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Review paper

In vivo monitoring of fluoropyrimidine metabolites: magnetic resonance spectroscopy in the evaluation of 5-fluorouracil

Michael PN Findlay and Martin O Leach

Cancer Research Campaign Clinical Magnetic Resonance Research Group and Department of Medicine, Institute of Cancer Research and The Royal Marsden Hospital, Downs Road, Sutton, Surrey SM2 5PT, UK. Tel: (+44) 81 642 6011; Fax: (+44) 81 643 3812.

Since 5-fluorouracil (5-FU) was synthesized in the late 1950s it has become an important component of many anticancer treatment regimens. The increasing volume of literature accumulating about this drug is evidence that the optimal administration schedule and its combination with modulators has yet to be determined. Much of the investigation of 5-FU, particularly in the clinical setting, has been in the development of administration schedules based on plasma pharmacokinetic data. Particularly with the development of modulators of 5-FU, investigators are looking more closely at its intracellular tissue pharmacology and metabolism. To study the tissue metabolism of 5-FU (and other drugs), patients often have to be willing to undergo invasive procedures, sometimes with significant discomfort, usually with little direct benefit to their management. The ability to conduct an investigation of the cellular effects of a drug in both tumor and normal tissue non-invasively will not only be more acceptable to patients, resulting in better compliance to protocols, but will give information about the *in situ* tissue which is not subject to the problems of invasive sampling techniques. Magnetic resonance spectroscopy is a non-invasive technique that has recently started to show potential in the area of investigating 5-FU metabolism and its impact on tumor and patient outcome. Further development of this method may ultimately have an impact on the investigation of any new anticancer agent.

Key words: Cancer, 5-fluorouracil, *in vivo* ^{19}F magnetic resonance spectroscopy.

Introduction

Nuclear magnetic resonance (NMR) was first demonstrated in 1937 by Lazarew and Schubnikow; however, it was not until 1966, with the development of Fourier transform NMR by Ernst and Anderson,[1] that the technique became available to biochemists. Further development of magnet technology led to NMR of intact biological tissues, which then progressed independently to imaging and spectroscopy. The first whole body image was demonstrated in 1977 while the first clinical demonstration of *in vivo* spectroscopy occurred in 1981.[2] Since then the development of imaging and spectroscopy have converged with the intention of a wider application of these two similar techniques. Following the introduction of NMR into the clinic the nomenclature was modified slightly to remove the word 'nuclear' and thus any connotations of radioactivity. Thus much of the basic physics and *in vitro* biological literature refers to NMR while clinical reference to this technique is to MRS (MR spectroscopy) or MRI (MR imaging).

MRS is possible with a variety of nuclei that exhibit magnetic properties, one of which is fluorine-19 (^{19}F). When this is substituted for a hydrogen atom in the pyrimidine ring of uracil, it forms 5-fluorouracil (5- FU), which exerts a cytotoxic antimetabolite effect in cells. 5-FU has now been widely used in cancer chemotherapy for over 30 years, yet its metabolism and mechanisms of action on target tissues are incompletely understood, thus further refinement of administration strategies is still desirable. The plasma pharmacokinetics of 5-FU generally do not accurately predict its antitumor effects,[3] whilst analysis of tumor biopsies for 5-FU is often not clinically feasible nor representative in a large heterogeneous tumor mass. ^{19}F MRS, however, is able to identify 5-FU and its metabolites in both tumors and normal tissues *in situ*, non-invasively obtaining kinetic and metabolic information in real time. Positron emission tomography (PET), another technique with considerable potential for monitoring the tissue distribution of drugs, can provide more sensitive detection of the short half-life

Correspondence to MO Leach

(110 min) positron emitting ^{18}F isotope when administered as a component of 5-FU. However in contrast to ^{19}F MRS, ^{18}F PET cannot separate 5-FU from its metabolites. The short half-life of ^{18}F further limits studies of long-term infusional metabolism.

This article will review the *in vivo* ^{19}F MRS literature after providing the reader with a basic introduction to the principles of NMR and a review of the relevant 5-FU pharmacology. The use of ^{19}F MRS for investigating the normal tissue and tumor metabolism of 5-FU, and its modulation will be discussed both from a preclinical and clinical perspective.

Basic principles of NMR

The aim of this discussion is to provide the reader with sufficient insight into the principles of NMR to enable an understanding of its biological applications. The discussion is not designed to be comprehensive as there are several excellent reviews elsewhere.[4–6] All nuclei are positively charged but only those with an odd number of neutrons or protons demonstrate a net spin. The combination of charge and spin results in a magnetic moment which is specific to that nucleus. The spin of a nucleus is a vector composed of the sum of the spins of the individual neutrons and protons comprising the nucleus. In the absence of an external magnetic field, the magnetic moments of these nuclei in a sample will have random orientations and thus have no resultant net magnetic moment. However, if the sample is placed in a magnetic field, the magnetic moments will tend to align themselves with or against the direction of the magnetic field. The nuclei used widely in biological NMR, ^{1}H, ^{31}P, ^{13}C and ^{19}F, have a net spin of $\frac{1}{2}$, such that the magnetic moment of the nuclei will be oriented either in the direction (spin up) or opposite the direction (spin down) of the external field. The spin up and spin down states represent two energy levels of the nucleus, low (with the field direction) or high (opposite the field direction), and a transition between the two states can be induced by radiofrequency signals at the Larmor frequency, having an energy equal to the difference between these two energy levels. This energy is small and proportional to the strength of the external magnetic field. The net sample magnetization is the very small difference between the populations of the spin up and spin down states. For this reason magnetic resonance is a weak phenomenon, utilizing the signal only from some three in every million nuclei.

To begin to illustrate the behavior of magnetic nuclei in an external field the example of a single proton will be used. Initially the net magnetic moment (**m**) of this proton is oriented in the same direction as the external magnetic field (**Bo**), which by convention is labeled as the z (or vertical) axis and is represented by an arrow in Figure 1. If an oscillating magnetic field of appropriate frequency is applied perpendicular to this vertically oriented magnetic moment then there will be precession about the equilibrium position. Precession can be illustrated by imagining the arrow representing the magnetic moment tipping away from the vertical and describing an ever increasing angle, while continuing to rotate—effectively spiralling down into the xy plane around the z axis. For the sake of clarity, Figure 1 omits the rotational motion—describing behavior as viewed by an observer rotating at the Larmor precession frequency. The net result is that the arrow tips downwards towards the horizontal plane. The horizontal component of the magnetic moment will continue to increase until it equals **m**, thus at that time there will be no magnetic moment in the vertical or z axis direction (the direction of **Bo**). The angle at which the moment is tipped away from the vertical depends on the amplitude and duration of the horizontally applied oscillating magnetic field. As the frequencies of these oscillating fields are in the radiofrequency region of the spectrum (10^6 cycles/s or MHz), they are referred to as RF pulses. When the RF pulse is switched off the magnetic moment will gradually return to the original equilibrium position along the z direction. The horizontal component of the magnetic moment will induce a current in a receiving coil providing the NMR signal which is the basis of the NMR experiment.

At this point it is convenient to consider a collection of identical nuclei in an external magnetic field (**Bo**). The sum of the magnetic moments of these nuclei will give a net moment, **M**, in the z axis. As with a single proton, if a suitable RF pulse tips this moment into the horizontal (xy) plane, the net moment will precess in that plane. In practice, however, the individual nuclei contributing to the summed moment, **M**, will not precess at the same frequency for two reasons. Firstly, the external magnetic field is not perfectly homogeneous throughout the sample resulting in minor variations in its strength. Secondly, many molecules, particularly dipolar water molecules, exhibit a weak magnetic field. The z component of this field will fluctuate in strength affecting neighboring nuclei. Both these factors will alter the Larmor frequency of individual nuclei, eventually resulting in the direction of the

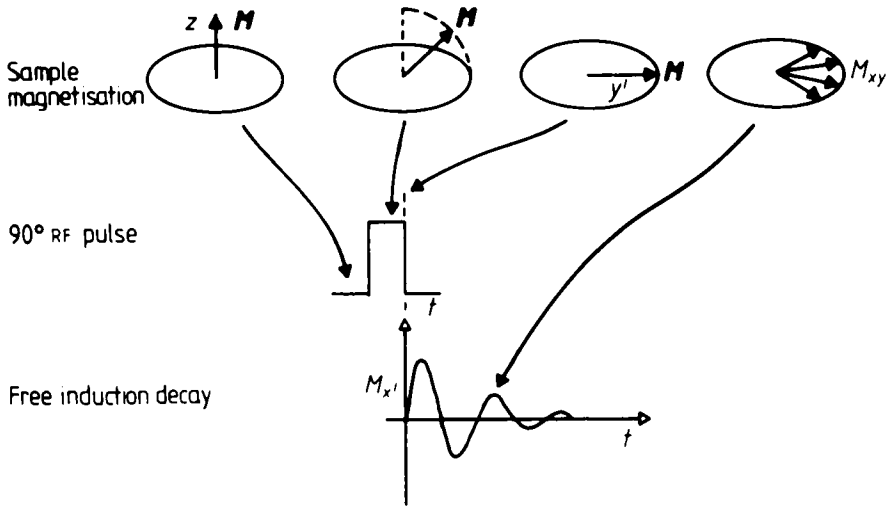

Figure 1. The sequence of events illustrating the effects of a 90° RF pulse at the Larmor frequency, on the magnetization **M** of a sample in an external magnetic field **Bo**, parallel to the *z* axis. The RF pulse tips **M** away from the *z* axis while continuing to rotate around it, thus describing a spiralling course down until **M** is completely in the *xy* plane. As the NMR signal is derived from the *xy* component of **M**, the FID signal will be of maximum amplitude at this point. Because **M** is a collection of individual nuclear components (**m**), which are subject to slight variations in **Bo** and to the effects of surrounding molecular fields, these components will all rotate in the *xy* plane at different rates, ultimately dephasing and cancelling the net moment **Mxy** to zero. This results in an exponential reduction in the envelope of the FID signal, the rate being governed by T_2 decay and by additional dephasing due to sample susceptibility. During this decay process in the *xy* plane the sample magnetisation **M** simultaneously returns to the equilibrium position in the *z* axis due to the continuing effect of **Bo**. The time taken to return to this equilibrium, known as the T_1 time, is longer than the T_2 time. Figure reproduced with permission.[5]

