. Because of the duration of each experiment, good haemostasis was essential as even a small injury to the omental vessels led to significant vascular changes over the period of the experiment.
3.1.6. Blood Pressure Measurement

Blood pressure was monitored by means of an 18g Teflon cannula (Critikon Ltd) inserted into the right carotid artery and blood pressure measured continuously using a pressure transducer (FCO 11, Furness Ltd, UK). The results were recorded to a personal computer using Metrabyte (DAS8-PG) signal processing hardware.

Figure 8 Cannula being inserted into a rat gastroduodenal artery
3.1.7. Preparation and Storage of Vasoactive Agents

Arginine vasopressin, endothelin I, angiotensin II and histamine, were obtained from Sigma Chemical Company as desiccated powders. They were then dissolved in a small volume of distilled water (variable according to the quantity of vasoactive agent and the required storage concentration) and micropipetted out in 100μl aliquots into ependorf tubes and stored at -20°. They were removed from the freezer and allowed to thaw before being diluted to the required concentration with 0.9% NaCl. L-NAME (Sigma UK) was stored at -5° in a desiccator and was freshly diluted in 0.9%NaCl shortly before use. Leukotriene C4 (Sigma UK) is supplied as a solution which is unstable in air. It was stored at -20° in a silanized glass vial. The solution was diluted in an oxygen free environment and drawn into a 0.5ml syringe and the end stoppered. This was then replaced in the freezer until shortly before the start of each experiment.
3.2. Methods for Laser Doppler Flowmetry Study

3.2.1. MBF3D Laser Doppler Flowmeter

The MBF3D Laser Doppler Blood Flow Monitor (Moor Instruments) was used for all experiments. It has dual channels allowing the simultaneous measurement of flow at two sites or from two tissues. The laser light is 780-820nm wavelength with a power output of approximately 1.8mW from the optic connector and 1.2mW from each of the probe tips and is a class 3B laser. The bandwidth processed is 20Hz-15kHz and the processing incorporates a lineariser. The readings are displayed on an LCD screen and there is an integral plotter device. There is also an RS 232 serial computer port through which all data recorded was transferred to an Elonex 80286 personal computer and recorded as an ASCII file.

As laser output power may vary over time the instrument periodically requires calibration against a standard which uses the Brownian motion of polystyrene microspheres in water at 20-22°C. In order to do this the probe was held motionless in the calibration fluid using a clamp, having first ensured a temperature of 20-22°C using a hand held digital thermometer. The calibration routine sequence was followed on the integral menu system for each probe in turn. Calibration was carried out every 1-2 weeks.

Figure 10  MBF3D Laser Doppler Flowmeter
3.2.2. Optical Probe Type

Three types of laser Doppler probe were evaluated.

1. Right angle probe, in a black acetal disc, 8 mm diameter, 5 mm high which was held in 20 mm acetal flattened disc like holder. Although primarily designed for the measurement of skin blood flow it has been used with some success in the measurement of liver blood flow. The principle was to attempt to attach the disc holder to the surface of the liver and allow the probe to sit snugly within. The main difficulty was finding a substance with which to adhere the disc to the liver. A variety of agents such as a tissue glue and a 'plastic skin' (nobecutane) were used, however the arrangement was generally unsatisfactory and there were also difficulties ensuring the appropriate degree of light contact between the probe surface and the raised tumour surface.

2. Dual fibre 30 mm needle probe, 1 mm diameter was used. This probe design was used throughout the laser Doppler experiments here and was used in conjunction with a specially designed probe holder (see below)

3. Multifibre needle probe This was of a similar shape to the dual fibre needle probe. Light delivery was through a single central optical fibre while there were 9 further fibres through which the returning scattered light was detected. Readings obtained were less variable than the dual fibre version as the broader area of measurement meant that a wider area of liver was being assessed. However the main disadvantage was the accurate placement of the probe over tumour without allowing adjacent liver to influence the reading. For this reason this probe type was not used for experimental measurements.

3.2.3. Probe Holder

Previous use of a probe holder for the measurement of liver surface flux has been described by Ackerman et al. (1989). This idea was modified in order to develop two holders that would sit side by side and enable simultaneous measurement of liver and tumour flux. A narrow metal tube of a diameter that would allow the passage of the probe through was cut to length to allow the probe to protrude slightly from the end. This tube was then supported by three struts arising from a metal washer at their base. Lighter weight holders which were easier to place, made from rigid polythene tubing were also used.
3.2.4. **Probe Placement**

Interstitial placement of the probe within the substance of the liver or tumour led to an increased respiratory artefact. In addition the slow leakage of blood amounted to a significant proportion of the rats circulating volume over the period for which the experiment ran.

Ultrasound coupling gel was used in an attempt to maintain a better signal from the probe tip which was maintained just above the surface of the liver. However once again there was a significant respiratory artefact and the gel did not appear to confer any advantage.

The aim of the use of the probe holder was to allow both probe and holder to move with the excursions of the liver without any relative movement between probe tip and liver surface. In order to do this it was sometimes necessary to place a retractor on the costal margin to prevent the edge of the costal margin moving the probe when the rat inspired. This was only necessary if the only available tumour was high on the surface of the liver lobe. Measurements from tumours in the left or middle lobe were more satisfactory as the probe was easier to place with a minimum of artefactual movement. In order to allow
the probe to move freely, the fibres were suspended above the probe in a clamp so that the fibres did not restrict probe movement nor lie heavily upon it. With careful probe placement the respiratory artefact could be limited to 5 - 15% of the flux readings.

A further artefact was discovered from the fibreoptic light source used with the dissecting microscope to insert the gastroduodenal cannula. While the incident light had little effect on readings, the fan within the light box caused an otherwise imperceptible vibration in the table which led to a considerable artificial elevation of the flux readings. Therefore before any readings were made the light source was switched off.

![Image of two laser Doppler probes on liver surface](image)

**Figure 12** The positioning of two laser Doppler probes sitting on the liver surface. A probe holder is used to hold the probe lightly in contact with the liver surface and to decrease movement artefact

**3.2.5. Reproducibility**

The most accurate and reliable laser Doppler method is to take readings continuously from the same place on the liver surface without moving or interfering with the probe between readings. If the probe is removed and replaced into approximately the same area
then the readings will vary according to the spatial variation of that tissue (i.e. the variation in readings as one moves across a non uniformly perfused tissue) and whether the probe is placed in an identical manner to the previous measurement. Spatial variation was seen within the liver but to a greater extent within tumour. Measurement of central tumour areas had lower flow than more peripheral areas although the very peripheral readings may have been influenced by the surrounding liver parenchyma.

3.2.6. Recording Rate and Time Constant

The MBF3D takes data readings at 40Hz but has the capacity to convert this to display at 0.25 Hz - 40 Hz with a time constant of 0.1 - 3.0 seconds. Use of a high rate of recording with a low time constant produces the most sensitive readings. This allows any slight variation in flow to be detected, including; pulsatile flow, respiratory movement artefact and any other movement artefact. Such high recording rates are not practical over longer recording periods as the large amount of data generated (144000 flux readings per channel per hour) is not easily stored or processed. However these settings are of use at the start of the experiment to ensure that the respiratory artefact is at a minimum and this was rechecked at the end. Readings throughout the experiment were performed at 0.25 Hz with a 3.0 second time constant which enabled a relatively smooth trace. To avoid the incorporation of extraneous movement artefact into the traces, once readings were commenced the experimental set-up was not disturbed.

3.2.7. Data Recording and Processing

Recordings were stored in the MBF3D and later transferred via the RS232 port to an Elonex 80286 personal computer using a programme GSOFT supplied with the MBF3D. Using this programme, data could be displayed graphically on the screen and averages could be calculated for selected areas. An additional ASCII file was also generated which was then stripped of unwanted characters and data and imported into a graphical package and spreadsheet; Fig P (Biosoft Software Corporation, USA).

3.2.8. Biological Zero

A biological zero was recorded from each probe at the end of the experiment. As flow could not easily be stopped with the animal alive without disturbing the probe placement, this was recorded after the animals were killed by an intravenous bolus of KCl with the probe position unchanged. In all cases this reading was then subtracted from all flux values.
blood pressure measurement

Figure 13 and 14 Experimental system used for laser Doppler experiments
3.2.9. Experimental Design

After the induction of anaesthesia, an initial laparotomy was carried out to assess tumour growth. The animal was only used if there was a tumour situated on the anterosuperior aspect of either the middle or left lobes of the liver and if a similarly situated tumour free area was also available. Tumours on the underside or lateral aspect of the liver were not suitable for adequate probe positioning and could therefore not be used. It is unlikely that this introduced a significant bias particularly as readings were made of the relative change from one area throughout the experiment.

Following cannulation of the carotid artery for measurement of blood pressure, the gastroduodenal artery was cannulated as described in section 3.1.5. The two laser Doppler probes were placed on the surface of the liver and tumour and their positions adjusted with reference to the laser Doppler monitor to place them in a way to allow free movement of the probe with the liver but no relative movement to occur between them.

An initial baseline period of 5-10 minutes of stable flux readings was allowed before the vasoactive infusion was commenced. Infusions into the gastroduodenal artery were administered at 50μl/min for 30 minutes using a Harvard infusion pump. Following the infusion a second period of 30 minutes was allowed to pass before the animal was killed with a bolus of KCl and 5 minutes allowed before a biological zero reading taken.
3.2.10. Data Analysis and Statistical Methods

Each experiment resulted in a continuous liver and tumour flux trace. The following parameters were derived from this trace:

- Average tumour and liver perfusion over the period before the vasoactive infusion was commenced (baseline).
- Average and maximal tumour and liver perfusion throughout the 30 minute infusion and at the end of the infusion period (endpoint).
- Average blood pressure during the preinfusion (baseline) and infusion periods.
- Average and maximal tumour and liver conductance\* before, during and at the end of the 30 minute infusion period.
- Average and maximal tumour to normal (TNR) perfusion ratios\† during the baseline and 30 minute infusion periods.

\* Vessel conductance was calculated by division of the flux reading by the blood pressure at each time point and provides a measure of vascular changes after allowing for changes of blood pressure (conductance = 1/resistance).

\† Tumour to normal ratios were calculated for each experiment by division of the tumour by liver flux at each of the corresponding time points.

Results are expressed as flux units and where percentage values are given these are the percentage change from the baseline period. The flux unit itself is an arbitrary unit related to the output of the laser Doppler probe in mili volts. It can not be directly translated into absolute flow or perfusion units.

Analysis of changes in one reading between different infusion periods was made using a paired T test. Comparisons between groups were made using an unpaired T test.
3.3. Radiotracer Measurement of Hepatic Artery Flow

3.3.1. Introduction

Measurement of tumour blood flow is more difficult than for normal tissue due to generally low blood flow as well as marked vascular heterogeneity. As a result of certain limitations in the interpretation of results from laser Doppler flowmetry (section 5.1.2) it was decided to use a second method to evaluate the hepatic arterial blood flow changes and the changes in the TNR. Several methods were comparatively evaluated in the experimental system before one was selected for use in the further studies. The initial studies in which each method was examined and the suitability for experimental measurement are included within this methods section.

3.3.2. Microspheres

The use of radioactive microspheres is a well established method in the measurement of blood flow (as described above (section 1.9.2)). Using multiple tracers it is possible to measure blood flow at two or more time points. Absolute blood flow measurements are possible using the reference organ technique (Bartrum et al. 1974) and portal and hepatic arterial flow can be estimated separately at a single time point (Groszmann et al. 1982). In view of this, the microsphere technique was selected as the method of choice and the initial pilot studies are described below.

3.3.2.1. Preparation of microspheres.

Two types of labelled polymeric spheres were used; tin-113 ($^{113}$Sn), and cobalt-57 ($^{57}$Co) (NEN Dupont) with a mean diameter of 15μm. The commercially supplied microspheres suspended in saline were mixed using a vortex mixer for 10 minutes. The total activity required was dispensed into a small test-tube. The microspheres were allowed to settle and the supernatent withdrawn and discarded. The microspheres were resuspended in 0.9% NaCl with 0.05% Tween 80 (Sigma UK), allowed to settle and the process repeated. Following the second wash, a sample of microspheres was taken for activity estimation. The microspheres were then resuspended in a warm 83.9% glucose solution with 0.05% Tween 80 and mixed vigorously. Even distribution of microspheres without clumping was checked visually under a dissecting microscope.

3.3.2.2. Microsphere dose

To accurately estimate flow, the aim was to deliver approximately $5 \times 10^5$ spheres to each animal. Above $1 \times 10^6$ the spheres may alter circulatory haemodynamics and introduce errors into the results. To obtain adequate precision, a minimum number of 300 microspheres per sample was required. The specific activity of the microspheres supplied by the manufacturers was checked to allow for decay, using a method previously described. A 10μl sample of dilute washed microspheres was pipetted onto a small square of graph paper. This was then counted in the scintillation counter and then the number of
microspheres visually counted using a microscope (x 20 magnification). The activity per microsphere was calculated and used to check to both microsphere dosage and the number of spheres per tissue sample.

3.3.2.3. Experimental sequence

The animal was anaesthetised as previously described. The carotid artery was cannulated with polythene tubing (i.d 0.40 od 0.81mm) (Portex) which was advanced a premeasured distance to lie within the left ventricle of the heart. This position was required to ensure adequate microsphere mixing in order to prevent streaming of flow. This position was checked visually at the end of the experiment when the animal had been killed. The results were only accepted if the tubing was correctly placed.

A second length of tubing (i.d. 0.28mm o.d. 0.61mm) was inserted into the femoral artery and secured in place with two 6/0 silk ligatures (Ethicon) This was used to draw off the reference organ sample and was attached to a syringe held in a withdrawal pump (Harvard). The gastroduodenal artery was then cannulated as previously described and the abdomen the closed with 4/0 chromic catgut sutures (Ethicon) with the end of the gastroduodenal catheter protruding from the abdomen and connected to an infusion pump for the administration of the vasoactive infusion.
Figure 15 and 16 Experimental system used in the double labelled microsphere experiments.
The pump withdrawing blood from the femoral artery at a fixed rate (0.5 ml/min or 8mm/minute using a 2.5ml syringe) was commenced 15 seconds before the microsphere injection and continued for 120 seconds. The microspheres were injected through the tubing into the left ventricle at a steady rate over 30 seconds. A vasoactive infusion (or control saline) was commenced into the gastroduodenal artery and allowed to run for 30 minutes before a second injection of microspheres was given and a second reference sample taken. A further 5 minutes were allowed to ensure the complete removal of microspheres from the circulation before the animal was killed with a bolus of KCl. The exact injected microsphere dose was calculated by weighing the injection syringe before and after the microsphere injection.

3.3.2.4. Sample preparation and counting

The magnitude of free label (i.e. unbound radioisotope) was assessed by counting a venous blood sample, obtained post-mortem. The uniformity of microsphere mixing was assessed by counting the two kidneys separately and discounting animals in which there was a difference of greater than 10% from the mean (approximately 1 in 10 animals need to be discounted for this reason). The liver was removed, divided into lobes and the tumours carefully dissected out ensuring no liver remained on their surface. Samples were weighed and placed in counting vials with up to 800mg in each vial. Samples from most other organs were also weighed and placed in counting vials although these results are not directly relevant to these studies and are not reported here.