individual nuclear moments being completely dephased and evenly distributed. As a result the horizontal component of **M** returns to zero. Remembering that the measured NMR signal results from the horizontal component of **M**, following an RF pulse, the envelope of the signal will show an exponential reduction as the horizontal components of **M** dephase and gradually cancel each other out. The signal is known as the free induction decay (FID). The exponential decay of this signal is a result of both T_2 relaxation processes and additional dephasing due to magnetic field inhomogeneities. As the name implies the FID signal is the result of progressive phase incoherence (in the horizontal plane), in the absence of the RF pulse, with resultant decay in the signal. The vertical component of **M**, after initially dropping to zero following the 90° RF pulse, will also return to equilibrium (i.e. with **M** aligned with **Bo**). This return to equilibrium is slower than the development of complete phase incoherence of the horizontal components. Again this decay is exponential characterised by a constant T_1 (or T_1 relaxation).

Different elements or isotopes have characteristic Larmor precession frequencies, determined by their gyromagnetic ratio and the magnetic field strength. At 1.5 Tesla, the field strength commonly used for *in vivo* clinical spectroscopy, the resonant frequencies of ^1H, ^{31}P and ^{19}F are about 64, 26 and 59 MHz, respectively. Different molecular forms of a particular isotope have characteristic small 'chemical shifts' in frequency compared with a reference frequency. This chemical shift is a result of the molecular electron cloud generating a small induced screening field that modifies the magnetic field experienced by the nucleus. By measuring the different frequencies excited by the NMR experiment, the different molecular forms of an isotope can be distinguished and monitored.

The FID signal observed in an NMR measurement is a variation in signal amplitude with time. Whilst the signal may be comprised of different frequencies these cannot be readily distinguished; however, the mathematical operation, known as a Fourier transform, converts this time signal to a frequency spectrum. In order to standardize the observed resonant frequencies of various nuclei at different external magnetic field strengths a calculation is performed using the formula:

$$\text{chemical shift} = \frac{10^6\,[\text{frequency of molecule} - \text{reference frequency}]}{[\text{reference frequency}]}$$

Because the difference in the numerator will be in Hz and the denominator is MHz the standardized frequency will have no unit but is expressed as parts per million (p.p.m.) because of the convention to multiply the value by 10^6 to obtain a sensible number. This value is referred to as the chemical shift. The example spectra included in this review are drawn from published work where authors have used different reference frequencies, depending upon the equipment and experiment. Therefore, although the relative separation of peaks (in p.p.m. is constant, the absolute frequencies are arbitrary. The most easily detected signal is that from ^1H in NMR. The NMR sensitivity of the other nuclei can be expressed relative to the ^1H sensitivity. The NMR sensitivity of ^{19}F is 0.83, nearly that of ^1H (1.0), and greater than ^{31}P (0.066) and ^{13}C (0.016). Where the natural isotopic abundance is less than 100% the sensitivity is further reduced. ^{19}F is the naturally abundant form of fluorine and the NMR visible levels of fluorine in the body are very low. This makes MRS an attractive method for monitoring injected ^{19}F-compounds such as the anticancer fluoropyrimidines.

Anticancer fluoropyrimidines

With its synthesis by Heidelberger and colleagues[7] in the 1950s, 5-FU became the first rationally designed anticancer drug to have a role in the clinic. Since then it has become the most widely used cytotoxic agent with significant roles in the treatment of most gastrointestinal malignancies, squamous tumors of the head and neck, and adenocarcinoma of the breast. Because it remains the only drug with significant activity in colorectal adenocarcinoma, much of the investigational work done with 5-FU has been in patients with this tumor type. Despite over three decades of experience, however, there is still much to be learnt about its mechanism of action and optimal delivery. 5-FU is derived from the naturally occurring pyrimidine uracil, by substituting the hydrogen at the 5-carbon position in the pyrimidine ring, with a fluorine atom. It is this fluorine substitution that has made 5-FU and its deoxyribonucleoside derivative 5-fluorodeoxyuridine (FdUrd), of interest to investigators in the field of MRS. The pharmacology and metabolism of 5-FU and its modulators are briefly reviewed as a background to the later discussion of the MRS experience. For further information the reader is referred to other reviews on this specific topic.[8,9]

Metabolism of 5-FU

The activation or anabolism of 5-FU can occur via two main pathways to form either ribonucleotides or deoxyribonucleotides (see Figure 2). Formation of ribonucleotides can occur by two different pathways. The first is by conversion of 5-FU to fluorouridine monophosphate (FUMP) after addition of a ribose phosphate [donated by phosphoribosylpyrophosphate (PRPP)] which is catalyzed by orotic acid phosphoribosyltransferase (OPRTase). The other is by conversion via a two-step path, initially to fluorouridine (FUrd) via uridine phosphorylase and then by uridine kinase to form FUMP. Although allopurinol inhibition of OPRTase reduces toxicity to bone marrow and gastrointestinal mucosa,[10] suggesting that this pathway is important in the normal tissue effects of 5-FU, there is no clear evidence that one or the other pathway is specific for antitumor activity. FUMP is further metabolized into fluorouridine diphosphate (FUDP) and triphosphate (FUTP) by uridine monophosphate (UMP) kinase and uridine diphosphate (UDP) kinase, respectively. Ultimately FUTP is misincorporated into RNA by RNA polymerase.

The second main activating pathway for 5-FU is the formation of deoxyribonucleotides. 5-FU is converted to FdUrd by thymidine phosphorylase after which thymidine kinase (TK) converts it to fluorodeoxyuridine monophosphate (FdUMP). FdUMP can also be formed from FUDP in the RNA directed pathway which, after conversion to FdUDP by ribonucleotide reductase, is dephosphorylated. From FdUMP is produced FdUDP and FdUTP by pyrimidine monophosphate and triphosphate kinases, respectively. FdUTP is then misincorporated into DNA by DNA polymerase. The more important effect of the deoxyribonucleotide pathway, however, is the effect of FdUMP on thymidylate (thymidine monophosphate) synthase (TS). The fluorine substitution enables binding to TS but the reaction does not proceed to the formation of thymidine monophosphate, thus a stable binary complex is formed, effectively blocking the enzyme. This complex becomes even more stable in the presence of excess 5,10-methylene tetrahydrofolate (CH_2THF) thus forming a ternary complex. Much of the current research into 5-FU cytotoxicity is directed at altering its schedule of administration and its combination with various modulators to optimize one or more of these different mechanisms of action.

Although catabolism of 5-FU occurs in a variety of tissues as a result of the enzyme dihydropyrimidine dehydrogenase (DHPD), the most important site of

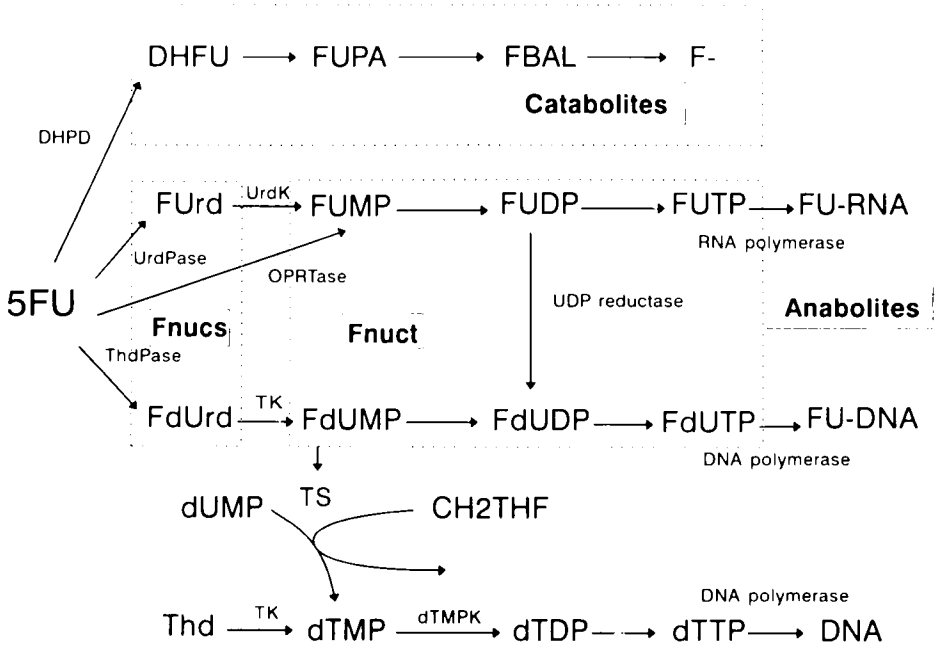

Figure 2. 5-FU metabolism. The various routes by which 5-FU is metabolized: (i) by catabolism to non-cytotoxic components DHFU, FUPA, FBAL and F^-; (ii) anabolism to FUMP with eventual incorporation into RNA; (iii) anabolism to FdUMP with inhibitory effects on enzyme TS; and (iv) incorporation into DNA. Abbreviations: DHFU, dihydrofluorouracil; FUPA fluoro-ureido propionic acid; FBAL, fluoro-β-alanine; DHPD, dihydropyrimidine dehydrogenase; ThdPase, thymidine phosphosylase; UrdPase, uridine phosphosylase; TK, thymidine kinase; UrdK, uridine kinase; OPRTase, orotate phosphoribosyltransferase; FUMP, fluorouridine monophosphate; FUDP, fluorouridine diphosphate; FUTP, fluorouridine triphosphate; FdUMP, fluorodeoxyuridine monophosphate; FdUDP, fluorodeoxyuridine diphosphate; FdUTP, fluorodeoxyuridine triphosphate; dUMP, deoxyuridine monophosphate; TS, thymidylate synthase; CH_2THF, methylene tetrahydrofolate; dTMP, deoxythymidine monophosphate; dTMPK, dTMP kinase; dTDP, deoxythymidine diphosphate; dTTP, deoxythymidine triphosphate.