Samples were γ counted in a 1272 Clinigamma auto-gamma counter (LKB Wallace). This machine incorporates two 2 inch sodium iodide crystals. Specific standardisation programmes were written for simultaneous measurement of Co-57 and Sn-113 activity using a dual energy method. Full automatic correction was made for background activity and cross talk between the two detector crystals. Sample tubes were placed in racks of 10 and placed on a conveyor. Samples with expected high counts (e.g. activity estimates or kidneys), or expected low counts (e.g. tumours or blood samples) were counted separately to minimise influence of or by a neighbouring sample.
Figure 17  1272 Clinigamma auto-gamma counter
3.3.2.5. Results of microsphere study

Ten animals were studied during which time, significant methodological problems became apparent which led to the abandonment of this method.

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<th>tot tum cts/min</th>
<th>ref sample cts/min</th>
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<th>flow tumour ml/min/g</th>
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Table 2 The results obtained during the dual label microsphere pilot study
The main problem which arose occurred due to the hypovascularity of the tumour and the difficulty encountered in achieving the required number of microspheres in each tumour specimen to ensure adequate precision. As a result, tumour counts had to be summed to provide an overall tumour to liver ratio for the animal rather than for each tumour or lobe. Following administration of endothelin I blood supply to the liver was markedly reduced at 30 minutes but even by summing the tumour counts, too few microspheres reached the tumour. Experiments were carried out in which the number of microspheres was increased to $1 \times 10^6$. Although this did increase the microspheres within the tumours, the number was still well below the 300 spheres per sample required. Furthermore, central cardiovascular effects became apparent with some animals experiencing temporary respiratory arrest. In addition higher microsphere concentrations increased the likelihood of microsphere clumping which led to uneven microsphere distribution and introduced a further error.

In animals receiving saline infusion the liver blood flow ranged from 0.041 to 0.539 ml/min/g. Tumour blood flow could only be calculated by summation of all tumours for reasons described above. Tumour blood flow varied between 0.003 to 0.073 ml/min/g. Following endothelin I infusion liver and tumour blood flow were both reduced by between 20 to 40 fold. However in no animal was there adequate numbers of microspheres, even after summation of the tumours. The percentage uptake of microspheres into the liver (including tumours) was 3.37% (s.d. 0.71%).

In previous studies microspheres have been successfully used in the measurement of tumour blood flow (Burton et al. 1985, Hafstrom et al. 1980, Hemingway et al. 1991b, Young et al. 1979). In some cases this was due to the use of a more vascular tumour model (sometimes more vascular than the host tissue) than the one used in these experiments. A second reason for the poor circulation of microspheres was the marked degree of vasoconstriction caused by endothelin infusion. An alternative strategy would have been to change the tumour model to a more vascular one such as the Walker 256 carcinosarcoma although it was felt more appropriate to use a hypovascular tumour as a model of human liver metastases and to maintain the same model throughout the studies.
As a result of the above experience with microspheres it was decided to consider a non particulate radiolabelled tracer molecule which could be delivered in adequate amounts to tumour tissue due to its solubility and high specific activity.

Three radioactive tracer molecules were considered before one was selected for use.

3.3.3. Iodoantipyrene

Iodoantipyrene (IAP) is a small (m.w. 310) highly diffusible compound, which when labelled with iodine-125 ($^{125}$I) or carbon-14 ($^{14}$C) may be used for the measurement of visceral blood flow. The advantage of using $^{125}$I-IAP is the relative ease with which autoradiographs can be prepared to study intra-tumoural blood flow distribution, in addition to counting the overall tumour activity in a gamma counter. A difficulty with regional IAP infusion into the liver is that it diffuses easily. Consequently, it is necessary to stop the circulation within a very short period of time after IAP injection in order to avoid diffusion equilibrating levels between tumour and liver. Prolonged infusion to allow a steady state to be reached would have given a falsely low tumour to normal ratio as the mesenteric organs would extract the IAP resulting in a high portal vein concentration and consequent liver uptake via the portal route. As the portal blood flow does not distribute equally between tumour and liver parenchyma, this would lead to an underestimation of tumour blood flow.

\[ \text{Figure 18 Iodoantipyrene molecule} \]

In a pilot study, $^{125}$I-IAP was used to evaluate the tumour to normal ratio and to examine intra-tumoural blood flow distribution with and without the administration of endothelin. CBH/cbi rats were used with hepatic HSN tumours as previously described. Iodoantipyrene was labelled on site and the lipophilicity of the compound assessed using partition coefficient (log p) measurement through extraction with octanol.
The gastroduodenal artery was cannulated by the method previously described. An initial animal study in which a 20μl bolus of methylene blue was administered into the gastroduodenal artery, indicated retrograde flow down the common hepatic artery. In order to prevent retrograde flow of 125I-IAP, a silk ligature was used to sling the proximal common hepatic artery and to briefly occlude it at the moment of tracer injection. Had retrograde flow been allowed to occur, perfusion of the mesenteric arteries may have occurred resulting in some portal presence of the radiotracer. In addition the dose administered into the hepatic arterial system would be unknown and dose calculation and autoradiograph exposure times would have been unpredictable.

Two groups of rats (n=4) were studied either with a 30 minute endothelin I infusion 25ng/minute at 25μl/min, or a control saline infusion prior to radiotracer injection. The 125I-IAP was delivered as a rapid 20μl bolus and the animal was killed after 5 seconds by rapid iv injection of KCl into the right ventricle via a catheter in the jugular vein.

Liver and tumour were then rapidly removed and the tumours excised from the liver. Some of the tumours were bisected and one half placed in a freezing bath, of isopentane precooled to -80°C by placing within a larger beaker of ethanol and dry card ice (CO2), to rapidly freeze the tissue before the iodoantipyrine had diffused. The remaining samples including the remaining tumour halves were then weighed and placed in counting vials and activity counted in the gamma counter. The counts were then decay corrected.

Autoradiographs were prepared from the frozen half of the tumours by cutting 20μm cryostat sections on a microtome (Bright Instruments) and placing the sections on autoradiography film (Amersham β-max). These were then placed in a metal light proof film cassette and exposed for 21 days and then developed and fixed (see fig 19 and 20).

**Results of iodoantipyrine study**

The findings of this experiment showed a mean TNR of 0.199, s.d. 0.130 in tumours from the animals receiving a saline infusion and 0.109, s.d. 0.107 in the group receiving endothelin (t test p<0.01). The results for the individual tumours are shown in table 3 below.

An example of a blood flow autoradiograph produced by this technique is shown in figure 19. It shows the hypervascular rim at the edge of the tumour. For comparison an autoradiograph showing the uptake of 3H-5FU is included (figure 20 ). This was prepared using a similar technique to that described although the autoradiograph exposure time was increased to allow for the lower energy β emissions.
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<th>rat No.</th>
<th>tum. wt.</th>
<th>tum. cpg</th>
<th>liver cpg /lobe</th>
<th>TNR</th>
<th>rat No.</th>
<th>tum. wt.</th>
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Table 3  Tumour to normal ratios (TNR) for each individual tumour
Figure 19 a and b  Autoradiograph of blood flow distribution (above) in an HSN tumour and rat liver parenchyma, from the uptake of $^{14}$C-IAP, shown with the same histological section stained with H and E below (x8).
Figure 20 a and b  Autoradiograph of 5FU uptake in an HSN tumour and rat liver parenchyma, from the uptake of \(^3\)H-5FU, shown with the same histological section stained with H and E below (x8).
These results show a significant fall in the tumour to normal blood flow ratio when endothelin is infused compared with saline. However, there are certain limitations to the validity of the technique:

Occlusion of the hepatic artery at the time of injection may influence flow and the distribution of blood flow. This may persist beyond the period of traction on the vessel if arterial spasm is induced.

Owing to the short period between radiotracer injection and the animal being killed, the $^{125}$I-IAP may not have had sufficient time to circulate throughout the tumour vasculature. As a result this would have underestimated tumour flow and would be expected to be more significant in animals receiving endothelin where the circulatory time through the whole liver would be expected to be slowed. This may partially explain the fall in tumour perfusion in animals receiving endothelin I infusion.

As a result of the uncertainties about radiotracer circulation through tumour areas and the necessity to occlude the hepatic artery at the time of the injection, it was felt that the results were difficult to interpret and therefore an alternative radiotracer was sought which had greater tissue retention and lower diffusibility within the tissue of interest.

3.3.4. $^{99m}$Tc-hexamethyl propyleneamineoxime ($^{99m}$Tc-HMPAO)

$^{99m}$Tc-HMPAO was developed as a cerebral blood flow agent for single photon imaging (Nowotnik et al. 1985) and has also been used to demonstrate blood flow in human soft tissue sarcomas (Sinnett et al. 1990). It has a reported extraction efficiency of approximately 80% and has been used for the measurement of tumour blood flow, although, due to high hepatobiliary clearance, showed a higher liver uptake than rubidium-81 chloride ($^{81}$RbCl) (Hammersley et al. 1987).

Eight HSN hepatic tumour bearing CBH/cbi rats were studied. The uptake of $^{99m}$Tc-HMPAO was measured in both tumour and liver after regional administration. From these measurements tumour to normal ratios were determined and compared to values obtained from systemic microsphere injection carried out in the same animal.

Animals bearing HSN hepatic tumours were anaesthetised as previously described. A laparotomy was performed and the gastroduodenal artery was cannulated. The right carotid artery was also cannulated and the cannula advanced to sit in the left ventricle of the heart. Femoral artery cannulation was not required as only relative values for tumour blood flow were sought and no reference sample was needed.

$^{99m}$Tc-HMPAO (Amersham Int. PLC) was prepared according to manufacturers instruction. 113-Tin microspheres were prepared and used as previously described.

$^{99m}$Tc-HMPAO was infused at a rate of 50μl/min into the gastroduodenal artery over a 2 minute period (2μCi/animal). The microspheres were injected over 30 seconds half way
through this infusion. Animals were killed 4 minutes after the start of the 99mTc-HMPAO infusion. Tumour and liver samples were carefully dissected out and weighed. Dual channel counting was used to count 99mTc activity in the same day. Radioactivity due to 113Sn was counted 4 days later after complete decay of 99mTc. The kidneys were counted to ensure adequate microsphere mixing.

**Results of 99mTc-HMPAO / microsphere study**

The TNRs for individual tumours are shown in the table. As with the previous microsphere experiment most tumours had less than the required number of microspheres and summation of different tumours was necessary. Tumour to liver ratios were calculated for individual tumours by dividing the counts per gram of each individual tumour by the counts per gram of the liver lobe in which the tumour lay.

![Graph showing the correlation between the tumour to normal ratios (TNR) obtained by the two techniques of regional 99Tc-HMPAO and systemic microsphere injection.](image)

**Figure 21** Graph showing the correlation between the tumour to normal ratios (TNR) obtained by the two techniques of regional 99Tc-HMPAO and systemic microsphere injection.
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<th>TNR - microspheres</th>
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Table 4 The tumour to normal ratios, as measured by regionally infused $^{99m}$Tc-HMPAO or by systemically administered tin microspheres (7 rats studied).
Table 5  Counts administered regionally and the counts detected in the liver, from which the extraction fraction is calculated.

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The mean percentage uptake of $^{99m}$Tc-HMPAO into the liver (including tumour) was 44.1% (s.d. 9.6%)

The comparison of TNRs between the microspheres and $^{99m}$Tc-HMPAO show some correlation. However the microsphere readings cannot be considered satisfactory due to the low numbers of spheres within them. The low uptake of $^{99m}$Tc-HMPAO into the liver was disappointing and indicated that the first pass uptake was not high and the tracer was recirculating. In an organ with only one blood inflow this would not matter. However because of the dual portal / hepatic arterial inflow to the liver and the differences in blood supply of the tumours, such a low uptake might lead to significant levels of tracer in the portal blood leading to an overestimation of liver flow as measured by radiotracer uptake. The net effect would be a reduction in the TNR.

Compared to $^{125I}$-iodoantipyrine, and microspheres, $^{99m}$Tc-HMPAO is a non particulate chemical trapping tracer which is commercially available at low cost. However, our results have shown that the uptake in the tumour and liver was only 44% which is inadequate to prevent significant portal recirculation.
3.3.5. Copper (II)-pyruvaldehyde bis(N-4 methyl thiosemicarbazone) (Cu-PTSM)

3.3.5.1. Introduction

Copper (II)-pyruvaldehyde bis(N-4 methyl thiosemicarbazone) (Cu-PTSM) was originally investigated for its anti-tumour activity over 30 years ago (Petering et al. 1964) and more recently has been suggested as a tracer for tissue blood flow estimation (Green et al. 1988). It can be labelled with a number of copper isotopes including $^{62}$Cu ($t_{1/2} = 9.7$ minutes), $^{64}$Cu ($t_{1/2} = 12.7$ hours) and $^{67}$Cu ($t_{1/2} = 62$ hours). $^{64}$Cu has been used throughout these experiments. The short half-life of $^{62}$Cu allows for repeated measurement of tissue perfusion using positron emission tomography (PET). Cu-PTSM (m.w. 308 Da) is a highly lipophilic molecule (log p 1.5-2.0) with a high extraction fraction and prolonged tissue retention. Tissue concentration of Cu-PTSM is determined by the relative proportion of cardiac output delivered to the tissues and may be used as a measure of the relative blood flow between different tissues (Young et al. 1994), (Green et al. 1988) including tumours (Mathias et al. 1991). Tissue extraction and retention is thought to occur as a result of the reductive decomposition of the copper (II) complex by intracellular sulphhydryl groups with release of copper (I) and entrapment by binding to intracellular macromolecules.

![Molecular structure of Copper (II)-pyruvaldehyde bis(n-4 methyl thiosemicarbazone) (Cu-PTSM)](image)

Figure 22 Molecular structure of Copper (II)-pyruvaldehyde bis(n-4 methyl thiosemicarbazone) (Cu-PTSM)
3.3.5.2 Radiotracer preparation

'No carrier' added $^{64}$Cu was produced by the $^{64}$Ni(d,2n)$^{64}$Cu reaction (Zweit et al. 1991) using the Nuffield cyclotron at the University of Birmingham. Following irradiation, the nickel-64 target was transported to the laboratory and the $^{64}$Cu extracted using ion exchange chromatography. Radionuclide purity was assessed using $\gamma$-ray spectroscopy. The radiolabelling was carried out based on a previously described method (Zweit et al. 1992). In brief, $^{64}$Cu-PTSM was prepared by buffering an aqueous solution of $^{64}$CuCl$_2$ in 0.3M HCl with two equivalents of 3M sodium acetate (pH 4.6). To this was added an ethanolic solution of $H_2$-PTSM ligand (0.1µg/µl). The radiochemical yield averaged 94% and the octanol water partition coefficient (log p) of this labelled product was on average 1.6. The final $^{64}$Cu-PTSM solution was diluted with 0.9% NaCl to a radioactive concentration of 200-400 µCi/ml (7.4-14.8MBq/ml) containing 5% ethanol to increase solubility.

3.3.5.3 Radiotracer hepatic retention

In order to establish that the $^{64}$Cu-PTSM taken up into the liver was not redistributed during the time course of the study, an experiment was designed to establish

- the regional uptake of $^{64}$Cu-PTSM after hepatic arterial delivery
- the retention of the radiotracer at various time points compatible with the duration of the study and a potential clinical study using PET imaging.

Sixteen hepatic tumour bearing rats were studied and were divided into 4 groups. The rats were anaesthetised and the gastroduodenal artery cannulated. $^{64}$Cu-PTSM was prepared as described above and 10µCi in 100µl was infused into the gastroduodenal artery over a 2 minute period. Animals were then killed at either 2, 5, 10 or 20 minutes post infusion. Liver and tumour were then removed, weighed and placed in vials for counting.

The counts were decay corrected and the uptake and retention of the radiotracer within the liver calculated.
This study illustrates that like microspheres the radiotracer is retained within the liver and not washed out as would be the case with a diffusible radiotracer such as iodoantipyrine. Consequently, the radiotracer can be infused slowly without need to rapidly stop the circulation.

The % uptake of $^{64}\text{Cu}$-PTSM into both the liver and tumour (combined) is shown for each of the 4 animals studied at each time point. The mean and standard deviations are also shown.

The results of this experiment showed a $^{64}\text{Cu}$-PTSM retention within the liver and tumour of 69.1% (s.d. 7.3%).

Retention within the liver and tumour was high at all time points although there was some decrease in the retention over the 20 minute period (ANOVA $p=0.04$ see figure 23).
3.3.6. Choice of Radiotracer Method for Measuring Flow

Based on the experience gained from these pilot studies, $^{64}$Cu-PTSM was selected for use as a blood flow tracer in subsequent experiments. The main advantages were:

- High specific activity (unlike microspheres the specific activity could be increased to ensure adequate counting statistics, even in tumours).
- Prolonged tissue retention.
- High first pass extraction, for a non particulate tracer and therefore negligible effects of recirculation.
- The ability to carry forward encouraging preclinical results into a clinical PET study to accurately quantify tissue blood flow.

Its main disadvantages when compared with the microspheres were:

- The inability to obtain absolute flow values.

This limitation would be present with any regionally administered tracer. However in this study it is the tumour flow relative to the liver (i.e. the TNR) which is the critical value and this is acceptable as a ratio.

- Only one measurement could be made per animal studied.

In theory this could have been circumvented by the dual labelling of the compound by a second copper isotope such as $^{67}$Cu. However practical considerations prevented this as the production of $^{67}$Cu requires the use of a high energy cyclotron which is not available in the U.K. Therefore a single reading was made after vasoactive infusion and this was compared to the value obtained in a different group of animals receiving a control saline infusion.

- Limited isotope availability

$^{64}$Cu is not commercially available. The lack of an on site cyclotron at Sutton necessitates using the machine at the University of Birmingham. It was then transported by special licensed courier to the laboratory in Surrey. This had to be co-ordinated with the availability of tumour bearing animals. In order to obtain more isotope, the target had to be cooled and reprocessed and sent back to the cyclotron centre. These practical problems limited the availability of the isotope.
3.4. Measurement of Tritiated 5-Fluorouracil Uptake

Initial assays of 5FU in plasma were bioassays (Clarkson et al. 1964). Later direct measurements of drug and metabolite levels were performed using gas chromatographic-mass spectrometry. Now most analysis of plasma levels is by high performance liquid chromatography (Stein et al. 1990). These measurements can be performed on tissue samples as well as plasma but require a complex procedure of tissue hydrolysis.

Both $^3$H and $^{14}$C labelled 5FU can be purchased commercially with labelling at the 2 or 6 position on the pyrimidine ring. For this work, 5-[$^6$H]-fluorouracil ([6-$^3$H]-5FU) was purchased (NEN Dupont) which has a high radiochemical purity (99% with a decomposition rate of 1% over 6 months), and long shelf life.

Preparation of the [6-$^3$H]-5FU for infusion into the animals was performed by pipetting out the required volume of stock solution into a small test tube. As the 5FU was supplied in ethanol, this was blown off and the radiotracer resuspended in 0.9% saline. [6-$^3$H]-5FU was used at a concentration of 1 mCi/ml (8.7 μg/ml). Forty μCi was administered to each animal over a 5 minute period representing a total dose of 348 ng of [6-$^3$H]-5FU at an estimated circulating concentration of 70 ng/ml within the tumour bearing liver. This 5FU concentration is of the same order as is obtained clinically with systemic 5FU infusion.

3.4.1. Tissue Sample Preparation and Liquid Scintillation Counting of ([6-$^3$H]-5FU)

[6-$^3$H]-5FU uptake was determined by liquid scintillation counting in which the scintillant is in direct contact with the radiotracer. Measurement of coloured tissue samples such as liver and tumour require careful sample preparation to avoid colour quenching artefacts. Tissue samples were weighed and placed in polypropylene liquid scintillation vials with between 150-200mg per counting vial. The samples were then each individually homogenised manually using a modified aluminium spatula which had been filed and sharpened to give a sharp flat blade at one end. The tissues were homogenised to the consistency of a thick paste and the samples then solubilized in a 50:50 mixture of soluene 350 (Packard) and isopropyl alcohol by leaving them at 35°C for 12 hours. Samples were then cooled in a refrigerator before being bleached by the addition of 0.5ml of 30% v.v. hydrogen peroxide (Sigma). Samples were left for a further hour and then 10ml of the liquid scintillant, Hi-Ionic Fluor (Packard) added to each vial which was then capped and mixed.
Figure 24  Sample preparation and counting; firstly in a gamma counter to count $^{64}$Cu and later in a liquid scintillation counter to count $^3$H.

Pilot studies adding hydrogen peroxide to scintillation vials shortly before counting showed that several thousand counts could be generated without any radioactivity being present. 24 hours was therefore allowed to elapse to allow chemiluminescence from the addition of the peroxide and photoluminescence from the addition of the liquid scintillant fluid to subside before counting in the liquid scintillation counter.

The liquid scintillation counter used was a Tricarb 2000 CA (Canberra Packard). The counter was set with a 1 min sample counting programme for $^3$H. An external standard technique was used for the determination of efficiency of each sample counted. With the information about counting efficiency, results were expressed as disintegrations per minute (dpm) based on the calculation:

\[
\text{disintegrations per minute} = \frac{\text{counts per minute}}{\text{efficiency}}
\]
Figure 25  Tricarb liquid scintillation counter used to measure the [6-\textsuperscript{3}H]-5FU

The linearity of response of the liquid scintillation counter was tested over several orders of magnitude by making dilutions of the stock solution of [\textsuperscript{3}H]-5FU to which were added solublised liver extract with or without peroxide. This showed a linear response over a wide range of activity for the counter. It also showed that the internal calculation to convert counts per minute (cpm) into disintegrations per minute (dpm) helped to minimise the luminescence errors associated with the presence of the liver tissue or the peroxide. Therefore the dpm readings were used for all subsequent experiments.
Figure 26  This study shows the linearity of response of the counter with some standard samples of the $[3^\text{H}]-5\text{FU}$. Three standard samples were taken from the stock solution of $[6-^3\text{H}]-5\text{FU}$. Liver homogenate was added to two samples and peroxide used to bleach the colour out of one of these. A series of dilutions were made of each solution and these were counted on the Packard liquid scintillation counter. These are shown in the figure as counts per minute (cpm) and disintegrations per minute (dpm). The calculation of disintegrations per minute is made within the counter using a series of external standards. The values for the theoretical dpm are calculated from the data sheet for the amount of $[3^\text{H}]-5\text{FU}$ added.
Figure 27  The biodistribution of [3H]-5FU after regional administration in a single animal. This study illustrates the high first pass uptake of regionally administered 5FU into the liver. The wide variation between different lobes (lt - left, rt - right, caud - caudate, med - median) reflects not only differing flow but also a degree of streaming of the radiotracer between lobes. The error bars show the standard deviation for each tissue.
Figure 28  This graph shows the results of a validation study in which blood levels of $[^3\text{H}]$-5FU before and after a 5 minute regional infusion of $[^3\text{H}]$-5FU were measured. Blood samples were taken through a catheter into the jugular vein and allowed to drop into a series of sample tubes held in a rotating drop counter.

The results show an increase in the blood 5FU level over the period of the infusion but that this level is maintained at a steady state for several minutes after the infusion ends.
3.5. Experimental Sequence for Dual Tracer Experiments (\(^{64}\)Cu-PTSM and \(^{3}\)H-5FU)

As it was not possible to measure blood flow ratios at more than one time point in each animal, it was decided to measure both blood flow ratios and 5FU uptake ratios in the same animals. In order to minimise errors which may have occurred due to variation in flow over time, both isotopes were administered simultaneously. Thus, the blood flow conditions would be identical for both radiotracers.

A 30 minute infusion of either normal saline (25µl/minute, \(n=20\) animals) or vasoactive agent at 25µl/minute, \(n=10\) animals/agent) was commenced into the hepatic arterial circulation via the gastroduodenal artery. A longer period of infusion would have been desirable in order to match experimental conditions more closely with clinical hepatic artery infusion. However, this infusion period was chosen to allow a period of stabilisation following the initial vasoactive changes while keeping the cardiovascular changes of prolonged anaesthesia and laparotomy to a minimum. The laser Doppler studies had indicated that by 30 minutes the rapid changes in flow had settled and more stable flow conditions were present. This was felt to provide a model closer to that of infusional therapy than bolus administration, within the practical constraints of the animal model used. Longer infusion periods might have been possible using an implantable pump system and allowing the animal to recover consciousness although this was not undertaken within this study.

The blood flow tracer \(^{64}\)Cu-PTSM and the \(^{3}\)H-5FU were administered simultaneously using an infusion pump (25µl/min) via the side arm of a T-piece over 5 minutes starting 25 minutes after onset of the vasoactive / saline infusion.

![Diagram showing the technique of infusing the two radiotracer molecules and the vasoactive agent through a T piece into the gastroduodenal artery.](image)

Figure 29 showing the technique of infusing the two radiotracer molecules and the vasoactive agent through a T piece into the gastroduodenal artery.
Higher infusion rates were found to increase the likelihood of retrograde flow in the hepatic artery and unwanted spillover into the systemic circulation. Five minutes was allowed to elapse after the end of the tracer infusions to ensure maximal uptake from the circulation before the animals were killed by rapid injection of potassium chloride. The tumour and liver were then separated for weighing and counting.

For all experiments the entire liver and each individual tumour were counted. Tissue samples were initially prepared for gamma counting as described in section 3.3.2. After all samples were counted the $^{64}$Cu activity was allowed to decay for a period of a week before sample preparation for liquid scintillation counting was commenced.

As both $^{64}$Cu-PTSM and [${}^3$H]-5FU were administered together, the octanol partition coefficient of the radiotracers were checked to ensure that the stability of 5-FU did not affect the $^{64}$Cu-PTSM stability.

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**Figure 30** Diagramatic representation of the sequence of events in the dual isotope uptake experiments of blood flow and 5FU uptake
4. Results

4.1. Results of Laser Doppler Studies

4.1.1. Introduction

This section addressed the following questions:

- How does liver and tumour perfusion change with the infusion of different vasoactive agents over a 30 minute infusion period?
- What is the effect of these vasoactive agents on the tumour to normal ratios?
- How do the resistance changes in the vessels vary in response to vasoactive agents?
- How does the effect vary when an agent such as endothelin I is administered systemically?
- Can nitric oxide inhibition prolong the duration of effect of a vasoconstrictor?

4.1.2. Doses and Agents tested

The vasoactive agents assessed were:

**Vasoconstrictors**

Vasopressin (0.5μg/minute for 30 minutes) \( n=9 \)

Angiotensin II (4μg/kg/minute for 30 minutes) \( n=8 \)

Endothelin I (50ng/min regionally infused for 30 minutes) \( n=8 \)

Endothelin I (50ng/min systemically infused for 30 minutes) \( n=7 \)

L-NAME (0.5mg/min for 30 minutes) \( n=6 \)

Vasopressin (0.5μg/min for 30 minutes) + L-NAME (0.5mg/min for 30 minutes or 0.7mg/min for 5 minutes at the onset of recovery from vasoconstriction) \( n=7 \)

**Vasodilators**

Calcitonin gene related peptide (CGRP) (0.5μg/minute for 30 minutes) \( n=7 \)

**Control infusion**

0.9% NaCl (50μl/min for 30 minutes) \( n=8 \)
Infusions were carried out at 50μl/min using an infusion pump (Harvard).

Doses were selected based on previous literature for each agent and a series of pilot studies in which the aim was to achieve a visible change in perfusion while keeping the average systemic blood pressure change to under 25% and avoiding other adverse systemic changes.

All agents were administered by hepatic artery infusion. In the case of endothelin a further group was studied in which it was administered systemically to establish whether a similar effect could be achieved.

The values for the laser Doppler readings are expressed as flux units. As previously described these are arbitrary units based on the strength of the electrical output of the photodetector cell of the laser Doppler and are calibrated against a reference standard sample (see section 3.2.1). Results are expressed either as flux units or as the percentage change from the baseline value.

Results for the average values for each group are shown in both graphical and tabular form along with a typical trace obtained with each agent. The graphs show the mean and standard deviations.

The variables derived in the results are described in the methods (section 3.2.10). The statistical analyses in this section (paired and unpaired T tests) are comparisons of the average and the maximal change throughout the infusion period with the average value obtained during the preinfusion baseline period. A further comparison is made between the values obtained during the last minute of the infusion (end value) with the baseline values. The maximal value is the greatest fall obtained in flux and conductance or the maximal rise in the TNR.
4.1.3. Vasopressin

Vasopressin produced a rapid and profound fall in hepatic perfusion. This response diminished by 15 minutes and returned to baseline levels (+/- 15%) in 4/7 (57%) cases with a mean perfusion fall of 20 flux units (8%) at the end of the infusion. There was no significant difference (p=0.081) between liver perfusion at the end of the infusion period (mean 211 flux units, s.d. 30) compared with the baseline period (mean 231 flux units, s.d. 26). There was a rapid and sustained rise in blood pressure throughout the infusion (mean rise 26 mmHg, s.d. 7). Despite the absence of a significant flux change at the end of the infusion, hepatic vessel conductance was significantly (p = 0.002) reduced at the end of the infusion (mean 2.308, s.d. 0.361) compared with the baseline period (mean 3.176, s.d. 0.493) indicating increased vessel resistance despite a return to baseline flux levels.

There was a significant fall in maximal (p= <0.002) (mean 19 flux units, s.d. 15) and average (p=0.008) (mean 31 flux units, s.d. 20) tumour perfusion values compared with baseline (mean 47 flux units, s.d. 28). The tachyphylaxis effect was not as marked within the tumour as in liver. Unlike liver, tumour perfusion at the end of the infusion was still significantly (p=0.03) reduced (mean 35 flux units, s.d. 20) compared with baseline values with only 2/7 (29%) returning to baseline with a mean end perfusion fall of 20%.

The tumour to normal ratio was significantly elevated during the infusion (p=0.013, mean 0.445 (115%), s.d. 0.310) although not so when comparing the average (p=0.286, mean 0.210, s.d. 0.118) or end values (p=0.286, mean 0.167, s.d. 0.087) with baseline (mean 0.200, s.d. 0.111).

Regionally infused vasopressin appeared to be a potent hepatic arterial pressor agent but this effect was of a limited duration. Despite normal flux values, the conductance change at the end of the infusion indicates that vessel resistance is increased throughout. Similarly there was a significant rise in the TNR although also of short duration.
Effect of 30 min vasopressin infusion into GDA

Figure 31 The effects of a 30 minute vasopressin infusion into the gastroduodenal artery, on liver and tumour flux and conductance and blood pressure and TNR
Summary graph (figure 32) and table (6)* of changes in perfusion, blood pressure and conductance following a 30 minute vasopressin infusion (n=9). Mean and standard deviation shown.