inactivation is in the liver. This enzyme, which becomes saturated above 5-FU concentrations of 50 μM, catalyzes formation of dihydrofluorouracil (DHFU).[9] Further catabolism results in the formation of fluoroureidopropionic acid (FUPA) which in turn forms fluoro-β-alanine (FBAL) then carboxy-fluoro-β-alanine (CFBAL) and ultimately fluoride (F^-)

^{19}F MRS appearances of 5-FU metabolites

In NMR terms ^{19}F has a wide chemical shift range, which means that different molecular forms will have resonant frequencies covering a wide range relative to its natural frequency. Despite this wide chemical shift there are still problems with specificity. MRS studies can be performed in a variety of samples *ex vivo* (or *in vitro*) and in living systems *in vivo*. The quality of the spectrum obtained from *ex vivo* analysis of an extracted biological sample is superior to similar analysis of non-extracted plasma, urine or other tissues (see Figure 3; showing *in vivo* and non-extracted *ex vivo* spectra). The reason for this is that the extraction technique removes most of the proteins and their associated electron clouds, reducing the effect which they have on the local homogeneity of **Bo**. This results in a signal with a narrow peak width which is more easily separable from peaks with closely similar peak frequencies. MRS of plasma and non-extracted tissues is associated with an increased line width, although urine is generally free of a significant protein content, so peaks are usually well defined and narrow (see Figure 4). Animal and patient studies are further complicated by the need for a magnet bore large enough to accommodate the subject. The increased bore is associated with greater variations in the achievable external magnetic field homogeneity even before the subject is introduced. This, in addition to the local inhomogeneities of **Bo** induced by the electron cloud variations in the subject, leads to further widening of the peaks. Although the aim is to use this technique *in vivo*, because of these increases in magnetic field inhomogeneities, specificity is reduced. Figure 5 shows the position of the frequencies of the 5-FU metabolites that have been

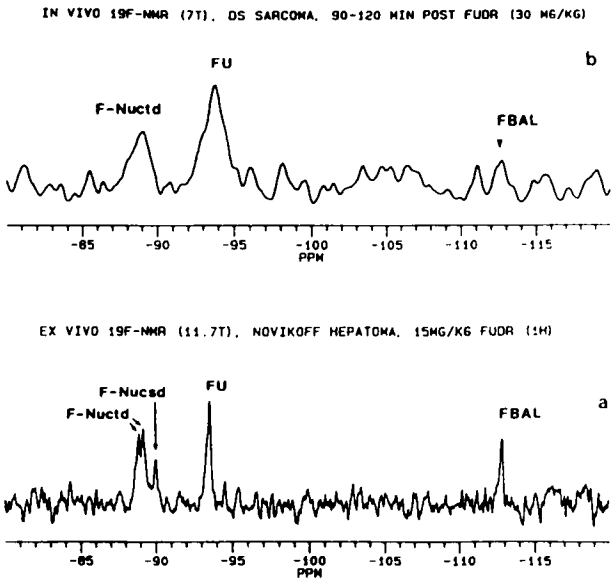

Figure 3. Examples of ^{19}F MRS studies of experimental tumors following FdUrd (FUDR) administration: (a) *ex vivo* at 11.7 T study of an excised tumor at 11.7 T; (b) *in vivo* study of a tumor in the hind leg of a Wistar rat at 7 T.[75] Absolute frequencies are arbitrary. Reproduced with permission.

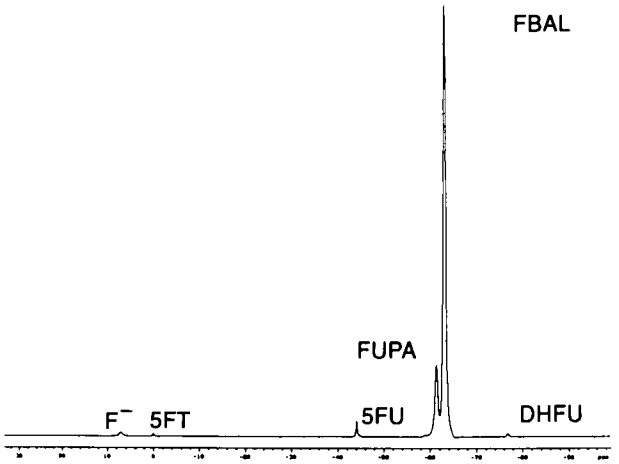

Figure 4. ^{19}F MRS examination (11.7 Tesla magnetic field) of a sample of urine from a patient receiving 5-FU as a continuous i.v. infusion (300 mg/m^2/day). The urine was spiked with a known quantity of 5-fluorotryptophan (5-FT), to enable quantitation of the metabolites. Absolute frequencies are arbitrary. Analysis performed by R Maxwell and P McSheehy.

detected using *in vitro* ^{19}F MRS in this case with 5-FU as the reference frequency at 0 p.p.m.[11]

The catabolites are readily distinguished from 5-FU with chemical shifts as follows; FUPA, −17.2 p.p.m.; FBAL, −19 p.p.m.; FUH2, −32.5 p.p.m.; and F^-, +50 p.p.m. compared with 5-FU. The anabolites are more difficult to distinguish from each other as they give signals in only two distinct chemical shift regions: the ribose and 2′-deoxyribose analogs of 5-FU give signals at 3.8 p.p.m. (referred to as Fnucs) and all the phosphorylated ribose and deoxyribose analogs (FUMP, FUDP, FUTP, FdUMP, FdUDP and FdUTP) have ^{19}F resonances at about 5 p.p.m. from 5-FU (referred as Fnuct) i.e. *in vivo* ^{19}F NMR cannot distinguish between the two cytotoxic metabolites, FdUMP and FUTP.[12] As the distinction between Fnuc and Fnuct is frequently not made by some investigators they are referred to here collectively as Fnuc.

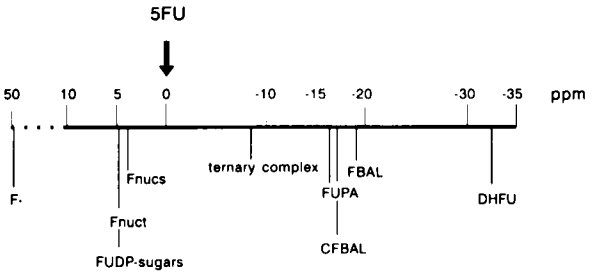

Figure 5. The chemical shifts of the fluoropyrimidines and their metabolites at pH 7, expressed as parts per million (p.p.m.), using the 5-FU frequency as the reference value. (Modified after McSheehy and Griffiths[11] with permission.)

A measure of the amount of ternary complex formed between FdUMP and TS in relation to the amount of free TS may provide a true indicator of the cell's response to FdUMP. This ternary complex, studied by *in vitro* NMR, was shown to have a chemical shift 12.4 p.p.m. upfield from FdUMP [13] and so can be resolved from the Fnuc and 5-FU peaks. A broad peak was observed in a human pancreatic cell line following incubation of the cells with high concentrations of 5′FdUrd by Malet-Martino *et al.*[14] The detection of a peak that may correspond to ternary complex formation, indicates that the complex may be present in sufficiently large quantities to be identified with MRS; however, this peak has not been reported by other workers and may only occur in that particular cell line. Other workers have reported levels of TS in the nM range[15,16] whereas the amount of FdUMP–TS complex found by Malet-Martino *et al.* [14] must have been at least 0.1–1.0 mM.

Ribonucleotides are generally present in cells in concentrations about two orders of magnitude higher than their deoxyribonucleotide counterparts.[17] This would suggest that the majority of any NMR detectable Fnuc *in vivo*, would consist of FUMP, FUDP and FUTP. McSheehy *et al.*[12] found in the Walker carcinosarcoma model that 50% of the Fnuc

signal was due to FUTP. The significance of these results is that NMR is most likely to be able to assess the extent of tumor toxicity mediated through FUTP.