Liver and tumour flux are expressed as flux units and blood pressure (BP) in mmHg. Conductance (cond) is derived by division of the individual flux values by the blood pressure and the tumour to normal ratio (TNR) by division of the tumour by the liver flux values.
4.1.4. Angiotensin II

Hepatic perfusion fell significantly for the average (p=0.016) (mean 154 flux units, s.d. 32) and maximal fall (p=0.007) (mean 135 flux units, s.d. 40) during the infusion period compared with baseline (mean 167 flux units, s.d. 38). Tachyphylaxis developed to the vasoconstrictor effect and again there was no significant (p=0.128) fall in hepatic perfusion at the end of the infusion (mean 156 flux units, s.d. 31). Perfusion returned to baseline (+/-15%) in 6/9 (67%) cases with a mean perfusion fall at the end of the infusion of 10%. Angiotensin also produced a rapid and sustained rise in blood pressure (mean increase 18 mmHg, s.d. 11). Hepatic conductance was significantly (p=0.003) decreased at the end of the infusion (mean 1.787, s.d. 0.569) compared with baseline (mean 2.170, s.d. 0.546).

Tumour perfusion followed a similar pattern to hepatic perfusion with significantly reduced flow for the maximal (p=0.032 mean 38 flux units, s.d. 23) and average change (p<0.018) (mean 40 flux units, s.d. 27) but not for the end change (p=0.49 mean 40 flux units, s.d. 28) when compared with baseline (mean 44 flux units, s.d. 27).

Tumour conductance showed a significant (p=0.01) decrease throughout the infusion (mean 0.466, s.d.0.366 ) but unlike normal liver was not significantly reduced at the end of the infusion (mean 0.540, s.d. 0.452) compared with baseline (mean 0.635, s.d. 0.357). Thus the tachyphylaxis effect was also apparent within tumour.

As with vasopressin, there was a significant although smaller rise in the TNR for the maximal value (p=0.004 mean 0.329 (32%), s.d. 0.175) compared with baseline (mean 0.252, s.d. 0.134) although no significant difference between the baseline and average (p=0.249) (mean 0.245, s.d. 0.141) or end values (p=0.429) (mean 0.247, s.d. 0.152).
Figure 33  The effects of a 30 minute angiotensin II infusion into the gastroduodenal artery, on liver and tumour flux and conductance and blood pressure and TNR (the troughs seen at the start and end of the infusion period on the tumour traces are event markers i.e. arterfactual)
Summary graph (figure 34) and table (7) of changes in perfusion, blood pressure, and conductance following a 30 minute angiotensin II infusion (n=9).

Mean and standard deviation shown.

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<th>sd</th>
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<td>14</td>
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Maximal fall was less than average fall due to a marked rise in tumour flux in one animal

Liver and tumour flux are expressed as flux units and blood pressure (BP) in mmHg. Conductance (cond) is derived by division of the individual flux values by the blood pressure and the tumour to normal ratio (TNR) by division of the tumour by the liver flux values.
4.1.5. Endothelin I

4.1.5.1. Regional

The pattern of response to endothelin was different to the previous two vasoconstrictors. Vasoconstriction occurred more gradually leading to a prolonged vasoconstriction with little evidence of tachyphylaxis. Hepatic perfusion was significantly reduced throughout (p=0.0001) (mean 150 flux units, s.d. 38) and at the end (p=0.0009) (mean 120 flux units, s.d. 48) of the infusion compared with baseline (mean 236 flux units, s.d. 42). Perfusion returned to baseline in only 1/8 (13%) cases with a mean perfusion reduction of 48% at the end of the infusion. Endothelin I caused a mild elevation of systemic blood pressure (mean change 10mmHg, s.d. 8mmHg). Liver conductance was also reduced throughout (p=0.0002) (mean 1.715, s.d. 0.353) and at the end (p=0.0003) (mean 1.184, s.d. 0.449) of the infusion compared with baseline (mean 2.932, s.d. 0.622).

Tumour perfusion was also significantly reduced throughout (p=0.008) (mean 42 flux units, s.d. 21) and at the end of the infusion (p=0.001) (mean 32.3 flux units, s.d. 20.4) compared with baseline (mean 56 s.d. 24). Tumour conductance was significantly less (p=0.001) at the end of the infusion (mean 0.461, s.d. 0.233) than for the average value throughout (mean 0.644, s.d. 0.267).

The TNR increased more gradually with endothelin although the increase was more prolonged. The maximal TNR (mean 0.473, s.d. 0.234) was significantly greater than baseline values (mean, 0.231, s.d. 0.096) causing a doubling of the TNR (105%). However the average (mean 0.305, s.d.0.162) and end (mean,0.299 s.d.0.240) values were not significantly increased. (p=0.051 and p=0.192 respectively) although there was a rise in both groups (34% and 31% respectively).
Figure 35  The effects of a 30 minute endothelin I infusion into the gastro-duodenal artery, on liver and tumour flux and conductance and blood pressure and TNR
Summary graph (figure 36 and table (8)) of changes in perfusion, blood pressure, and conductance following a 30 minute regional endothelin I infusion (n=9). Mean and standard deviation shown.

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</tr>
<tr>
<td>tumour flux</td>
<td>56</td>
<td>26</td>
<td>42</td>
<td>24%</td>
<td>↓</td>
<td>22</td>
<td>0.600</td>
<td>30</td>
<td>49%</td>
<td>21</td>
<td>0.001</td>
</tr>
<tr>
<td>BP</td>
<td>84</td>
<td>12</td>
<td>94</td>
<td>12%</td>
<td>↑</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>liver cond</td>
<td>2.932</td>
<td>0.665</td>
<td>1.715</td>
<td>40%</td>
<td>↑</td>
<td>0.377</td>
<td>0.002</td>
<td>0.922</td>
<td>68%</td>
<td>0.369</td>
<td>0.000</td>
</tr>
<tr>
<td>tumour cond</td>
<td>0.885</td>
<td>0.393</td>
<td>0.644</td>
<td>25%</td>
<td>↓</td>
<td>0.286</td>
<td>0.003</td>
<td>0.436</td>
<td>49%</td>
<td>0.213</td>
<td>0.002</td>
</tr>
<tr>
<td>TNR</td>
<td>0.231</td>
<td>0.096</td>
<td>0.305</td>
<td>34%</td>
<td>↑</td>
<td>0.162</td>
<td>0.051</td>
<td>0.473</td>
<td>105%</td>
<td>0.234</td>
<td>0.003</td>
</tr>
</tbody>
</table>

* Liver and tumour flux are expressed as flux units and blood pressure (BP) in mmHg. Conductance (cond) is derived by division of the individual flux values by the blood pressure and the tumour to normal ratio (TNR) by division of the tumour by the liver flux values.
4.1.5.2. Systemic

Systemically infused endothelin also caused a slower onset and more prolonged vasoconstriction than for vasopressin or angiotensin. Hepatic perfusion values fell significantly for average (p=0.0005, mean 172 flux units, s.d. 29), maximal (p=0.0002, mean 136 flux units, s.d. 32) and end values (p=0.0003, mean 140 flux units, s.d. 32) compared with baseline values (mean 226 flux units, s.d. 42). Perfusion returned to baseline in 2/8 (25%) cases with mean reduction in the flux value at the end of the infusion period of 37%.

Blood pressure again rose with endothelin with a mean increase of 7mmHg (s.d. 5mmHg). Average conductance values throughout the infusion were reduced for liver (p=0.0006, mean 1.889, s.d. 0.325) compared with the baseline (mean 2.585 s.d. 0.432) and for tumour (p=0.0005, mean 0.543, s.d. 0.166), compared with baseline (mean 0.644, s.d. 0.185).

The TNR rose significantly for the average value during the infusion (p=.041, mean 0.257, s.d. 0.090) and at the end (p=0.022, mean 0.281, s.d. 0.086) of the infusion compared with the baseline level (mean 0.228, s.d.0.087).
Figure 37 The effects of a 30 minute endothelin I infusion into a systemic vein, on liver and tumour flux and conductance and blood pressure and TNR
Summary graph (figure 38) and table (9) of changes in perfusion, blood pressure, and conductance following a 30 minute systemic endothelin I infusion (n=9). Mean and standard deviation shown.

<table>
<thead>
<tr>
<th></th>
<th>baseline</th>
<th>sd</th>
<th>ave</th>
<th>sd</th>
<th>p</th>
<th>max value</th>
<th>s.d</th>
<th>p</th>
<th>end value</th>
<th>s.d</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>liver flux</td>
<td>226</td>
<td>42</td>
<td>172</td>
<td>29</td>
<td>0.000</td>
<td>136</td>
<td>32</td>
<td>0.000</td>
<td>140</td>
<td>32</td>
<td>0.000</td>
</tr>
<tr>
<td>tumour flux</td>
<td>57</td>
<td>28</td>
<td>51</td>
<td>23</td>
<td>0.01</td>
<td>36</td>
<td>17</td>
<td>0.003</td>
<td>40</td>
<td>18</td>
<td>0.005</td>
</tr>
<tr>
<td>BP</td>
<td>88</td>
<td>5</td>
<td>94</td>
<td>94</td>
<td>8%↑</td>
<td>105</td>
<td>6</td>
<td>0.000</td>
<td>105</td>
<td>1</td>
<td>0.252</td>
</tr>
<tr>
<td>liver cond</td>
<td>2.585</td>
<td>0.466</td>
<td>1.889</td>
<td>0.325</td>
<td>0.000</td>
<td>1.295</td>
<td>0.246</td>
<td>0.000</td>
<td>1.313</td>
<td>0.252</td>
<td>0.000</td>
</tr>
<tr>
<td>tumour cond</td>
<td>0.644</td>
<td>0.185</td>
<td>0.543</td>
<td>0.166</td>
<td>0.000</td>
<td>0.420</td>
<td>0.124</td>
<td>0.000</td>
<td>0.430</td>
<td>0.127</td>
<td>0.000</td>
</tr>
<tr>
<td>TNR</td>
<td>0.228</td>
<td>0.087</td>
<td>0.257</td>
<td>0.090</td>
<td>0.041</td>
<td>0.295</td>
<td>0.092</td>
<td>0.01</td>
<td>0.281</td>
<td>0.092</td>
<td>0.022</td>
</tr>
</tbody>
</table>

* Liver and tumour flux are expressed as flux units and blood pressure (BP) in mmHg. Conductance (cond) is derived by division of the individual flux values by the blood pressure and the tumour to normal ratio (TNR) by division of the tumour by the liver flux values.
4.1.5.3. Comparison of systemic and regional endothelin

Systemically infused endothelin led to a similar pattern of response to that when regionally administered in that there was a gradual onset of vasoconstriction which was prolonged. However, the changes were more marked with the regional infusion group. The fall in hepatic perfusion was significantly greater (p<0.02) for the average fall in hepatic perfusion following regional administration (mean 36%, s.d. 12%) compared with systemic administration (mean fall 23%, s.d. 10%). Maximal change in liver flux was also greater for regional administration (see table 10). The increase in the average TNR value throughout the infusion was 34% (s.d.42%) for regional and 14% (21%) for systemic although the difference was not statistically significant (p=0.139).

<table>
<thead>
<tr>
<th></th>
<th>average % (s.d.)</th>
<th>maximum % (s.d.)</th>
<th>end value % (s.d.)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>liver flux</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>regional</td>
<td>36 ↓ (12)</td>
<td>65 ↓ (11)</td>
<td>48 ↓ (23)</td>
<td>0.15</td>
</tr>
<tr>
<td>systemic</td>
<td>23 ↓ (10)</td>
<td>39 ↓ (14)</td>
<td>37 ↓ (14)</td>
<td></td>
</tr>
<tr>
<td>tumour flux</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>regional</td>
<td>24 ↓ (17)</td>
<td>49 ↓ (19)</td>
<td>46 ↓ (20)</td>
<td>0.047</td>
</tr>
<tr>
<td>systemic</td>
<td>11 ↓ (13)</td>
<td>35 ↓ (15)</td>
<td>28 ↓ (17)</td>
<td></td>
</tr>
<tr>
<td>B.P.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>regional</td>
<td>12 ↑ (11)</td>
<td>105 ↑ (61)</td>
<td>31 ↑ (67)</td>
<td>0.44</td>
</tr>
<tr>
<td>systemic</td>
<td>8 ↑ (6)</td>
<td>33 ↑ (35)</td>
<td>-27 ↓ (33)</td>
<td></td>
</tr>
<tr>
<td>TNR (rise)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>regional</td>
<td>34 ↑ (42)</td>
<td>105 ↑ (61)</td>
<td>31 ↑ (67)</td>
<td>0.44</td>
</tr>
<tr>
<td>systemic</td>
<td>14 ↑ (21)</td>
<td>33 ↑ (35)</td>
<td>-27 ↓ (33)</td>
<td></td>
</tr>
</tbody>
</table>

Table 10  The comparison between regional and systemically infused endothelin. Flux values and blood pressure are shown as percentage fall from baseline, while the tumour to normal ratio (TNR) is shown as a percentage rise. Groups were compared using a paired t test.
4.1.6. CGRP

Despite its vasodilator properties, CGRP led to a significant (p=0.035) fall in hepatic perfusion over the infusion period (mean 179 flux units, s.d. 68) compared with baseline values (mean 219 flux units, s.d. 39). This appeared to be due to a systemic hypotensive effect with an average blood pressure fall over the infusion period of 24mmHg (s.d.5mmHg).

There was a significant (p=0.004) rise in the maximal liver conductance (mean 3.806, s.d. 1.250) compared with baseline (mean 2.603, s.d. 0.643) although the conductance rise between the baseline period and the infusion period (mean 3.076, s.d. 1.150) was not significant (p=0.079).

There was no significant (p=0.23) fall in tumour perfusion between the baseline period (mean 48 flux units, s.d. 22) and the infusion period (mean 44 flux units, s.d. 14) although there was a significant (p=0.034) conductance increase between baseline (mean 0.624, s.d. 0.250) and infusion periods (mean 0.831, s.d. 0.251).

The tumour to normal ratio did increase from the baseline value (mean 0.261, s.d. 0.168) although this was not significantly different to the maximal (p=0.127, mean 0.476, s.d.0.594), average (p=0.152, mean 0.400, s.d.0.471) or end values (p=0.151, mean 0.370, s.d.0.402).

CGRP thus did appear to decrease the vascular resistance in both tumour and to a lesser extent liver circulation although due to the systemic vasodilation this led to a fall in blood pressure and in hepatic perfusion. The TNR did rise although this did not reach statistical significance.
Effect of 30 min CGRP infusion into GDA

- B.P. (mmHg)
- flux (mV)
- conductance
- T:N ratio

Figure 39  The effects of a 30 minute CGRP infusion into the gastroduodenal artery, on liver and tumour flux and conductance and blood pressure and TNR.
Summary graph (figure 40) and table (11) of changes in perfusion, blood pressure, and conductance following a 30 minute regional CGRP infusion (n=9). Mean and standard deviation shown.

<table>
<thead>
<tr>
<th></th>
<th>baseline</th>
<th>sd</th>
<th>ave</th>
<th>sd</th>
<th>p</th>
<th>max value</th>
<th>s.d</th>
<th>p</th>
<th>end value</th>
<th>s.d</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>liver flux</td>
<td>219</td>
<td>43</td>
<td>179</td>
<td>20%</td>
<td>74</td>
<td>0.035</td>
<td>141</td>
<td>37%</td>
<td>82</td>
<td>0.008</td>
<td>186</td>
</tr>
<tr>
<td>tumour flux</td>
<td>48</td>
<td>24</td>
<td>44</td>
<td>3%</td>
<td>15</td>
<td>0.233</td>
<td>41</td>
<td>7%</td>
<td>19</td>
<td>0.448</td>
<td>46</td>
</tr>
<tr>
<td>BP</td>
<td>83</td>
<td>6</td>
<td>58</td>
<td>28%</td>
<td>3</td>
<td>51</td>
<td>3</td>
<td>38%</td>
<td>3</td>
<td>61</td>
<td>26%</td>
</tr>
<tr>
<td>liver cond</td>
<td>2.603</td>
<td>0.694</td>
<td>3.080</td>
<td>15%</td>
<td>1.25</td>
<td>0.08</td>
<td>3.806</td>
<td>43%</td>
<td>1.345</td>
<td>0.004</td>
<td>3.110</td>
</tr>
<tr>
<td>tumour cond</td>
<td>0.624</td>
<td>0.270</td>
<td>0.830</td>
<td>43%</td>
<td>0.271</td>
<td>0.034</td>
<td>1.053</td>
<td>77%</td>
<td>0.395</td>
<td>0.002</td>
<td>0.826</td>
</tr>
<tr>
<td>TNR</td>
<td>0.261</td>
<td>0.168</td>
<td>0.400</td>
<td>30%</td>
<td>0.471</td>
<td>0.152</td>
<td>0.476</td>
<td>52%</td>
<td>0.594</td>
<td>0.127</td>
<td>0.370</td>
</tr>
</tbody>
</table>

* Liver and tumour flux are expressed as flux units and blood pressure (BP) in mmHg. Conductance (cond) is derived by division of the individual flux values by the blood pressure and the tumour to normal ratio (TNR) by division of the tumour by the liver flux values.
4.1.7. L-NAME

L-NAME alone

By inhibiting the production of endogenous nitric oxide, L-NAME might be expected to have a vasoconstrictor effect on the liver and general circulation.

Liver perfusion was indeed significantly reduced (p=0.0003) throughout the infusion (mean 186 flux units, s.d. 45) compared with baseline (mean 230 flux units, s.d. 44) as well as at the end of the infusion period (p= 0.005) (mean 198 flux units, s.d. 41). Tumour perfusion was also significantly reduced (p=0.01) from the baseline (mean 30 flux units, s.d. 13) over the period of the infusion (mean 22 flux units, s.d. 14) but not at the end of the infusion period (p=0.349) (mean 28 flux units, s.d. 25), although tumour conductance was reduced at the end of the infusion period. Blood pressure was significantly (p=0.001) elevated throughout by an average of 28% from the baseline (mean 96 mm Hg, s.d. 6) over the infusion period (mean 102. s.d. 37 mm Hg)

The TNR was not significantly elevated from the baseline at any point during the infusion.
Figure 41 The effects of a 30 minute L-NAME infusion into the gastroduodenal artery, on liver and tumour flux and conductance and blood pressure and TNR
Summary graph (figure 42) and table (12) of changes in perfusion, blood pressure, and conductance following a 30 minute L-NAME infusion (n=9). Mean and standard deviation shown.

* Liver and tumour flux are expressed as flux units and blood pressure (BP) in mmHg. Conductance (cond) is derived by division of the individual flux values by the blood pressure and the tumour to normal ratio (TNR) by division of the tumour by the liver flux values.
4.1.8.  Saline

The overall effect of infusion of normal saline was very small. There was some fluctuation in resting levels of perfusion at all point of the infusion and this tended to reach significance when considering the maximal change in any one direction. There was however an overall significant increase (p=0.04) in liver flux from the baseline (mean 213 flux units, s.d. 35) over the infusion period (mean 223 flux units, s.d.34) in those seen when normal saline was infused. This may have been due to vasodilation as a result of a build up of halothane within the animal. Pilot studies also showed that the temperature of the infusion could induce some vasoactive changes in the liver although when infused slowly at room temperature, this effect did appear minimal. There was a maximal TNR increase of 11% from the baseline (0.227, s.d. 0.161) for the maximal change (mean 0.250, s.d. 0.181) although the average TNR was not significantly changed (p=0.28, mean 0.221, s.d.0.150).
Effect of 30 min saline infusion into GDA

B.P. (mmHg)

flux (mV)

conductance

T:N ratio

Figure 43  The effects of a 30 minute saline infusion into the gastroduodenal artery, on liver and tumour flux and conductance and blood pressure and TNR
Summary graph (figure 44) and table (13) of changes in perfusion, blood pressure, and conductance following a 30 minute saline infusion (n=9). Mean and standard deviation shown.

<table>
<thead>
<tr>
<th></th>
<th>baseline s.d</th>
<th>ave s.d</th>
<th>p</th>
<th>max value s.d</th>
<th>p</th>
<th>end value s.d</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>liver flux</td>
<td>213</td>
<td>35</td>
<td>223</td>
<td>5%</td>
<td>34</td>
<td>200</td>
<td>7%</td>
</tr>
<tr>
<td>tumour flux</td>
<td>46</td>
<td>29</td>
<td>46</td>
<td>1%</td>
<td>27</td>
<td>41</td>
<td>8%</td>
</tr>
<tr>
<td>BP</td>
<td>85</td>
<td>5</td>
<td>86</td>
<td>2%</td>
<td>3</td>
<td>90</td>
<td>6%</td>
</tr>
<tr>
<td>liver cond</td>
<td>2.494</td>
<td>0.385</td>
<td>2.587</td>
<td>4%</td>
<td>0.327</td>
<td>2.312</td>
<td>7%</td>
</tr>
<tr>
<td>tumour cond</td>
<td>0.665</td>
<td>0.362</td>
<td>0.667</td>
<td>0%</td>
<td>0.372</td>
<td>0.612</td>
<td>9%</td>
</tr>
<tr>
<td>TNR</td>
<td>0.227</td>
<td>0.161</td>
<td>0.221</td>
<td>1%</td>
<td>0.150</td>
<td>0.250</td>
<td>11%</td>
</tr>
</tbody>
</table>

* Liver and tumour flux are expressed as flux units and blood pressure (BP) in mmHg. Conductance (cond) is derived by division of the individual flux values by the blood pressure and the tumour to normal ratio (TNR) by division of the tumour by the liver flux values.
4.1.9. **L-NAME and Vasopresin**

When L-NAME was coadministered over or during the vasopressin infusion, the overall effect appeared to be to prolong the effects of the vasopressin. The fall in liver perfusion was significantly reduced from baseline (mean 205 flux units, s.d. 35) over (p<0.0002, mean 91 flux units, s.d. 57) and at the end of the infusion period (p<0.001, mean 105 flux units, s.d.73). Tumour perfusion was reduced from baseline (mean 38 flux units, s.d. 25) for the maximal fall (p<0.026) (mean 12 flux units, s.d. 4) but not for the average (p=0.068, mean 22 flux units, s.d. 8) or end values (p=0.123) (mean 23 flux units, s.d. 9). The blood pressure was elevated throughout and the liver and tumour conductance were also significantly reduced at all time points.

The tumour to normal ratio was significantly elevated (p=0.05) from the baseline (mean 0.212, s.d. 0.142) over the infusion period (mean 0.360, s.d. 0.295) and although still reduced, this was not statistically significant at the end of the infusion (mean 0.362, s.d.0.334).
Figure 45 The effects of a 30 minute combined L-NAME and vasopressin infusion into the gastroduodenal artery, on liver and tumour flux and conductance and blood pressure and TNR
Summary graph (figure 46) and table (14) of changes in perfusion, blood pressure, and conductance following a 30 minute combined vasopressin and L-NAME infusion (n=9). Mean and standard deviation shown.

<table>
<thead>
<tr>
<th></th>
<th>baseline</th>
<th>s.d.</th>
<th>ave</th>
<th>s.d.</th>
<th>p</th>
<th>max value</th>
<th>s.d.</th>
<th>p</th>
<th>end value</th>
<th>s.d.</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>liver flux</td>
<td>205</td>
<td>35</td>
<td>91</td>
<td>59%</td>
<td>57</td>
<td>0.000</td>
<td>42</td>
<td>81%</td>
<td>&lt;0.00</td>
<td>105</td>
<td>52%</td>
</tr>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tumour flux</td>
<td>38</td>
<td>25</td>
<td>22</td>
<td>32%</td>
<td>8</td>
<td>0.068</td>
<td>12</td>
<td>61%</td>
<td>&lt;0.00</td>
<td>23</td>
<td>23%</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BP</td>
<td>76</td>
<td>7</td>
<td>105</td>
<td>37%</td>
<td>13</td>
<td>0.001</td>
<td>120</td>
<td>43%</td>
<td>&lt;0.00</td>
<td>120</td>
<td>23%</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>liver cond</td>
<td>2.788</td>
<td>0.631</td>
<td>0.942</td>
<td>68%</td>
<td>0.583</td>
<td>&lt;0.00</td>
<td>0.421</td>
<td>85%</td>
<td>&lt;0.00</td>
<td>1.039</td>
<td>65%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tumour cond</td>
<td>0.577</td>
<td>0.346</td>
<td>0.230</td>
<td>56%</td>
<td>0.096</td>
<td>0.018</td>
<td>0.138</td>
<td>73%</td>
<td>&lt;0.00</td>
<td>0.246</td>
<td>45%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNR</td>
<td>0.212</td>
<td>0.142</td>
<td>0.360</td>
<td>68%</td>
<td>0.295</td>
<td>0.050</td>
<td>0.526</td>
<td>156%</td>
<td>&lt;0.00</td>
<td>0.362</td>
<td>55%</td>
</tr>
</tbody>
</table>

* Liver and tumour flux are expressed as flux units and blood pressure (BP) in mmHg. Conductance (cond) is derived by division of the individual flux values by the blood pressure and the tumour to normal ratio (TNR) by division of the tumour by the liver flux values.
Comparison of the effects of L-NAME infused with Vasopressin

The tachyphylaxis effect on liver and tumour perfusion seen during vasopressin infusion (and to a lesser extent during angiotensin infusion) is unexplained. One possibility is that decreased perfusion is associated with an accumulation of nitric oxide in the tissues. In order to test if nitric oxide is involved with the tachyphylaxis effect L-NAME was infused either simultaneously with vasopressin or given as a short infusion at the onset of the tachyphylaxis effect. The results were then compared between vasopressin alone (some of this data previously described above 4.1.5), vasopressin and L-NAME and also with L-NAME alone and saline alone.

The percentage flux change between the baseline and average, maximal and end flux values were derived and comparisons made using an unpaired T test.

Vasopressin caused a marked vasoconstriction during the early infusion period although within 15 minutes this returned towards baseline values. Overall in the vasopressin only group, the flux fall from baseline seen in the first 15 minutes (mean 45.0%, s.d. 12.0%) was significantly (p<0.0001) greater than that seen in the second 15 minutes (mean 11.0%, s.d. 12.0%).

When L-NAME was coadministered with vasopressin the vasoconstrictor effect was prolonged and there was no significant (p=0.06) difference in the flux fall from baseline for the first 15 minutes of the infusion (mean 63.1%, s.d. 15.4%) compared with the second (mean 54.0%, s.d. 24.6%) reflecting a more prolonged vasoconstrictor effect than that seen with vasopressin. At 30 minutes the flux fall was significantly greater (p < 0.002) for the combined vasopressin/L-NAME group (mean 54.0% s.d. 25.8%) than for any other group.

If an infusion of L-NAME was commenced at the time when the vasoconstrictor effect to vasopressin was decreasing (n=3), then the tachyphylaxis effect was reversed and the full vasoconstrictor response to vasopressin restored (figure 47).

L-Name administered alone caused a small but significant (p<0.001,) fall in perfusion (mean flux fall 9.0%, s.d.5.6%) which was maintained over the infusion compared with the saline group (mean flux increase 4.6%, s.d. 5.7%).
There was a significant fall in tumour perfusion at 30 minutes which was significantly
(p<0.05) greater for both vasopressin (mean 20.4%, s.d.20%) and the combined
vasopressin/L-NAME group (mean 36.0%, s.d.26.7%) compared with saline (mean flux
increase 3.5%, s.d.13.7%) at the end of the infusion period (figure 48).

The vasopressin induced tumour perfusion fall was significantly (p<0.005) greater during
the first half of the infusion period (mean 37.3%, s.d. 16.5%) compared with the second
half (mean, 23.5%, s.d. 17.4%). This was in contrast to the group receiving combined
vasopressin/L-NAME in which there was no significant (p=0.25) difference in the
perfusion fall in the first half of the infusion (mean, 46.7%, s.d. 14.6%) compared with
the second (mean 40.3%, s.d. 24.2%). There was a significant (p<0.05) flux fall in the L-
NAME alone group (mean 17.5%, s.d. 20.3%) compared with the saline control group
(mean rise 0.9%, s.d. 10.2%).

The average tumour to normal flux ratios over the entire infusion period were not
significantly (p=0.20) changed for the vasopressin group (mean increase 10.5%, s.d.
34.7%) compared with saline (mean fall 1.7%, s.d. 12.4%). However this masked a
significant (0.0002) rise in TNR for the first half of the infusion (mean increase 30.0%,
s.d. 37.0%) which was significantly greater than the second half in which the TNR fell
(mean fall 11.4%, s.d. 27.3%).

Combined vasopressin/L-NAME did produce a significant rise (p<0.02) in the average
infusion TNR (mean 67.6%, s.d. 62.5%) compared with saline (mean fall 1.7%, s.d.
12.4%). There was no significant (p=0.10) difference in the rise seen in the first half
(mean 85.6%, s.d. 87.8%) of the infusion compared with the second half (mean 49.6%,
s.d. 40.0%).
Figure 47  The effect of a short infusion of L-NAME (LN) commenced during a longer infusion of vasopressin at the point where escape from hepatic vasoconstriction was occurring. L-NAME acts to reverse the tachyphylaxis to vasopressin induced constriction.
Figure 48 Summary graph showing the flux change with L-NAME, saline, vasopressin and combined L-NAME + vasopressin at the end of a 30 minute infusion period showing a datapoint for each animal studied.
4.1.10. Summary of Key Findings

Vasopressin produced a profound vasoconstriction in both liver and tumour of limited duration and a short lived improvement in the TNR.

Angiotensin led to hepatic and tumour vasoconstriction. The maximal effect being short lived although some vasoconstriction persisted. The increase in TNR was also short lived.

Regional endothelin produced a profound vasoconstriction in tumour and liver, with a slower onset but prolonged duration without evidence of tachyphylaxis. The increase in TNR was also more prolonged.

Systemic endothelin produced similar effects to regional infusion although the changes were significantly greater following regional infusion.

Despite a fall in vascular resistance, there was an overall fall in tumour and liver perfusion as a result of CGRP infusion.

Inhibition of NO production by L-NAME led to a prolongation of vasopressin induced vasoconstriction in liver and tumour. L-NAME infusion reversed the tachyphylaxis seen in response to vasopressin infusion.