Cellular transport

5-FU appears to use the same transport system as uracil; however, as there is no evidence of temperature or energy dependence, it does not appear to be an active process. Despite this, 5-FU will accumulate in human tumors at up to 10 times the plasma 5-FU level between 4 and 48 h after i.v. bolus administration.[18] This accumulation may be related to the pH-dependent transmembrane passage of the ionized form of 5-FU (the pK of 5-FU is 8.0).[19] FdUrd, unlike 5-FU, uses the nucleoside transport system. This step is rapid and not rate-limiting, with intracellular phosphorylation the critical step in further nucleotide formation.[20]

Pharmacology

5-FU has been administered using a variety of routes—oral, intravenous (systemic and portal), intra-arterial and intraperitoneal. Oral administration results in peak plasma concentrations within 1 h; however, bioavailability can be erratic and dependent on the dose administered, suggesting saturable first pass liver metabolism.[21] This is reflected in the inferior antitumor activity seen in studies comparing this to the intravenous route.[22] For this reason the oral route is no longer used while the intravenous route is favored. Following intravenous bolus injection of 5-FU at conventional doses (400–600 mg/m^2) the primary elimination half-life shows interpatient variability in the range of 10–20 min.[23,24] The clearance of 5-FU reduces with increasing dose of administration suggesting saturation of anabolic or catabolic metabolism.[24]

An estimated 90% of 5-FU elimination is through metabolism while less than 5% is excreted via the kidney.[25] However, only 50% of metabolism occurs in the liver while metabolism at extrahepatic sites such as kidney and lung have been reported.[23] The initial step in the catabolism of 5-FU—reduction of the pyrimidine ring by DHPD to form 5′,6′-dihydrofluorouracil (DHFU), which is saturable above 50 μM—may explain the dose-dependent pharmacokinetics of 5-FU.[9]

There is an increasing interest in altering the schedule of i.v. administration of 5-FU from bolus to short- or long-term infusions. Cell line studies have shown greater inhibition of colony formation with prolonged exposure to 5-FU.[14] Clinical studies have shown less myelosuppression despite the development of equivalent mucositis in patients treated with 5-FU at 1100 mg/m^2/day for 4 days when compared with a bolus schedule.[21] Seifert *et al.*[26] randomized patients with colorectal cancer to either 5-FU (450 mg/m^2 days 1–5 q 28 days) or 5-FU 1100 mg/m^2/day, day 1–5 infusion. The tumor response rate (44 versus 22%) and patient survival (8 versus 2 months) favored infusional 5-FU. Another clinical trial in patients with advanced colorectal cancers compared the IV bolus 5-FU (450 mg/m^2 days 1–5) schedule to i.v. protracted infusion (300 mg/m^2/day).[27] The results show less myelosuppression and stomatitis but increased erythroderma of the hands and feet in the infusion arm. Moreover, the tumor response rate was higher in the infusion arm (30 versus 7%). These studies suggest the mechanisms of 5-FU induced toxicity may differ in different normal tissues and that schedule modifications may improve anti-tumor activity.

Hepatic arterial infusions of 5-FU and FdUrd have been used in an attempt to enhance delivery of drug to tumors while minimizing systemic exposure. Available data suggest that rapid i.a. infusion of 5-FU (1g/m^2/day) results in 20–60% hepatic extraction and high systemic availability while slower rates of infusion (780 mg/m^2/day) result in hepatic extraction of 90% and low systemic availability.[28,29] The concept of regional i.a. treatment which also allows therapeutic systemic exposure is attractive; however, to date the results of randomised studies are mixed.[30] Intrahepatic infusions using the portal vein have been used as adjuvant treatment for colon cancer. The assumption is that early and small volume metastases to the liver will derive blood supply via this route as opposed to larger lesions which will be supplied from the hepatic artery supply. Again the benefit of this manoeuver is yet to be consistently demonstrated. Intraperitoneal infusions of 5-FU have the advantage of high drug exposure to the peritoneum and liver (via the portal vein) relative to systemic exposure. A 2–3 log difference between peritoneal and systemic plasma 5-FU concentrations has been documented; however, when the dose of i.p. 5-FU was escalated, clearance decreased and systemic exposure rose.[31]

Modulation of 5-FU metabolism

A variety of agents, referred to here as modulators, have been used in conjunction with 5-FU with the intention of either enhancing tumor cytotoxicity or

reducing normal tissue effects of 5-FU. Some of these modulators, those that have been studied using ^{19}F MRS, will be discussed here; however more comprehensive reviews of 5-FU pharmacology and modulation are found elsewhere.[8,9]

Leucovorin. Maintaining high intracellular levels of reduced folates will minimize their dissociation from the ternary complex and thus maximize the TS directed effects of 5-FU. Folinic acid (leucovorin; N^5-formyltetrahydrofolate; CHO–FH_4), following intravenous injection, is rapidly metabolized to 5-methyl-FH_4 (CH_3–FH_4), transported into the cell by the reduced folate carrier and subsequently polyglutamated by folylpolyglutamyl synthase. Ultimately, 5,10-methylene tetrahydrofolate (CH_2–FH_4), the cofactor for TS, is formed. There is now a large volume of clinical data regarding this modulator, particularly in colorectal cancer. This combination appears to show greater antitumor activity than 5-FU alone, however, the impact on survival in colorectal cancer patients is less clear.[32]

Methotrexate. Methotrexate has been combined with 5-FU in various schedules in an attempt to enhance cytotoxicity. When methotrexate is given prior to 5-FU it increases formation of FUTP by expanding the intracellular pool of PRPP thus enhancing conversion of 5-FU to FUMP by OPRTase.[33] However, through its inhibition of dihydrofolate reductase (DHFR), methotrexate prevents the conversion of dihydrofolate (FH_2) to CH_2–FH_4, required for TS-FdUMP stability. In addition the resultant increased intracellular dUMP competes with FdUMP for TS.[34] The reduction in reduced folates, however, is not thought to be complete and so these negative effects are outweighed by the enhancement of FUTP formation. Reversal of the sequence such that 5-FU is given before methotrexate results in an initial inhibition of thymidylate synthesis by FdUMP. The impact of the methotrexate induced block in the conversion of FH_2 to FH_4, which inhibits *de novo* purine synthesis, is then reduced because the 5-FU induced inhibition of thymidylate synthesis coincidentally results in a reduction in FH_2 levels.[34]

Interferon (IFN)-α. Despite a recent increase in interest in the use of IFN-α to modulate 5-FU metabolism, as yet no well defined mechanism has been agreed. Current evidence suggests IFN may: increase intracellular FdUMP levels,[35] perhaps by favoring its formation or interfering with its catabolism; impair thymidine transport and thymidine kinase activity;[36] prevent 5-FU induced upregulation of TS;[37] enhance incorporation of FdUTP into DNA.[38] Alterations in 5-FU pharmacokinetics with IFN-α have been documented although this may be schedule dependent.[39–41] Phase II studies suggest increased antitumor activity and reversal of 5-FU resistance;[42–45] however, preliminary reports of phase III studies reveal no significant impact on 5-FU activity.[46]

Thymidine. Thymidine, after being metabolized by thymidine phosphorylase to thymine, competes with 5-FU for the catabolic enzyme DHPD. This results in decreased 5-FU breakdown and clearance, thus increasing 5-FU toxicity. In addition, thymidine replenishes the thymidylate pools, bypassing the block in production induced by FdUMP. This and the negative feedback of dTTP on ribonucleotide reductase diminishing FdUMP formation from FUDP, effectively shifts the metabolism of 5-FU to the RNA directed pathway.[47,48] Clinical studies have to date confirmed a significant increase in 5-FU induced patient toxicity with thymidine addition.[49]

Allopurinol. Allopurinol, by inducing a rise in orotic acid levels, blocks conversion of 5-FU to FUMP by competing for the enzyme OPRTase. Preclinical models have found normal tissues but not tumor tissues dependent on this step for 5-FU induced effects. The rationale for clinical investigation was that blockade of 5-FU conversion to FUMP via OPRTase would save patient toxicity but the tumor would still be able to convert 5-FU, initially via uridine phosphorylase to FUrd then uridine kinase to FUMP. Clinical studies have reported mixed results with some showing an increase in tolerable 5-FU dose with allopurinol,[50] while others found increased neurotoxicity and an alteration in 5-FU half- life.[51] With no conclusive evidence of a maintained antitumor activity in patients when allopurinol is added to 5-FU systemically, the focus of this drug's use is as a mouthwash to minimize oral stomatitis thus reducing systemic exposure.[52] This particular example of 5-FU modulation is ideally suited to further investigation with *in vivo* ^{19}F MRS, both to examine the intra-tumoral effects and to study normal tissue effects, e.g. in the brain.

Uridine. The use of uridine to rescue the RNA directed effects of 5-FU is based on a rationale that there are differences in its uptake and activation in normal tissues as compared with tumor. The expanded UTP pool induced by uridine has been

associated with increased clearance of 5-FU from RNA and DNA in both normal and tumor cells. Because of a short half-life, uridine has been administered as an i.v. infusion and by mouth, showing evidence of reversal of 5-FU induced toxicity.[53,54] A recent clinical study in upper gastrointestinal tumors found uridine rescue enabled a 60% increase in the tolerated dose of 5-FU (modulated by methotrexate).[55] The initial protocol started the uracil 2 h after the 5-FU; however, no tumor responses were seen in the first 12 patients. Subsequently the 5-FU–uridine interval was increased to 24 h and responses were then seen without any reduction in normal tissue protection. This is an example of the type of clinical investigation where ^{19}F MRS could have more accurately defined the uridine salvage in tumor and normal tissue before formally testing the dose-intensity hypothesis.

Monitoring 5-FU in normal tissues with ^{19}F MRS

^{19}F MRS can increase the understanding of fluoropyrimidine tissue metabolism, its kinetics and the distribution of metabolites in patients. Ultimately this may assist in developing new scheduling strategies, in optimizing the use of modulators and individualizing treatment for a particular patient. The majority of current studies in this field have focused on monitoring the tumor metabolism of 5-FU. Most available normal tissue metabolism data, both preclinically and clinically, relate to the liver because of its primary role in fluoropyrimidine metabolism. There is, however, the potential to use this technique to examine tissues subject to specific 5-FU toxicities with the aim of identifying the offending metabolite and subsequently countering its action with a new strategy. Examples of this include the 5-FU related cerebellar toxicity which is the dose-limiting adverse effect in some 5-FU schedules and possibly the differentiation of coincidental ischemic heart disease from 5-FU cardiac effects which seldom respond to anything other than drug discontinuation.