In control animals infused with saline, there was some variation in perfusion over time as well as a small but significant increase in liver flux over the infusion period.
4.2. Relationship Between Blood Flow and Drug Uptake

4.2.1. Introduction

In this experiment the relationship between blood flow and drug uptake was investigated in a dual tracer experiment by the simultaneous infusion of the blood flow tracer $^{64}$Cu-PTSM and the $[^3]$H-5FU.

The section addresses the following questions:

- What is the relationship between blood flow and the 5FU uptake?
- How does the tumour to normal ratio for blood flow and drug uptake vary between host animals, with tumour weight and tumour burden?

4.2.2. Experimental Numbers and Statistical Methods

Twenty one animals, each receiving a control saline infusion, with a total of 147 tumours (median 2.0, IQR 1-11 tumours per animal) were studied. A number of smaller tumours (<10mg) had been grouped and counted together. These were discounted from the individual tumour analysis leaving 117 individual tumours (median 2.0, IQR 1-11 tumours per animal) available for study.

Analysis of the data showed a skewed distribution of tumour to normal ratios and therefore non parametric analysis were used. Results were described by the median and interquartile ranges and comparison between groups was performed using a Wilcoxon rank sum test. Differences between the individual animals was assessed using analysis of variance.

Graphical representation of the tumour to normal ratios is sometimes shown on a log scale for clarity. The Pearson's correlation coefficient and test of significance are performed on the original data values.

4.2.3. Inter and Intralobe Range

In order to assess the distribution of the radiotracers both within and between lobes, a ratio was derived between the highest and lowest samples measured for the lobe or between the four measured lobes for each animal. The range of values between the highest and lowest $^{64}$Cu-PTSM samples within a lobe was significantly greater (p<0.005) within tumour (median 7.9 IQR 3.9 - 10.5) than within liver (median 3.3 IQR 2.1 - 5.0). Similarly the intralobe range of $[^3]$H-5FU uptake was significantly greater (p<0.05) within tumour (median 3.3 IQR 2.0 - 6.7) than liver (median 2.3 IQR 1.6 - 3.6).

The median range in counts between lobes within individual animals was 7.5 (IQR 4.5 - 11.4) for $^{64}$Cu-PTSM and 5.0 (IQR 3.3 - 5.7) for 5FU. This could either reflect blood flow variation between lobes or streaming of infusate between lobes. For this reason tumour : liver ratios were derived on an individual lobe basis.

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4.2.4. Correlation Between Blood Flow and 5FU Uptake

TNR blood flow and the TNR for 5FU uptake closely correlated both for individual tumours \((r=0.939, p<0.0001)\) when calculated per animal \((r=0.942, p<0.0001)\).

Figure 49 a and b: Tumour to normal ratios for blood flow \((^{64}\text{Cu-PTSM uptake})\) against \([^{3}\text{H}]\)-5FU uptake for saline control groups shown for individual tumours (above) and for individual animals (below) (shown on a log axis)
4.2.5. Range of Variation of Tumour to Normal Ratios

There was a wide variation between the TNRs for blood flow (median 0.115, IQR 0.051-0.247) and 5FU uptake (median 0.185, IQR 0.100-0.321) although over 90% of the TNRs were below 1 (i.e. the tumours were less well perfused or had lower 5FU uptake than surrounding tissue). In an attempt to identify factors which might give rise to this variation, the effects of tumour weight, tumour burden and individual host animal have been individually examined.

4.2.6. Effect of Tumour Weight

There was no significant correlation in the TNR for blood flow for tumours of differing weights ($r=-0.05$, $p=0.608$) (figure 50). Similarly there was no significant correlation between tumour weight and the TNR for 5FU uptake ($r=-0.031$, $p=0.756$) (figure 51).

4.2.7. Effect of Tumour Burden

The median percentage hepatic replacement (PHR) by tumour was calculated according to the formula

$$\text{Percentage hepatic replacement} = \frac{\text{tumour weight}}{[\text{tumour} + \text{liver weight}]} \times 100.$$  

In animals receiving normal saline, the median PHR was 3.60% (IQR 1.1 - 12.7). When the individual tumours from animals above and below the median PHR value were compared there was no significant difference ($p=0.386$) in the TNR blood flow between animals with a PHR below the median (median blood flow TNR 0.121) compared with those above (median 0.126). Similarly there was no significant difference ($p=0.189$) in the TNR for $^3$H-5FU uptake between those tumours from animals below the median PHR (median 5FU TNR 0.120) compared with those above (median 0.195).

When the average (the average of all the TNRs for all the tumours in any one animal) TNR per animal was plotted against the overall PHR for the animal there was no significant correlation for blood flow ($r=0.011$, $p=0.961$) or 5FU uptake ($r=0.017$, $p=0.940$) with tumour burden.

These findings show no evidence to support a decrease in tumour vascularity or 5FU uptake in animals with higher tumour burdens within the range tested.

4.2.8. Effect of Interanimal Variation

A further possibility examined was that the TNR within any one animal might vary from the TNRs of other animals. Tumours from animals with 5 or more tumours within the liver were plotted individually (figure 52 and 53). The variation in TNR was not attributable to interanimal variation (ANOVA $p>0.05$)
Figure 50 and 51  Relationship between tumour weight and the TNR for blood flow (above) and 5FU uptake (below)
Figure 52 and 53  Graphs of the blood flow TNR (above) and 5FU uptake (below) for 10 individual animals with >5 tumours each. The TNRs for each tumour of each animal are shown in each column. Medians and IQRs shown.
4.2.9. Summary of key points

There was a wide variation between the individual TNRs for blood flow and 5FU uptake for any one animal.

TNR was not directly related to tumour weight or total tumour burden.

There was a very close correlation between blood flow and 5FU uptake.
4.3. Effect of Vasoactive Agents on Hepatic Arterial Flow Ratio and 5FU Uptake Ratio

4.3.1. Introduction

Having established, in the preceding section, a close relationship between blood flow parameters and the uptake of 5FU, one would predict that any manipulation which increased the proportion of blood flow to the tumour would also increase the uptake of 5FU.

This section addresses these following questions:

1. What is the effect of the vasoactive manipulation following agents on the TNR for blood flow and 5FU uptake compared with the control animals receiving normal saline infusions?

2. Is the relationship between blood flow and 5FU uptake, noted in the previous section maintained during vasoactive manipulation?

4.3.2. Experimental Design

The design of these experiments was identical to those of the previous section apart from the fact that a vasoactive agent was substituted for the saline infusion into the gastroduodenal artery. Sample preparation and counting were also the same.

The following agents and flow rates were tested:

<table>
<thead>
<tr>
<th>agent tested</th>
<th>No. animals</th>
<th>No. tumours</th>
<th>dose</th>
<th>infusion rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>angiotensin II</td>
<td>10</td>
<td>102</td>
<td>1μg/min</td>
<td>25μl/min</td>
</tr>
<tr>
<td>endothelin I</td>
<td>10</td>
<td>133</td>
<td>25ng/min</td>
<td>25μl/min</td>
</tr>
<tr>
<td>L-NAME</td>
<td>12</td>
<td>40</td>
<td>750μg/min</td>
<td>25μl/min</td>
</tr>
<tr>
<td>histamine</td>
<td>9</td>
<td>35</td>
<td>100μg/min</td>
<td>25μl/min</td>
</tr>
<tr>
<td>leukotriene C4</td>
<td>9</td>
<td>45</td>
<td>250ng/min</td>
<td>25μl/min</td>
</tr>
</tbody>
</table>

Statistical analysis was performed using Wilcoxon rank sum tests. Comparison of the two slopes was made using an a confidence interval analysis for the difference between two slopes.
4.3.3. Vasconstrictors

4.3.3.1. Angiotensin II

Angiotensin II produced a significant (Wilcoxon p=0.001) increase by 124% in the blood flow TNR (median 0.258, IQR 0.114-0.670) compared with tumours from animals in the saline infusion group (median 0.115, IQR 0.051-0.247). The TNR [3H]-5FU uptake ratio was also significantly increased (Wilcoxon p=0.034) in the angiotensin infusion group (median =0.245, IQR 0.129-0.375) by 32% compared with the saline group (median =0.185, IQR 0.100-0.321) (figure 54 and 55).

As with the saline control group there was a significant correlation between the TNR for blood flow and that for 5FU uptake. However, slope of the linear relationship between blood flow and drug uptake was significantly (p<=0.05, 95% C.I. of the difference 0.137 - 0.374) lower in the angiotensin group (slope = 0.199) than in the saline infusion group (slope = 0.455) (figure 49).

4.3.3.2. Endothelin I

The TNR blood flow was significantly elevated (p=0.05) by 25% for animals receiving an infusion of endothelin (median 0.144, IQR 0.064-0.311) compared with the control group (median 0.115, IQR 0.051-0.247). The TNR for 5FU uptake was also significantly elevated (p=0.0005) by 26% in animals receiving endothelin (median 0.234, IQR 0.161-0.542) compared to controls (median = 0.185, IQR 0.100-0.321).

The slope of the relationship between blood flow and 5FU uptake was significantly (p<0.05, 95% C.I. of the difference 0.045 - 0.253) different to the control group with the slope of the blood flow 5FU uptake being steeper with endothelin (slope = 0.604), compared with the control saline infusion group (slope =0.455).

4.3.3.3. L-NAME

L-NAME infusion caused an increase in blood flow TNR (median 0.229, IQR 0.063-0.330) although this did not reach statistical significance (p=0.66) compared with the control group (median 0.115, IQR 0.051-0.247). There was also no significant difference (p=0.349) in the TNR for 5FU uptake (median 0.180, IQR 0.080, 0.373) compared with the control group (median =0.185, IQR 0.100-0.321).

As with endothelin the relationship between blood flow and 5FU uptake was significantly (p<0.05, 95% C.I. of the difference 0.098 - 0.224) steeper for L-NAME(slope = 0.619) than for the saline control group (slope =0.455).
4.3.4. Permeability Agents

4.3.4.1. Histamine

Infusion of histamine led to a non significant fall in the TNR blood flow (median 0.081, IQR 0.048-0.182) compared with controls (median 0.115, IQR 0.051-0.247). There was a significant fall (p=0.004) of 31% in the TNR 5FU uptake (median 0.114, IQR 0.053-0.188) compared with controls (median =0.185, IQR 0.100-0.321).

The slope of the correlation between drug uptake and blood flow following histamine infusion (slope 0.427) was not significantly different (p>0.05, 95% C.I. of the difference -0.267 - 0.326) from the saline control group (slope 0.455).

4.3.4.2. Leukotriene C4

The TNR blood flow after leukotriene C4 infusion (median 0.121, IQR 0.063-0.405) was not significantly different (p=0.125) to controls. Similarly there was no significant difference (p=0.092) in the TNR 5FU (median 0.128, IQR 0.080-0.276) from controls. The slope of the relationship between blood flow and drug uptake ratios following leukotriene C4 infusion (0.604) was significantly steeper (p<0.05, 95% C.I. of the difference 0.067 - 0.231) than the saline control group (slope 0.455).
Figure 54 and 55  Graphs showing the tumour to normal blood flow (above) and 5FU uptake ratio (below) for the individual tumours in the saline and experimental groups. (p = in comparison with saline control group)

(n.sal - saline, Ag - angiotensin II, Et - endothelin I, L.C4 - leukotriene C4, Hist - histamine) (medians and IQRs shown)
Figure 56 and 57  Relationship between the TNR for blood flow and 5FU uptake in tumours of animals receiving a control saline infusion (above) and angiotensin II infusion (below). (The relationship from the saline group is shown in this and subsequent graphs as a broken line)

\[ \text{saline control} \]
\[ r = 0.939 \]
\[ y = 0.455x + 0.144 \]

\[ \text{angiotensin} \]
\[ r = 0.295 \]
\[ y = 0.199x + 0.288 \]
Figure 58 and 59  Relationship between the TNR for blood flow and 5FU uptake in tumours of animals receiving a regional endothelin I infusion (above) and L-Name (below)
Figure 60 and 61  Relationship between the TNR for blood flow and 5FU uptake in tumours of animals receiving a histamine infusion (above) and leukotriene C4 infusion (below)
4.3.5. Summary

The vasoconstrictors; angiotensin and endothelin produced increases in the TNR for blood flow and 5FU uptake.

The capillary permeability agent and vasoconstrictor leukotriene C4 produced no increase in blood flow or 5FU uptake ratios while histamine produced a fall in the TNR for 5FU uptake.

Under conditions of vasoactive manipulation, the relationship between blood flow and 5FU uptake may vary.
5. Discussion

5.1. Discussion of Methodology

5.1.1. Animal Tumour Model

The experimental tumour system was selected on the basis of its close resemblance to the known vascular features of human colorectal metastases and practical considerations concerning cell and tumour availability. Initial experiments were carried out using tumours arising following subcapsular tumour cell injection. However these were abandoned in favour of intraportally injected tumours. The former were much easier to grow requiring only 7 days to reach the same size and were more reliable. Despite this, the appearance of the tumours was of an amalgam of several small superficial tumours rather than a single tumour nodule. Because of concern that this would reflect a difference in tumour vasculature this method of tumour implantation was not used. Intraportal injection of tumour cells does not make the resultant tumours true metastases as they have not migrated from a primary tumour site, although they provide a reasonable model of the metastatic process entering the liver via the portal blood stream. Tumour growth was reasonably reliable although on occasions tumour take was poor for reasons which were not clear but may have been related to both cell passage techniques and the animals environment.

As a result of the variability in tumour take, some experimental groups in the radiotracer studies had more tumours than others. The results of the saline control group (in which there was a wide range of tumour numbers) showed no relationship between the TNR for blood flow or drug uptake with tumour size (weight) or total tumour burden within any animal. Therefore one would not expect any bias to be introduced to the results from the variation in tumour numbers within any one liver, although the power to show a small difference between the study group and the control group would be reduced. Despite these attempts to make the model as close to the clinical situation as possible, there are other physical differences with human tumours which are likely to be larger at the time of diagnosis and treatment. The results obtained in this study therefore relate to small tumours and similar results cannot be assumed to apply to large tumour masses. A rat sarcoma cell line rather than a human colorectal line was used. This was largely for practical reasons as growth of human tumour lines requires to be carried out in immuno-suppressed ("nude") rats which are less hardy and more costly to maintain. Furthermore there is no evidence of a difference in the response of the tumour vasculature between different cell lines.

Measurement of blood flow parameters in an animal tumour model is always open to the criticism that either the animal or the tumour is not representative of the human situation.
In many respects this is true and must be borne in mind in the interpretation of the results. However, all of the vasoactive agents tested are essentially common across most mammalian species, albeit with minor peptide variations. One can thus expect that the type of change initiated by such compounds and their duration of action might be similar.

5.1.2. Laser Doppler Flowmetry

Laser Doppler flowmetry has only recently been developed to a point where it can reliably be used to monitor perfusion within biological systems and provides a unique and valuable technique for the continuous monitoring of tissue perfusion. As has been demonstrated in section 1.9 there is no other direct technique which can be applied to the continuous measurement of tumour and liver tissue perfusion in response to a vasoactive infusion. Ackerman has used a similar system to measure the short term flow changes following intraportal adrenaline but no previous study has looked at the changes seen in response to a variety of vasoactive agents over a similar period of time. Despite the lack of absolute flow units, the ability to continuously monitor perfusion in the same subject gives reliable information on the direction of change over a period of time. This alone clarifies some of the confusion seen in the literature over the response to vasoactive agents where studies have made measurements at one or two time points only. These results have frequently drawn conflicting conclusions as the authors have failed to appreciate that there is a continuum of vasoactive change following injection of a vasoactive compound and that it is not always possible to dichotomise into a "before and after" situation. However, as with any measurement there are some limitations and errors inherent in its use.

Measurements are relative and despite the attempts of some manufacturers to calibrate the machine in absolute flow units, this is not generally accepted. Perfusion measurements themselves are complex and relate to the volume of blood passing through a volume of tissue as compared with flow measurements which are generally applied to flow through a single vessel and can be measured in ml/min. The volume of tissue sampled is also uncertain and varies between different tissues according to the optical properties of the tissue. A low sampling volume is acceptable in a uniformly heterogeneous tissue however in less heterogeneous tissues such as tumour it may not be possible to assume that the sampled volume reflects flow in the total volume of the tissue of interest. In these experiments, the area of liver sampled by the probe was tested by placement of a piece of silver foil beneath the liver and progressively moving the probe closer to the liver edge where the liver thinned. At the point where the light was passing through the liver, an increase in the scattered light could be noted by observing the "DC" reading on the blood flow monitor. In our system the thickness of tissue sampled appeared to be approximately 1mm in the liver, which is in keeping with previous work. However this could not be tested in tumour as there was no similar wedge shaped
tumour occupying the edge of the lobe and therefore the area of tissue sampled may have varied.

The laser Doppler probes are highly sensitive to flow change and when recording at high frequency with a low time constant, can detect the fluctuations of pulsatile flow. However the sensitivity of the probe also makes it vulnerable to incorporation of movement artefacts into the readings. There was no method by which the probe could be rigidly fixed to the liver to avoid any relative movement between the two surfaces. Use of a more sedative anaesthetic agent did make probe placement easier with smaller respiratory excursions although these agents were not used because of the profound hypotension and concern about the vasoactive changes associated with respiratory depression. Movement artefact with respiration was monitored in each study and was found to vary between 5-15%. Assuming that this value was constant throughout the experiment, the effect of this would be to decrease the likelihood of a small change in perfusion reaching significance. However the size of the change in perfusion is in most cases likely to outweigh the potential error involved.

In some of the studies it was difficult to obtain a "level trace" for the baseline period before the infusion commenced. This reflects the continuous vasoactive changes that occur naturally over a period of time. The fact that these variations are unlikely to have a major effect on the results can be concluded from the lack of overall change seen when saline was infused.

Flow measurements within the liver reflect the input from both hepatic arterial and portal systems. However as already mentioned the blood supply to the tumours is predominantly arterial, and for the delivery of hepatic artery infusional chemotherapy the portal component of the liver blood supply is of lesser importance. The information obtained from the laser Doppler study indicated the extent and duration of perfusion change. However it was not able to reflect changes in the tumour to normal ratio for hepatic artery flow alone compared with total hepatic flow. As the clinical application of this work is to enhance the delivery of cytotoxic agents delivered through the hepatic artery, the tumour to normal ratio for this component of total hepatic blood flow was of interest. Intra-arterial vasoactive infusion may have led to shifts in the balance between the arterial and portal components of the flow which may not have been reflected as a change in total flow.

Calculation of tumour to normal ratios has been performed as part of the results analysis. There are however certain errors inherent in such a calculation. Firstly because of potential differences between the optical properties of different tissue these values can only considered as relative values rather than absolute. Secondly, as already mentioned, an assumption is made that the laser Doppler readings of tumour perfusion reflect total
tumour perfusion. This may not be justified, particularly in view of the uncertainty of the volume of tissue reflected by the laser Doppler measurements and also the marked vascular heterogeneity which we know exists (fig 19). Thirdly, because the flux readings obtained for the tumour were considerably less than for liver parenchyma, any errors incorporated into the readings might be expected to have a greater influence on tumour than liver.

For these reasons a second method of measurement of tumour flow was required which could use the information obtained from the laser Doppler studies but which would measure the blood flow changes at a fixed time point. The initial aim of this second part of the work was to measure the flow values before and after vasoactive manipulation in absolute flow units using radioactive microspheres. However for reasons previously described, this method could not be applied to this model and a further method was devised by which the hepatic artery tumour to normal blood flow ratio could be measured along with an assessment of the effects on the 5FU uptake tumour to normal ratios following hepatic arterial infusion.

5.1.3. Radiotracer measurement of blood flow and drug uptake

As can be seen from the methods section, the development of a suitable radiotracer method for the measurement of hepatic arterial flow was not without problem. A series of radiotracers were evaluated for use in the measurement of tissue and tumour blood flow. The technical challenges of such measurements, particularly under circumstances of marked vasoconstriction, are considerable. Radioactive microspheres would have enabled absolute flow measurements at two time points (e.g. before and after infusion) and were the original intended method. While the hypovascularity of the tumour model is similar to human colorectal metastases it raises practical difficulties about how to deliver a minimum number of spheres to the tumour to obtain valid results. The specific vascular conditions of the liver with a dual inflow system makes measurement more complex and a trapping tracer with a high extraction fraction and retention is required. $^{64}\text{Cu-PTSM}$ uptake was identified as the most suitable method available. The major limitation with its use in these studies is that it only yields information about the TNR and is not measured in absolute flow terms. If, however the information about the direction and extent of the flow change is deduced from the results of the laser Doppler studies, then it is reasonable to use these experiments to investigate the change in ratios.

The fact that the first pass uptake of $^{64}\text{Cu-PTSM}$ was not 100% might lead to some recirculation through the portal system with underestimation of the TNR. With an extraction of 70% this would leave 30% to recirculate. Some of the remaining $^{64}\text{Cu-PTSM}$ which returns to the left side of the heart will then be taken up into the lungs with the remaining amount redistributed by the left ventricle. The amount reaching the portal
organs would be estimated to be under 5% of the original total and the residual amount passing to the liver through the portal vein would be expected to be very small. For this reason the error involved due to recirculation of the tracer would be expected to be minimal and the high retention within the time scale of these experiments meant that little redistribution would be likely to occur.

Measurement of uptake of 5FU was more straightforward than measurement of a physiological parameter such as blood flow, as the cytotoxic agent is the same as might be given in the clinical situation and is administered by the same route. Nevertheless the solublisation and bleaching of the samples required careful consideration and planning. In liquid scintillation counting the scintillant fluid is in contact with the sample within the vial. The light emitted by the scintillant is detected by a separate detector outside the vial. Therefore any solid particles or strong colours within the vial itself will lead to an underestimation of the counts. It was therefore important to ensure that each sample was fully homogenised, solublised and bleached before counting. When the samples were solublized and transferred to the liquid scintillation counting vials, care was required to ensure complete transfer of the whole of the sample. The linear response of the liquid scintillation counter has already been demonstrated and photo and chemiluminescence were minimal providing that 24 hours was allowed to elapse after addition of the scintillant. Approximately 70 counting vials of either liver or tumour were generated per animal, making approximately 5000 samples which had to be processed for this study.

Within the animal itself streaming of flow between lobes was present but the effect of this was minimised by calculation of each of the tumour to normal ratios from the liver of the lobe in which the tumour lay rather the whole liver. A further error may have been introduced if there was streaming of blood flow within a lobe although this did not appear to be a significant problem when different samples within a lobe were compared. By injecting the two radiotracer simultaneously, identical flow conditions were present for both and streaming within a lobe would have applied to one radiotracer in the same way as the other. The strong correlation between the $^{64}$Cu-PTSM and the $[^3]$H]5FU molecule confirms the accuracy of the counting statistics as this would be unlikely if a random error were introduced. The chemical purity of the Cu-PTSM was checked throughout the experiments by periodic testing of the octanol partition coefficient. This confirmed that there was no chemical binding between the Cu-PTSM and the 5FU molecules.

In using a clinically applicable cytotoxic drug and measuring the entire liver and tumour samples the results should accurately reflect the TNR for the 5FU uptake into the liver. The dose of 5FU was estimated to give rise to a circulating 5FU level of 70ng/ml which is a similar order of magnitude to that one would expect in the clinical situation. A
significantly higher dose might give rise to concerns about saturable transport mechanisms in the 5FU uptake.

The coadministration of the blood flow and 5FU tracer together made it easier to demonstrate the close correlation between blood flow and 5FU uptake. The closeness of this relationship suggests that being a small molecule the majority of the 5FU is leaving the vascular channels into the tissue as quickly as it is delivered and implies that 5FU uptake is limited by flow and not by transport out of the circulation.

Probably the greatest potential sources for errors in the use of radiotracers to measure blood flow, are related to the sample preparation and counting. In this series of experiments, great care was taken in its design to keep these sources of error to a minimum. During the sample preparation all samples were treated in a similar manner. Tissues were carefully separated after the animal was killed and each individual tumour "shelled out" and cleared of any residual adherent liver tissue. This was important as the liver immediately surrounding the tumour is influenced by tumour angiogenic factors and is likely to be highly vascular. Thus the inclusion of a small amount of this tissue with the relatively hypovascular tumours could cause a large increase in tumour counts.

The ⁶⁴Cu has a high energy γ radiation. Therefore care was taken to minimise interference between samples in the γ counter and those nearby. The results were decay corrected because of the short half life of the ⁶⁴Cu (12.7 hours).

5.2. How Does Vasoactive Infusion Affect Liver Parenchymal Blood Flow?

All the vasoconstrictors used in this study led to a fall in hepatic perfusion. Perfusion changes were monitored continuously over the infusion period and it was therefore possible to characterise differing patterns of response between the different agents in terms of how the vasoconstriction varied with time.

Vasopressin had the most clear-cut and reproducible response (figure 31). The onset of vasoconstriction was rapid and profound. The vasoconstriction then eased slightly but persisted for 15 minutes after which time the effect returned towards baseline, despite the continued infusion of the agent. If the beneficial effects of vasopressin infusion depend on a fall in hepatic perfusion, then clearly the agent would appear to only have an effect for a short period of time. A similar clinical observation was made by Conn (1973) who observed that the hepatic arterial vasoconstriction rapidly recovered when vasopressin was administered to patients in the treatment of gastrointestinal haemorrhage. The blood pressure was raised throughout vasopressin infusion as was vascular conductance reduced suggesting that there was a prolonged effect on vascular tone, despite the return to near normal perfusion levels.
Angiotensin led to a less consistent vascular response (figure 33). Rapid vasoconstriction was again seen in all cases although this time the maximal response only persisted for 2-3 minutes before perfusion partially recovered. A much lesser degree of vasoconstriction then persisted throughout the infusion. As with vasopressin, blood pressure was elevated throughout the infusion and conductance was similarly increased. So despite a marked degree of tachyphylaxis to angiotensin within the first few minutes, there was, as with vasopressin, a more prolonged increase in vascular resistance.

Regional infusion of endothelin led to a very different pattern of response. Following the onset of the infusion there was a vasodilator response, associated with a transient period of hypotension. The vasodilation diminished within the first 5 minutes and there then followed a very profound degree of hepatic vasoconstriction. The onset of the vasoconstriction was gradual, taking at least 15 minutes to reach the maximal level of vasoconstriction which was then prolonged throughout the infusion period and persisted with little recovery for 30 minutes following the end of infusion. This is in keeping with observations by Withrington et al. (1989) who observed a biphasic response consisting of a short vasodilation followed by prolonged vasoconstriction. A similar response was seen when endothelin was infused systemically, with a slow onset but prolonged vasoconstriction following a transient vasodilation. The greater response to regional endothelin demonstrates the "first pass effect" in which regional infusion leads to higher local concentrations within the hepatic circulation.

Blood pressure changes with endothelin were less marked than with the angiotensin or vasopressin. There was a mild elevation of systemic blood pressure in the regionally infused group although the average blood pressure fell slightly when the endothelin was infused systemically.

Only one vasodilator was tested in this study having not been studied for this purpose before. CGRP is a powerful vasodilator with a role in the circulation in increasing the local formation of nitric oxide. It led to a fall in vascular blood pressure and resistance of both tumour and hepatic vasculature. Hepatic perfusion was increased in some animals studied although frequently the fall in blood pressure led to a fall in perfusion despite a fall in vascular resistance. The fact that such a fall in resistance of the tumour vasculature can come about is of interest as tumour vessels have been thought to be maximally dilated.

The evidence seen from the results shown so far indicate that no agent tested led to a rise in tumour blood flow. The secondary aim of vasoactive manipulation therefore becomes to achieve a rise in the tumour to normal ratio. On the assumption that to achieve a prolonged increase in TNR, prolonged parenchymal vasoconstriction is required, then all three agent tested increased vascular resistance over the infusion period but only...
endothelin caused a significant reduced hepatic parenchymal vasoconstriction at the end of a 30 minute infusion.

5.3. How Does Vasoactive Infusion Affect Tumour Blood Flow?

In the laser Doppler studies carried out in this work, no regionally infused vasoactive agent led to an absolute increase in tumour blood flow and there was a fall in tumour perfusion using laser Doppler flowmetry with all the vasoconstrictors tested.

It is not possible to say whether the vasoconstriction occurred within the common larger arterioles or whether it happened within the tumour precapillary arterioles. Controversy exists over whether tumour vasculature can directly respond to vasoactive agents. The apparent absence of a significant smooth muscle component leads some to regard the tumour vasculature as a totally passive vascular bed. Mattsson et al. (1978) studied the effect of intravenous noradrenaline infusion and noted a fall in tumour blood flow as measured by $^{133}\text{Xe}$ washout. He went on to try and distinguish whether the constriction was within the tumour or in the pretumour arterioles by directly injecting the vasoactive agent into the centre of the tumour and found a similar fall in perfusion implying there may be a direct vascular response within the tumour bed. Despite Mattsson's attempts, there is no clear way of knowing at what level vasoconstriction occurs. The issue is further clouded by the incorporation of host arterioles into tumours. These vessels do maintain some vascular tone although whether the blood which perfuses them, undergoes vascular exchange with the tumour or merely shunts through high flow vessels is not clear.

In previous studies, some investigators have felt it possible to induce an absolute increase in tumour blood flow by vasoactive manipulation. Ackerman demonstrated a short lived increase in tumour blood flow in a rat carcinosarcoma by a variety of methods, as well as demonstrating a redistribution of blood flow to the centre of the tumour, which he postulated was due to an opening of vascular channels. Hemingway et al. (1992) used a laser Doppler probe at operation to measure the effect of a 90 second infusion of angiotensin. He showed an absolute increase in tumour flow but failed to measure liver blood flow. Furthermore the increase in tumour flow was not maximal until after the end of the angiotensin infusion raising doubts about whether such an effect would be produced during a prolonged angiotensin infusion. The majority of investigators have either not shown an absolute increase in tumour blood flow or have only measured flow relative to the host tissue. When a bolus of vasoconstrictor was administered there was sometimes a rebound effect in which tumour blood flow rose slightly for a short period of time and it may be this which explains some of the findings of some previous observers.
Suzuki et al. (1981) postulated that the tumour vasculature is unable to autoregulate flow in response to hypertension and demonstrated an increase in tumour blood flow in response to systemically infused angiotensin. This approach, while valid as a potential therapeutic method, is fundamentally different from that in which vasoconstrictors are regionally administered, as the vasoconstrictor is not being used to directly affect the tumour or liver vasculature. The success of this approach depends on whether the vessels involved in autoregulation are situated in that part of the vascular bed common to both liver and tumour or whether they affect only the normal liver parenchyma. In the studies performed within this thesis, there was no increase in tumour blood flow when endothelin I was administered systemically although its central pressor effects are less clear-cut than with angiotensin II.