Preclinical studies of fluoropyrimidine metabolism and kinetics

In 1984 Stevens *et al.*[56] published the results of a series of experiments examining 5-FU metabolism in the C57 mouse using *in vivo* ^{19}F MRS (Figure 6). After placement of a surface coil directly on the liver, an i.v. injection of 5-FU (30 mg/kg) was given and its metabolism monitored. A signal from FBAL was observed along with a second peak (initially reported as probably being FUH_2 but later identified as FUPA) appearing at 22 min and decaying over the subsequent 3 h. No 5-FU signal was seen at this dose but after injection of 180 mg/kg 5-FU, a 5-FU signal was seen initially and decayed thereafter. At the higher dose of 5-FU, signals from FBAL and FUPA were both seen to increase during the same time period that the 5-FU signal was reducing. No active anabolic metabolites of 5-FU (Fnuct, Fnucs) were seen in either experiment. Prior *et al.*[57] assessed liver metabolism in Wistar rats with ^{19}F MRS following i.v. bolus injections of 5-FU, 2'FdUrd, and two 5-FU prodrugs 5'FdUrd and *R,S*-1-(tetrahydro-2-furyl)-5-fluorouracil (Ftorafur) which work by first being converted to 5-FU. Following injection of 5-FU, signals from both 5-FU and FBAL were seen; however, after 2'FdUrd injection, 2'FdUrd was not visible after the first 2 min acquisition, although FBAL was observed. Injection of the prodrug 5'FdUrd produced signals corresponding to 5-FU and FBAL. While injection of Ftorafur resulted in an FBAL signal, no data was presented suggesting the signals from 5-FU or Ftorafur were visible. An activity time course was plotted using the natural log of the peak areas with time and from this a first order rate constant for elimination was calculated, enabling a drug half-life in the liver to be obtained. At a dose of 0.46 mmol/kg (60 mg/kg) 5-FU, the $t_{1/2} = 4.7 \pm 0.6$ min while at a dose of 0.92 mmol/kg (120 mg/kg) 5-FU the $t_{1/2} = 9.5 \pm 1.6$ min, consistent with saturable kinetics. Following a 0.46 mmol/kg dose of 5'FdUrd its

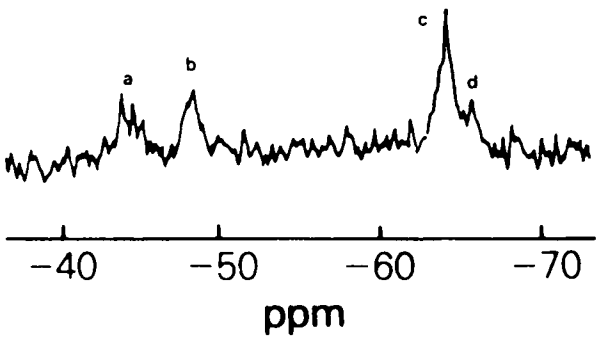

Figure 6. The first published example of a ^{19}F MR spectrum obtained *in vivo*.[56] Using a C57 mouse with a surface coil placed over the liver, the spectrum was collected 54 min after injection of 5-FU (30 mg/kg) with thymidine (180 mg/kg). Peak assignment as follows: (a) Fnuc, (b) 5-FU, (c) FUPA and (d) FBAL. Absolute frequencies are arbitrary. Figure reproduced with permission.

$t_{1/2}$ was 15.5±0.8 min which was significantly longer than the molar equivalent 5-FU dose. The FBAL production rate from 5-FU and 2'FdUrd appeared similar while that from 5'FdUrd was moderately delayed and that from Ftorafur was sustained for at least 500 min. These results suggested that the metabolism of 5'FdUrd and Ftorafur was limited by the rate of conversion to 5-FU, the resultant concentration of which was small as no 5-FU signal was seen. In addition to the metabolic observations, this study demonstrated that ^{19}F MRS can also provide kinetic data non-invasively.

Sijens *et al.*[58] studied C3H mice after i.p. injection of 5-FU. Using three different doses of 5-FU (65, 130 and 260 mg/kg), they demonstrated half-lives in liver of 23 ± 4, 28 ± 2 and 59 ± 7 min, respectively. At the two higher dose levels Fnuc were seen to accumulate for up to 205 min from the time of injection. FUPA also accumulated over 205 min at the highest dose, however; at the 130 and 65 mg/kg doses, it peaked after 65 and 45 min, respectively. The 5-FU kinetic data again demonstrated saturable kinetics with the rate limiting step of 5-FU metabolism to FUPA being the conversion of 5-FU to DHFU by DHPD, as there was no DHFU seen in any experiment. Similarly the efficiency of FUPA catabolism to FBAL increased with the smaller doses of 5-FU. An interesting observation in this study was of a small but significant reduction in liver pH (0.04–0.08 pH units) during the period of exposure to 5-FU. This was calculated from the observed reduction in the chemical shift difference between 5-FU and Fnuc based on a previously determined equation.[59] Although it has been previously shown *in vitro* that the chemical shift differences (relative to 5-FU) of many of the Fnuc signals obtained from 5-FU-substituted tRNA are smaller at lower pH,[60] the implications of this observation are still unclear. Determination of the differences between intracellular and extracellular pH changes in this setting would be helpful. These investigators[59] and others[61], have also examined tumor pH and its effect on 5-FU signal characteristics, with multi-nuclear MRS (see later section).

5-FU induced cardiac toxicity has been investigated by Lemaire *et al.*,[62] using isolated perfused rabbit hearts. The previously postulated toxicity mediated by fluoroacetate (FAC) formed by metabolism of FBAL was tested. The findings, however, suggested that FAC was formed, not from FBAL which was present in significant quantity in the heart, but from an impurity in the perfused 5-FU solution, and that the FAC was associated with increased cardiac effects. Urine collected in a parallel clinical study of 5-FU cardiac toxicity[63] revealed FAC was present both in patients with and without cardiac symptoms. Although modification of the 5-FU preparation protocol will reduce the presence of FAC, the observation that the urinary FAC excretion increased over the period of the 4–5 day 5-FU infusion suggests a possible schedule dependent variation in production. Discovery of an association between cardiac effects and the presence of FAC (or other metabolites), in any 5-FU schedule, could lead to the development of an *in vivo* method for monitoring this.

Preclinical studies of modulation of fluoropyrimidine metabolism in normal tissues

As mentioned in the earlier section on the biochemistry of fluoropyrimidines, their metabolism can be altered by agents to either enhance anti tumor activity or protect normal tissues. By studying fluoropyrimidine metabolism in the liver while under the influence of any of these modulating agents, ^{19}F MRS may be able to investigate the kinetics of the process. To date, however, few studies have set out to specifically look at the effects of modulation on normal tissue—particularly liver. Stevens *et al.*, [56] in their original report on *in vivo* ^{19}F MRS, were able to demonstrate that the addition of thymidine (180 mg/kg i.v.) to 5-FU (30 mg/kg i.v.) produced signals in the liver compatible with an increase in both 5-FU and Fnuc (Figure 6). Although the higher dose of 5-FU (180 mg/kg) alone does produce a larger 5-FU signal than 30 mg/kg 5-FU plus thymidine, it does not produce Fnuc (thymidine was not added to the higher 5-FU dose). In a more recent study,[64] the metabolism of 5-FU was examined in C3H mouse liver after i.p. injection of 5-FU ± thymidine. After 5-FU (65 and 130 mg/kg) administration, signals from 5-FU, FBAL and FUPA were detected, with Fnuc seen only at the higher dose. Pretreatment with thymidine (500 mg/kg i.p.) was associated with additional signals of Fnuct and Fnucs, but no FBAL. Thymidine pretreatment increases the 5-FU half-life in liver from 24 ± 2 to 126 ± 46 min at 65 mg/kg 5-FU dose and 28 ± 2 to 95 ± 22 min at 120 mg/kg 5-FU dose. Both these studies are consistent with a thymidine induced inhibition of 5-FU catabolism and enhancement of RNA directed anabolism (reviewed earlier). Using Wistar Kyoto rats, Harada *et al.*[65] found pretreatment with uracil (200 mg/kg i.p.) followed by i.v. 5-FU (100 mg/kg) resulted in a larger 5-FU signal

which had delayed clearance, reduced accumulation of FBAL and more Fnuc formation in liver, when compared to animals treated with 5-FU alone. From this data the uracil effect appears to be due to inhibition of 5-FU catabolism. Investigation of the effects of new and established modulators on the metabolism of 5-FU in liver using ^{19}F MRS will continue to improve the understanding of these interactions at the tissue level. Further application to the study of tissues that are known to suffer toxicity from 5-FU, e.g. the brain and heart, may enable investigation of intervention strategies that may ultimately enable further dose escalation.

Clinical studies of fluoropyrimidine metabolism and kinetics in normal tissue

5-FU metabolism was first observed *in vivo* in the liver of patients by Wolf *et al.*[66] from the University of Southern California in Los Angeles. After a 1.5 g short i.v. infusion (10–15 min), using a 15 cm diameter surface coil placed on the abdomen over the liver while the patient lay in a 1.5 T MR system, a 5-FU signal was seen initially which subsequently decayed while an FBAL signal increased (see Figure 7). Several reports from other institutions followed this study.[67–69] From Heidelberg in Germany, Semmler *et al.*[68] studied eight patients who received a bolus of 5-FU into the gastroduodenal artery to treat tumors of the liver. Using a 15 cm surface coil, signal was detected from the underlying liver. Both 5-FU and FBAL signals were seen in these patients, while in one an additional signal was seen at a frequency consistent with Fnucs and Fnuct. At that time, however, no spatial localization method was available so the signal detected came from both normal liver and tumor.

From the same group, Port *et al.*[70] monitoring using patients with liver metastases receiving short i.a. 5-FU infusions (10 min), applied resultant ^{19}F MRS data to a kinetic model. Without knowing the exact tissue being examined (i.e. normal liver or tumor or both) because of localization limitations, a liver 5-FU catabolism model was used. The 5-FU spectra and total catabolite (FBAL plus FUPA) spectra were found to fit a non-linear three compartment model. This method can provide absolute values for param-eters such as half-life and maximal velocity of metabolic conversion; however, because ^{19}F MRS only provides relative tissue concentrations (tissue concentration is proportional to peak area) other pharmacokinetic parameters are not absolute.

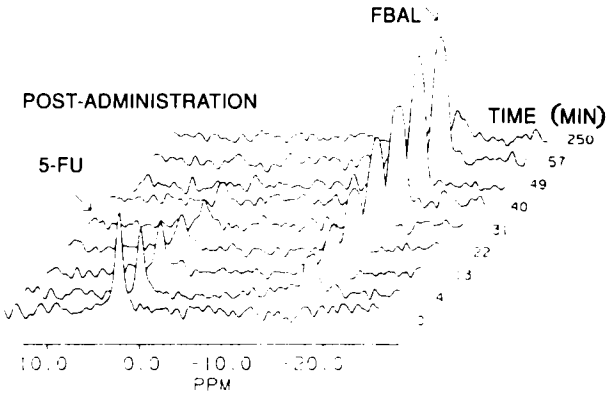

Figure 7. The first published example of a ^{19}F MR spectrum obtained from a patient *in vivo*.[66] Using a 1.5 Tesla MRI system and a surface coil placed over the patient's liver, a series of spectra were collected at intervals following i.v. injection of 5-FU and displayed as a three-dimensional stack-plot. Absolute frequencies are arbitrary. Reprinted from *Magnetic Resonance Imaging* (1987) with kind permission from Elsevier Science Ltd.

Further application of modeling to other infusion schedules will further increase the strength of the ^{19}F MRS data, particularly with improvements in localization.

Early work from our unit[67] was directed at examining the tissue pharmacokinetics of 5-FU in liver using ^{19}F MRS. Patients given i.v. 5-FU developed a corresponding signal in liver which in some cases decayed over 60 min. In contrast, intraperitoneal administration resulted in a delayed 5-FU signal which was still increasing at 60 min. The catabolite FBAL was seen to appear by 20 min after either route of administration. Because of the presence of 5-FU within the peritoneal cavity and its potential to lie between the 17 cm surface coil and the liver surface, contamination of the spectra was possible. For this reason a patient having i.p. 5-FU was studied using the 'fast rotating gradient selection' (FROGS) sequence[71] adapted for use with fluorine. This technique effectively suppressed signal from superficial areas (abdominal wall and 5-FU containing peritoneal space (see Figure 8). The results of this manipulation revealed that, in one case, intraperitoneal 5-FU was contaminating the detected liver signal. This was not a problem with the FBAL signal because its metabolism from 5-FU occurred only within the liver and not in the peritoneal cavity.

More recently our group has been investigating the monitoring of continuous ambulatory 5-FU infusions with ^{19}F MRS. We have studied 19 patients with normal livers every 2 weeks from the begin-

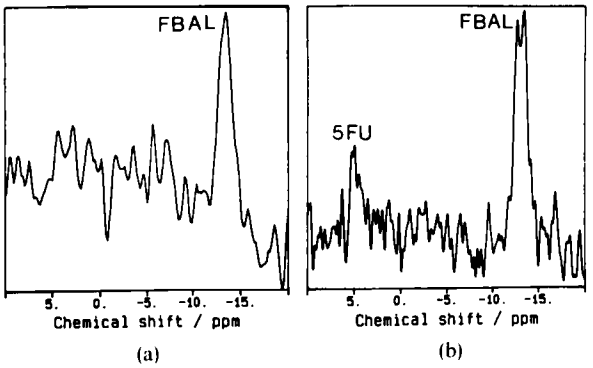

Figure 8. An example demonstrating the contamination of a ^{19}F MR spectrum by 5-FU lying within the intraperitoneal cavity, between the liver and the surface coil placed on the overlying abdominal wall (b). The FROGS technique used to collect data in (a) shows that 5-FU contamination can be suppressed. Figure reproduced with permission.[67] Absolute frequencies are arbitrary.

ning of a 5-FU infusion (300 mg/m^2/day) until chemotherapy related toxicity intervened. FBAL was repeatedly detectable in normal liver over the first 8 weeks of treatment. While there was significant interpatient variation there was an apparent plateau of FBAL signal intensity during this period. 5-FU was less frequently detected in liver than FBAL. No Fnuct or Fnucs have been seen in normal liver on this protocol. Preliminary data suggest that there is a relationship between liver 5-FU signal and 5-FU toxicity.

Honenberger *et al.*[69] studied 25 patients with colorectal liver metastases who received either i.v. or hepatic artery i.a. bolus or 16–24 h infusion of 5-FU, prior to surgical resection of these lesions. At the time of surgery normal liver tissue was excised and stored for *ex vivo* analysis in an 11.7 T system. In patients having continuous i.v. infusion 5-FU (550–700 mg/m^2) the only signal from the liver was of catabolites, similarly following i.a. infusion 5-FU (1 g/m^2) the catabolic signal accounted for almost all the metabolites; however, anabolites were seen in one patient. Following i.v. bolus 5-FU (550–700 mg/m^2), signals from 5-FU and metabolites were found to be 5- to 10-fold higher in liver tissue than in metastases, with two of 11 patients showing anabolites in liver as well. Bolus 5-FU (1 g/m^2) given i.a., resulted in anabolic signal in eight of nine liver samples representing over 75% of the total F signal. While comparisons of i.v. and i.a. routes are complicated by different administered doses of 5-FU, these results demonstrate a lower likelihood of developing 5-FU or Fnuc signals with infusions than with bolus treatment.

^{19}F-MRS has been used to examine blood and urine samples from patients during a 6 h infusion of 5'FdUrd.[72] Using this technique it was possible to identify the presence of FUPA, FBAL and F^-, establishing a catabolic fate similar to that of 5-FU. In addition, unlike 5- FU and 2'FdUrd, this study found 35–48% of 5'FdUrd was excreted unchanged in the urine. As the major toxicity of this drug is on the central nervous system, the investigators were able to examine the cerebrospinal fluid of six patients with this complication. Unfortunately, however, these studies revealed no signal.

There is, to date, no published clinical data on *in vivo* monitoring of alterations in normal tissue metabolism of 5-FU in response to modulators. Hull *et al.*[73] have examined the effects of methotrexate on 5-FU metabolism in patient plasma and urine samples, using *ex vivo* ^{19}F MRS. 5-FU catabolic products DHFU, FUPA, FBAL and F^- were all observed in plasma and urine. The methotrexate treated patients all had urinary alkalinization, under which conditions CFBAL was detected. There were, however, no differences in 5-FU catabolism detected in plasma or urine with methotrexate administration, nor was there any difference in catabolism between patients with responding tumors or unresponsive disease. The established mechanisms of methotrexate modulation, however, are intracellular and not known to affect plasma pharmacokinetics. *In vivo* study of the liver 5-FU–methotrexate interaction would be of interest. We have studied 10 patients with normal liver who, when refractory to a 5-FU infusion (300 mg/m^2/day), had IFN-α added to the 5-FU. Of these patients, three were seen to develop a 5-FU signal within 1 week of this, although no particular catabolite signal changes were noted.

Monitoring 5-FU metabolism in tumors with ^{19}F MRS

Until recently the only means of monitoring 5-FU (and other drug) metabolism in the tumors of patients has been by invasively obtaining tumor tissue for *ex vivo* analysis or by comparing plasma drug data to ultimate tumor outcome. Similarly, in order to have confidence that non-invasively obtained MRS observations are biologically relevant it is important to compare them to the effect of treatment on the tumor. This section will review both the pre clinical and clinical experience using *in vivo* ^{19}F MRS to monitor tumor metabolism of 5-FU.

Preclinical evaluation of tumor 5-FU metabolism

Evaluation of 5-FU metabolism *in vivo* using ^{19}F MRS has been conducted in a variety of preclinical animal models. Stevens *et al.*[56] implanted Lewis lung carcinomas subcutaneously in the sacral region of C57Bl/Cbi mice then studied the metabolism of 5-FU using a surface coil positioned over the tumors. 5-FU was injected intravenously at two doses (30 and 180 mg/kg) and spectra were acquired over 120 min. At the lower dose of 5-FU, a 5-FU signal was seen initially which decayed almost completely after 120 min; however, no catabolites were seen. This is in contrast to the liver metabolism studies (already discussed) where only catabolites were observed. At the higher dose of 5-FU, the tumor showed a significantly larger and prolonged 5-FU signal and at 112 min an Fnuc signal was also seen. Again, in comparison to liver, the tumor had more 5-FU signal, exclusively had Fnuc, but had no catabolites.