CGRP did lead to a fall in vascular resistance within the tumour circulation although the overall perfusion usually fell due to a fall in blood pressure. Once again the fact that a fall in resistance was possible implies that the tumour circulation is not in a state of maximal dilatation or at least that precapillary resistance vessels have been incorporated which maintain the capacity to vasodilate, as illustrated in figure 2.

From the current studies, it is clear that vasoactive infusion in our experimental system did not lead to an increase in tumour flow. If an absolute tumour blood flow increase is not feasible during vasoactive infusion, then it may be possible to increase the proportion of blood flow to the tumour i.e. to cause an increase in the tumour to normal blood flow ratio. This may come about either if the tumour circulation remains unaffected while the liver vasoconstricts or may occur if both constrict but the tumour by a lesser degree than the liver.

5.4. How Does Infusion of Capillary Permeability Agents Affect 5FU Uptake?

There was no increase in TNR for either of the permeability agents tested; leukotriene C4 or histamine and in fact there was a slight fall in the TNR following histamine. Neither of these agents were examined in the laser Doppler studies as it was thought that their effect was likely to occur through changes in vascular permeability. Despite this, histamine is known to have some vasodilator effects and leukotriene C4 to be in part a vasoconstrictor.

Because of the small size of the 5FU molecule and the close correlation with blood flow it appeared that its uptake was largely determined by perfusion rather than diffusion. This is in contrast to the situation in which antibodies were regionally administered in which histamine led to a 3 fold increase in uptake ratios (Hennigan et al. 1993). With this in mind it would appear that manipulation of capillary permeability is unlikely to be of any benefit when increasing uptake of small cytotoxic molecules although it may enhance the
uptake of larger cytotoxic molecules whose uptake from the circulation is diffusion limited.

5.5. What Were the Effects on the Tumour to Normal Ratio in the Laser Doppler Studies?

For reasons discussed in section 5.1.2, the laser Doppler studies provide information about the tumour to normal ratios for total hepatic blood flow rather than for the hepatic arterial component alone.

All the vasoconstrictors tested led to a significant increase in the tumour to normal ratio at some part of the infusion period, doubling it in the cases of vasopressin and endothelin. However it is clear that the duration of effect with angiotensin and vasopressin was short-lived and corresponds approximately although not precisely, to the period of maximal hepatic vasoconstriction. The maximal increase in the TNR appeared to occur when the hepatic parenchyma was actively constricting. Once the rate of constriction decreased, the TNR reduced again or returned to resting levels. Of the vasoconstrictor agents tested, only endothelin appeared to increase the average tumour to normal ratio over the whole infusion period on account of the profound hepatic vasoconstriction caused. Because of the gradual onset of this vasoconstriction, the increase appeared to be more apparent in the second half of the infusion than the first. Only endothelin had any significant prolonged effect apparent at the end of the infusion period. This finding suggests that endothelin would lead to a more significant and prolonged increase in the TNR had the infusion been prolonged further.

5.6. What Was the Relationship Between Blood Flow and Drug Uptake?

In these experiments, under control conditions a very close relationship in the ratios of blood flow and drug uptake was apparent. This relationship between tumour blood flow and cytotoxic uptake ratios has not previously been clearly described in such an experimental study although it has been generally assumed that poor vascularity leads to poor cytotoxic uptake. Sigurdson et al. (1986) has shown in a human study a correlation between the blood flow TNR and the TNR of radiolabelled F UdR in biopsy specimens of human hepatic tumours. Although not directly demonstrated by these experiments, the fact that tumours with higher blood flow have higher drug uptake was taken to imply that manoeuvres to increase the proportion of blood flow to tumour are likely to lead to similar increases in drug uptake. This finding is confirmed by the results of these studies.

The relationship between blood flow and drug uptake is important to the strategy of vasoactive manipulation. The finding that this relationship may vary under conditions of vasoactive manipulation was unexpected and the reason why this might be so is unclear. With angiotensin the slope of the correlation was significantly less than the saline control
group. Therefore at higher blood flows the $^3$H-5FU uptake ratio appears to be less than predicted. There was no significant difference in the relationship between blood flow and drug uptake with histamine while the slope of the correlation was steeper with endothelin and leukotriene C4 and L-NAME. This is in the opposite direction to the change with angiotensin and varied from the control group by a lesser amount. Why there should be a difference in the relationship between blood flow and drug uptake under conditions of vasoactive manipulation is not clear. Furthermore the reason why the slope of the line should be significantly flatter with angiotensin yet steeper with vasopressin, endothelin and L-NAME is also unclear. Because this experiment was performed by measuring flow at only one time point, it is not possible to say whether there is a change in 5FU uptake in tumours which have increased their flow. In order to answer this question it would be necessary to perform a paired study with blood flow and drug uptake measured before and after vasoactive manipulation.

5.7. What Were the Effects of Vasoactive Manipulation After a 30 Minute Infusion?

5.7.1. Vasoconstrictor Agents

Both angiotensin and endothelin led to rises in the TNR for blood flow and 5FU uptake following a 30 minute infusion period. There was no significant increase in blood flow or drug uptake ratios for L-NAME. Because of the nature of the experiment it was only possible to measure the ratios at one fixed point in time. Thirty minutes was allowed for the infusion as this was comparable to the laser Doppler studies and was longer than previous studies. Longer infusions produced physiological imbalances from the prolonged procedure under anaesthesia. It would have been of interest to know if these changes would be present if the infusion were prolonged further. Future studies of this might be carried out by implantation of osmotic pumps into the rats to deliver a vasoactive infusion over a period of days, although the longer term patency of the gastroduodenal catheters would need to be tested.

The fact that endothelin had a beneficial effect at 30 minutes was predicted from its long duration of action in the laser Doppler studies. Angiotensin had a more variable response shown by the laser Doppler and despite an initial tachyphylaxis effect, there were signs of a lesser degree of vasoconstriction lasting a little longer. Nevertheless it was surprising that this effect was still present at 30 minutes. One limitation of the laser Doppler studies is the inability to distinguish hepatic arterial from portal blood flow. It may therefore be that subtle alterations in flow between these systems was occurring which led to a change in the TNR as an increase in the proportion of hepatic flow through the hepatic arterial system rather than the portal is likely to increase the TNR.
5.7.2. What Limits the Duration of Effect of Vasoconstrictor Infusion?

Nitric oxide has been postulated as one of the endothelial cell derived compounds responsible for maintaining vascular tone, which opposes the vasoconstrictor tone from the adrenergic sympathetic innervation and the effects of local humoral vasoconstrictors. Several inhibitors of nitric oxide formation are currently in use. L-NAME was used for these experiments because of its relatively high solubility and ease of use. When L-NAME was regionally infused, it led to a gradual onset but well maintained degree of vasoconstriction (figure 41 and 42). The slow onset probably reflects the fact that L-NAME is a competitive inhibitor of nitric oxide synthase and requires a period of time to build up to its maximal effect. The finding that L-NAME would both prolong the vasoconstriction and reverse the tachyphylaxis in response to vasopressin suggests that the tachyphylaxis phenomenon seen in response to the peptide vasoconstrictors may be due to accumulation of nitric oxide. This is in keeping with other recent work showing that nitric oxide inhibition can potentiate the magnitude of angiotensin induced contraction of rabbit aortic ring (Zhang et al. 1994) or prolong the duration of contraction to angiotensin in rabbit renal arterioles (Yoshida et al.). Further studies in a more suitable long term model would be necessary to determine whether the effect seen in these studies is sustained over a period of hours or days.

Tachyphylaxis to vasopressin infusion was not as apparent within the tumour as in the liver parenchyma and consequently while the addition of L-NAME prolonged the constriction seen during the first half of the infusion, there was no significant difference at the end of the infusion compared with vasopressin alone. This suggests that this vasoconstrictor effect arose from tumour vessels where the role of nitric oxide in the regulation of vessel tone may not be the same as in normal vessels. Little work has yet been done to characterise differences in nitric oxide production between tumour and normal endothelium although significant differences may exist.

Despite the reduction in tumour flow with vasopressin there was an increase in tumour to normal flow ratio, offering the potential for therapeutic advantage by increasing the dose of the cytotoxic drug delivered. This effect was sustained by prolonging the effect of vasopressin infusion using L-NAME.

Although L-NAME infusion to inhibit nitric oxide production did lead to a gradual onset hepatic vasoconstriction in the laser Doppler studies, L-NAME infusion did not lead to an increase in blood flow or 5FU uptake ratios. This implies that at 30 minutes the effect of nitric oxide inhibition was similar within liver and tumour tissues resulting in no overall change in the TNR. This is in keeping with the findings from the laser Doppler studies in which there was no overall change in the TNR with L-NAME infusion. One further question of interest would be to establish if L-NAME was able to prolong the
effect of an otherwise short acting agent such as vasopressin such as was demonstrated in the laser Doppler studies.

5.8. What is the Significance of These Findings for the Treatment of Colorectal Liver Metastases?

Care must be used when drawing inferences from animal model studies and applying them directly to a clinical situation. Apart from the possibility of different responses between species, there are differences in tumour type, endothelial differences and a size difference of the tumours which in humans are physically much larger (and often necrotic) and require diffusion of molecules over greater distances. Nevertheless from these studies it would appear that a prolonged infusion of the vasoconstrictor peptides endothelin I and angiotensin II may have a beneficial effect in enhancing the proportion of hepatic arterial blood flow to tumour. A decrease in tumour perfusion may decrease 5FU uptake although by decreasing liver parenchymal flow to a greater extent, the cytotoxic dose can be increased and higher doses delivered to the tumour. The close correlation demonstrated between the TNR for blood flow and that for 5FU uptake is likely to hold true for human tumours within the size range tested. This relationship is important and underpins the strategy of vasoactive manipulation.

In these studies the magnitude of change appears to suggest that up to a doubling of the TNR may be achievable. Other studies looking at the short term effects of vasoconstrictors have also shown similar or greater degree of change although have predominantly looked at bolus administration of vasoactive agent rather than infusion.

There is evidence to say that a doubling of TNR, if accompanied by an increased delivery of drug to the tumour, may lead to an increased tumour response (Hyrmuk et al. 1987). Whether this change would be of sufficient magnitude to be a clinical benefit to the patient is unknown. However it must be noted that despite vasoactive manipulation the tumours studied here remained hypovascular with respect to the surrounding liver parenchyma. Therefore the dose limiting factor for regional chemotherapy is still likely to be hepatic parenchymal toxicity. If as Ackerman suggests there is a redistribution of blood flow within the tumour then responses may possibly be enhanced because of a more uniform delivery rather than merely an increased delivery of cytotoxic agent.

There are few studies looking at the actual tumour to liver blood flow ratios in human colorectal metastases. Sigurdson et al. (1986) showed TNRs for blood flow (as demonstrated by regional $^{99m}$Tc-macroaggregated albumin injection) of 0.79 +/- 0.99 and a TNR for the uptake of radiolabelled F UdR of 0.43 +/- 0.36. If these uptake ratios are a true representation of the TNR found in human metastases, then a doubling of this value, as was achieved in the work in this thesis, would increase the blood flow to a point where the tumour was better perfused than the liver. If this is achievable in the clinical
setting, then it may be possible to increase the cytotoxic dose significantly to increase the tumouricidal effect of the chemotherapy without increasing the hepatic toxicity.
5.9. Conclusions

Laser Doppler studies demonstrated that the infusion of a vasoactive agent was accompanied by a complex response in tumour and liver which varied with the agent used. This response varied with time and therefore measurements of blood flow that do not take this temporal change into account may be misleading.

The vasodilator CGRP did not increase tumour or liver perfusion although caused a fall in vascular resistance. All the vasoconstrictors investigated, decreased liver parenchymal and tumour perfusion. The agent with the most prolonged effect on liver parenchymal flow was endothelin I while the other agents seemed to be subject to a tachyphylaxis effect in which the vasoconstrictor effect diminished despite continued infusion of the vasoconstrictor agent. This effect is likely to be due to the nitric oxide release.

Both liver parenchyma and tumour reacted similarly to vasoactive infusion although the decrease in perfusion in response to a vasoconstrictor agent was more marked in liver parenchyma than tumour leading to a rise in the tumour to normal ratio.

There appeared to be a close linear relationship between the tumour to liver blood flow and 5FU uptake ratios across a wide range of tumour to normal ratios, although this relationship appeared less close after vasoactive manipulation.

It would appear that by giving a prolonged vasoconstrictor infusion, it is possible to nearly double the tumour to normal blood flow and 5FU uptake ratios.
6. Future Studies

The endpoint measured in these studies was the uptake of 5FU into tumour and liver. However, it is not possible to say from this what the tumouricidal effect of such an increased uptake would be. 5-fluorouracil has catabolic and anabolic pathways of metabolism. It is thought that the cytotoxic components are mainly produced by anabolic metabolism and it may therefore be valuable to establish how an increase in the TNR of 5FU was reflected by a change in the proportion of these two pathways. Alternative endpoints for measurement might therefore be the detection of catabolic products by NMR (deBrauw et al. 1991). Other studies have also looked at the inhibition of thymidylate synthetase (Houghton et al. 1981) or the overall effect of the drug on tumour growth (Bloom et al. 1987). All of these approaches are valid to study and may also provide valuable information on whether vasoactive manipulation can increase the tumouricidal properties of any one cytotoxic agent.

This study has shown that vasoactive manipulation has the potential to improve tumour chemotherapy response by reducing parenchymal cytotoxic dose and thus increasing the proportion reaching tumour. Studies which are required as a continuation of this work include:

- A study in an animal tumour model over a longer period of time (days) to assess the effects of intermittent or continuous infusion of a longer acting vasoconstrictor such as endothelin I on the hepatic parenchyma. L-NAME may be used to minimise the diminution of vasoconstrictor effect with prolonged vasoconstrictor infusion.

- Animal studies to assess the potential for an increase in the tumour drug uptake following long term vasoactive infusion.

- Clinical studies assessing the effect of regional vasoactive infusion on blood pressure, tumour and liver blood flow and tumour response in patients with colorectal liver metastases.

A protocol for this clinical study has already commenced. Patients with hepatic artery pumps for the treatment of colorectal hepatic metastases will be studied as there is access to the hepatic arterial system for vasoactive infusion and injection of the blood flow tracer. The isotope used to label the Cu-PTSM will be the positron emitting $^{62}$Cu which can be generated on site using a zinc copper radionuclide generator. The half life of $^{62}$Cu is 9.7 minutes which enables repeat measurements to be made. Measurement will be made using a Positron Emission Tomography (PET). Permission has been obtained to study the effects of infusion of angiotensin II although if the method is successful it will
be extended to include other compounds. If promising this work could be extended to
the study of drug uptake using either [F-18]-5FU or [F18]-FUDR combined with PET
imaging. Although the half life is longer and less measurements can be taken than for
blood flow, it will then be possible to investigate the effects of vasoactive manipulation in
a human study using repeated scans to measure the changes in blood flow and cytotoxic
uptake over a prolonged vasoactive infusion.
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