A subsequent study from the same group,[12] this time using Wistar rats with the Walker 256 carcinosarcoma subcutaneously implanted in one flank, compared the *in vivo* findings with *in vitro* ^{19}F MRS and HPLC studies of the freeze-clamped tumors. In addition, tumor regression was evaluated and correlated with these findings. Tumor regression was noted at a 5-FU dose of 50 mg/kg; however, weight loss was seen. No tumor reduction, but a reduced animal weight gain, was seen at 25 mg/kg dose. The spectroscopy studies used three dose levels of 5-FU (25, 50 and 120 mg/kg). *In vivo* evaluation was followed immediately by excision and freeze clamping of the tumors. Following i.v. injection of 25 mg/kg 5-FU (the dose producing no tumor reduction) very little Fnuc was seen, while after 50 mg/kg (the tumor reducing dose) and 120 mg/kg (a dose too toxic to the animal to have been tested for antitumor activity), increased Fnuc levels were observed. *In vitro* analysis of the tumor samples confirmed the presence of Fnuc and 5-FU. In addition it revealed an FBAL signal which was below the level of *in vivo* sensitivity. Analysis of the components of the Fnuc peak, performed by HPLC, showed that approximately 50% of this signal was due to FUTP. The results of this study show that the cytotoxicity of 5-FU is related to the level of the Fnuc in the tumor, and that this can be quantitated and characterized by *in vitro* analyses. In these experiments using 5-FU alone (5-FU plus allopurinol discussed in next section), the level of 5-FU signal in tumor also correlated with the cytotoxic effect.

Wolf *et al.*,[74] using VX2 tumors in New Zealand White rabbits, studied the 5-FU tumor retention times and compared them with an area of normal skin and subcutaneous tissue in a similar place in the non-tumor-bearing animals. They found that the half-life of free 5-FU in the tumors was approximately 1.05–1.22 h while the 5-FU half-life in an equivalent non-tumor-bearing region was 6.5–9 min, similar to that of the plasma half- life. Neither FBAL or Fnuc signals were detected *in vivo*; however, *in vitro* studies did reveal small anabolic signals. This model system has been extrapolated to the prediction of tumor response in patients (see later).

A study investigating the relative effects of equimolar doses of 5-FU and 2'FdUrd on the SG prolactinoma in Wistar-Furth rats produced several interesting results.[57] Growth inhibition studies showed 5-FU and 2'FdUrd had similar activity in this tumor. Intravenous bolus administration of 5-FU and 2'FdUrd (0.92 mmol/kg) gave similar levels of Fnuc while neither had any detectable FBAL. In contrast, when the same doses of both drugs were administered as an i.v. infusion over 4 h, the 5-FU gave signals of Fnuc, 5-FU and FBAL, while 2'FdUrd gave 5-FU and FBAL only. In an additional experiment a dose of i.v. FBAL, estimated to be equivalent to that produced from the 5-FU infusion, was infused and found to give a corresponding signal in the tumor. This suggests that FBAL may not be primarily produced in tumor but the tumor can take up FBAL which is produced in liver and released into the circulation.

In a study already discussed in the section on MRS of normal tissues, Sijens *et al.*[58] also studied C3H mice with RIF-1 tumors grown subcutaneously in the flank. At the two doses of i.p. 5-FU used (130 and 260 mg/kg) tumor shrinkage of 24 and 48%, respectively, was observed. Spectra were collected for 2 h following 5-FU injection. The 5-FU and Fnuc signals were larger after the higher dose injection; however, there was no significant difference between the two groups in their catabolite signals. The catabolites, FUPA and FBAL, were seen but were not completely resolved from each other so were measured collectively. Using linear regression analysis, a positive correlation between tumor shrinkage and tumor Fnuc levels was detected. In addition, a significant negative correlation between liver catabolite levels and tumor Fnuc was seen. Because there was no significant change in the total F signal over time, a closed system was assumed, thus the investigators were able to postulate a competition of 5-FU inactivation to FBAL in liver with 5-FU activation to Fnuc in tumor. This observation, if

translated to the clinic, may enable the investigator to predict the intratumoral Fnuc levels in microscopic tumor metastases by measuring the level of 5-FU catabolism in the liver. This model, however, is based on 5-FU doses higher than those used in patients and may therefore not be applicable.

Using multi-nuclear MRS with ^{31}P and ^{19}F in the same tumor model system, these investigators have also examined the relationship between tumor response, Fnuc levels and phosphate (P_i) levels, following i.p. 5-FU (260 mg/kg) injection.[59] They found correlations with: Fnuc and response; pretreatment P_i and Fnuc; and pretreatment P_i and response. They also noted a correlation between the pretreatment tumor pH, derived from the ^{31}P MR spectra, and the difference in the chemical shift between 5-FU and Fnuc—the lower the pH, the smaller the 5-FU–Fnuc chemical shift difference. Another study, using ^{31}P and ^{19}F MRS in Wistar rats with a fibrosarcoma implanted in the thigh,[61] found that by acidifying the tumor with glucose, the 5-FU tumor retention time was increased. Further study of these observations is needed to determine whether the intracellular or extracellular pH, or the actual pH gradient, is the significant factor, as 5-FU is thought to be transportable in or out of the cell in its non-ionized form, in which it exists below pH 8.[19]

Locoregional administration of 2'FdUrd has been investigated in Wistar rats with Novikoff hepatoma implanted in the thigh (75). At three doses of 2'FdUrd (12, 19 and 30 mg/kg) via the femoral artery, four different rates of infusion were given (bolus, 1, 5 and 24 h). The 30 mg/kg dose induced most tumor regression at all infusion rates; however, the 5 h infusion was the most active. Subsequently animals were treated at 15, 30 and 60 mg/kg i.a. for the same infusion times, then tumors were excised and studied *in vitro* with ^{19}F MRS. The total amount of F detected in tumor tissue increased with dose and decreased with infusion time. Fnuc levels correlated well, while FBAL levels correlated poorly, with the overall F signal in the tumors—the latter suggesting that the tumor FBAL may have recirculated from liver. An extension of this approach from the same group[76] examined the effects of systemic and locoregional 5-FU on both its tumor and liver metabolism. Using Sprague-Dawley rats with DS sarcomas transplanted i.m. into the thigh, 5-FU was administered at the same dose rates (bolus, 1, 5 and 24 h) at three doses (25, 50 and 100 mg/kg) either i.a. (iliac artery) or i.v. (iliolumbal vein). Subsequently excised tumor and liver samples were studied using *in vitro* ^{19}F MRS. The findings confirmed a linear correlation between total NMR visible F signal and Fnuc levels in tumor, but in liver there was an exponential correlation. The catabolic signal in liver had a linear correlation with total F signal; however, no clear correlation between tumor catabolites and total F signal was observed. Drug uptake was enhanced with the i.a. route, particularly at lower dose rates, when compared to the i.v. route. Despite the increased tumor uptake no apparent difference in liver drug uptake was noted between the i.a. and i.v. route, although the small sample size in some of the experiments may account for this. Tumor growth studies performed using five daily doses found i.a. 5-FU 25 mg/kg over 5 or 24 h were the most active and non-toxic schedules. These schedules resulted in the highest Fnuc to total F ratios in the tumors. The clinical experience with regional administration of 5-FU has found that the inadequate systemic drug exposure has resulted in tumor relapse outside the region treated. Further use of regional treatment should address this problem by either combining systemic and regional administration or by giving regional chemotherapy such that cytotoxic systemic drug levels are also achieved. Continued development of animal models using ^{19}F MRS may potentially play a significant role in the ultimate incorporation of regional 5-FU schedules into standard clinical practice.

Preclinical evaluation of 5-FU modulation in tumor

The resurgence in the use of 5-FU in recent years stems from the development of a variety of modulators of its metabolism. While ^{19}F MRS is not specific or sensitive enough to detect every metabolic component of 5-FU and thus accurately chart the drug's modulation, the real-time monitoring of the broad metabolite groups may reveal useful information. McSheehy *et al.*[12] studied the Walker carcinosarcoma in Wistar rats using both *in vivo* and *in vitro* ^{19}F MRS. In addition to treating rats with 5-FU (discussed in previous section), they pretreated with allopurinol at molar equivalent doses. Growth studies showed that allopurinol prevented the weight loss induced by 50 mg/kg 5-FU but also gave less tumor shrinkage from 5-FU alone. ^{19}F MRS experiments performed using 5-FU at 50 and 120 mg/kg doses with or without allopurinol showed that allopurinol reduced the amount of Fnuc and increased the amount of 5-FU signal at both dose levels. Further *in vitro* evaluation using

HPLC revealed the percentage of FUTP in the Fnuc signal was significantly reduced by allopurinol, consistent with the known mechanism of 5-FU–allopurinol interaction (discussed in pharmacology section).

These results illustrate the value of *in vivo* ^{19}F MRS in determining the effect of rescue or protection agents like allopurinol on both normal and tumor tissue. Conducting a clinical study using ^{19}F MRS, with the objective of documenting the tissue metabolism of 5-FU, would help clarify whether allopurinol gives any undesirable protection of the effects of 5-FU on tumor. This result would be obtained with less patient resource and more direct biochemical data than a clinical trial examining classic endpoints (toxicity, tumor response, patient survival).

The effect of methotrexate on 5-FU metabolism has been studied in CD8F1 mice with transplanted mammary tumors implanted in their flank.[77] 5-FU (150 mg/kg) was injected i.v. with or without methotrexate (300 mg/kg i.p.) administered 205 min earlier. The *in vivo* findings showed that methotrexate had no significant effect on 5-FU signal decay; however, it did significantly increase the Fnuc signal which peaked later than the same signal from 5-FU alone. HPLC analysis measuring FUMP in tissue extract showed that methotrexate pretreatment increased the total amount of 5-FU ribonucleotide per mg of protein. Subsequent experiments using 5-FU i.p. gave similar results although the magnitude of the Fnuc enhancement with methotrexate was smaller than in the i.v. experiments. This is likely to be due to the first pass effect of 5-FU liver uptake which would reduce the amount of drug available to the tumor.

El-Tahtawy and Wolf[78] studied the effect of methotrexate pretreatment (40 mg/kg either 5 or 22 h before 5-FU) in Sprague-Dawley rats bearing the Walker 256 adenocarcinoma, treated with 150 mg/kg 5-FU. Pretreatment with methotrexate induced a faster formation of Fnuc with a more rapid decline in 5-FU. This was estimated using a two compartment model. The formation of Fnuct and Fnucs was significantly greater with the methotrexate pretreatment although there was no detectable difference between the 5 and 22 h methotrexate pretreatment. Validation of the MRS findings by evaluating the RNA 5-FU content confirmed a similar increase in conversion of Fnuc into RNA with both methotrexate pretreatments although neither was superior.

McSheehy *et al.*,[79] using a Walker carcinosarcoma rat model, examined 5-FU modulation by methotrexate with the intention of obtaining information on dose schedules more comparable to the clinical setting. Administration of methotrexate (20 or 50 mg/kg), 3, 6, 12 or 24 h prior to 5-FU (50 mg/kg), did not measurably alter the 5-FU signal decay; however it did significantly increase the formation of Fnuc. No differences in the rate of Fnuc formation or tumor inhibition were detected between the various intervals of methotrexate pretreatment. Increased cytotoxicity was, however, noted when the order of drug administration was reversed, with the 5-FU–methotrexate sequence less active than the methotrexate–5-FU schedule. No ^{19}F signal was seen at 3–24 h after 5-FU administration regardless of subsequent addition of methotrexate. They suggest that the reason no difference between the various pretreatment intervals was seen is that this tumor may significantly polyglutamate the methotrexate, maintaining an intracellular effect regardless of the pretreatment interval.

These studies clearly demonstrate the modulatory effect of methotrexate on 5-FU with ^{19}F MRS with additional *in vitro* validation, although to date there is no evidence suggesting an optimal pretreatment interval. The ability to apply compartmental models demonstrated by El-Tahtaway and Wolf will further increase the understanding of the tissue metabolism of this drug.

Li and Jin investigated the use of hydralazine to modulate 5-FU metabolism.[80] Using RIF-1 tumors implanted in C3H/Km mice, an i.v. bolus of 5-FU (150 mg/kg) was administered and its metabolism studied *in vivo*. After 90 min all the signal was from Fnuc. At this stage hydralazine (5 mg/kg i.p.) was added which resulted in a reduction of the Fnuc signal and increased 5-FU signal after a further 60 min and by 4.5 h almost complete reconversion to 5-FU. Control animals showed no change in the Fnuc levels over that time. The investigators postulate that the hydralazine induced reduction in tumor energy levels favors the conversion of Fnuc to 5-FU.

Seymour and colleagues[81] have investigated the effect of IFN-α on 5-FU metabolism. Because IFN-α is known to increase cytotoxicity in the HT29 human colon cell line and is also thought to alter the pharmacokinetics of 5-FU in humans, they used nude mice with HT29 cells injected in the flank. The intention was to delete the potential pharmacokinetic effect by using human IFN in the mouse. Mice received pretreatment with IFN-α (50 000 IU i.p.) either 24–30 h or 1–4 h before spectroscopy. 5-FU (200 mg/kg) was injected i.p. and its metabolism monitored. Using data fitted to a pharmacokinetic model, absorption and elimination constraints were

generated. No difference in absorption in liver was noted with IFN-α, however in tumor, the elimination half-life doubled from 22 to 44 min with the use of IFN-α. The Fnuc and F catabolites did not conform to the model; however, no major differences in these were noted with IFN-α. These findings suggested that IFN-α alters the retention of 5-FU in HT29 cells without obviously enhancing the Fnuc conversion. This could be achieved by reduction of intratumoral catabolism (although not obvious from this experiment) or by altering 5-FU efflux from the cell. Further clinical studies of IFN-α modulation, performed in our institution, are referred to in the next section. The use of nude mice with a human cell line model in this MRS experiment is a positive step towards developing these preclinical studies as models of clinical conditions.

Clinical studies of intratumoral 5-FU metabolism

In 1990 Wolf *et al.*[74] published a report on the use of ^{19}F MRS to monitor 5-FU metabolism in patients and rabbits. Six patients with tumors at a variety of sites, receiving 5-FU based chemotherapy (5-FU and leucovorin; 5-FU, cyclophosphamide and methotrexate; 5-FU, cyclophosphamide and the anthracycline menogaril) were studied after the bolus of 5-FU. Four patients had visible 5-FU signal in tumor while two did not. All four with visible 5-FU signal had a tumor response as did one without this signal (this patient received combination chemotherapy and may have responded to the other drugs regardless of the 5-FU sensitivity). The one patient with progressive tumor did not have a 5-FU signal. This evaluation has been extended and presented as a comparison between tumor 5-FU 'trapping' and subsequent response.[82] Trapping of 5-FU was defined as a tumor 5-FU half-life greater than the plasma half-life. Eleven patients with a variety of tumors were studied, 10 were evaluable for response. Five patients' tumors trapped 5-FU and went on to respond while four who did not trap 5-FU, did not respond. One patient (from the earlier data) did not trap 5-FU but did respond to a combination of drugs including 5-FU. A further update of this work included 32 patients, 16 of whom were treated with 5-FU 600 mg/m^2 i.v. with leucovorin 500 mg/m^2. In this subgroup a highly statistically significant association between 5-FU trapping and subsequent response was noted, while in those having combination chemotherapy the association was less impressive.[83] Our own experience monitoring 22 patients on i.v. infusional 5-FU (300 mg/m^2/day) for colorectal cancer liver metastases reaches similar conclusions.[84] The presence of a 5-FU signal in tumor at any stage during the first 8 weeks of chemotherapy predicts tumor response. No Fnuc signals were seen during the 5-FU infusion; however, significant catabolic signals were observed in some tumors. Because of the tumors' size and their proximity to the surface coil, signal contamination from surrounding normal liver tissue was minimal so we regarded recirculation of catabolites from liver and their subsequent accumulation in the tumor as the most valid explanation for the findings. This has some support from preclinical observations discussed earlier.[57] Continued investigation of the optimal scheduling of 5-FU for clinical use can be potentially facilitated by ^{19}F MRS; however, further refinement of the technology is required.

Clinical studies of intratumoral 5-FU modulation

To date there is little published clinical experience in monitoring the metabolic consequences of adding a modulator to 5-FU. We have been investigating the use of IFN-α to modulate 5-FU given as an infusion. In 15 patients who had become refractory to infusional 5-FU (300 mg/m^2/day), IFN-α was added.[84] Metabolic changes observed included the appearance of new 5-FU signals (seven patients) and in three cases Fnuc signals were seen (Figure 9). A significant correlation between the 5-FU signal and subsequent tumor responsiveness was documented; however, too few cases of Fnuc signal were seen to confirm any significant correlation with response. These observations are consistent with the preclinical findings of Seymour *et al.*[81] More recently, preliminary results from two South Western Oncology Group studies have been presented.[85] Six patients with gastrointestinal malignancies were treated with i.v. 5-FU 600 mg/m^2 on cycle 1, then on cycle 2 the 5-FU was preceded by leucovorin 500 mg/m^2 (study 9006). A significant prolongation in the tumor 5-FU half-life was documented (3–26%; mean 18.2%; $p < 0.05$) using ^{19}F MRS. Another patient (study 9118) received 5-FU 1500 mg/m^2 on cycle 1 and then methotrexate 1500 mg/m^2 prior to the 5-FU on cycle 2. While the 5-FU half-life did not significantly alter with methotrexate, an Fnuc signal was identified. No tumor response correlations were available at the time in either study; however, the ability to observe measureable changes in 5-FU metabolism with

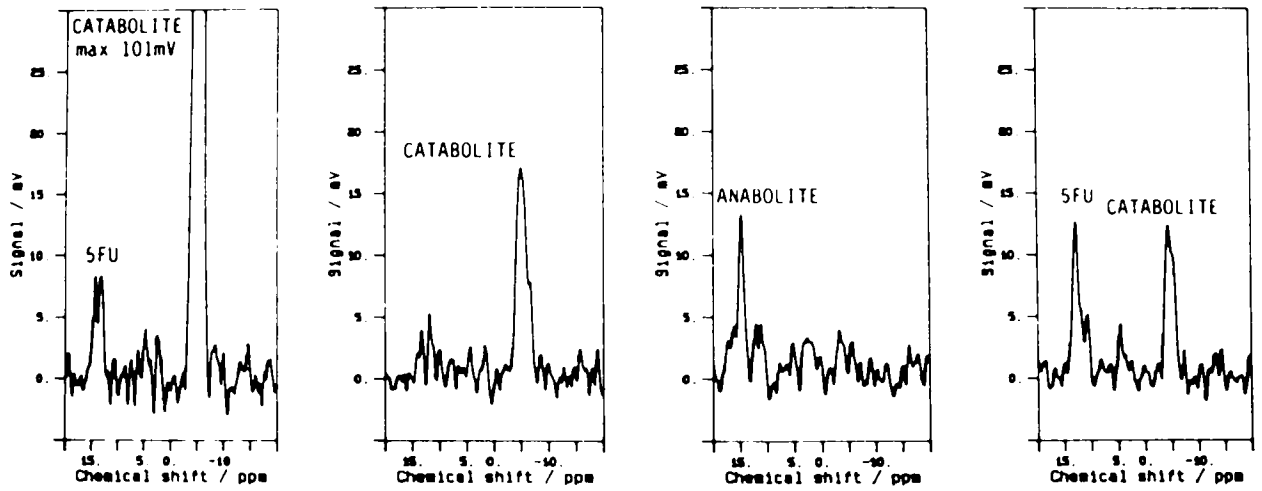

Figure 9. A series of ^{19}F MRS studies on the same patient at different stages of treatment[84] using i.v. infusional 5-FU (300 mg/m^2/day). The first MRS scan (MRS 1) was performed after 1 week of 5-FU infusion. The patient went on to have an objective partial response but later developed progressive disease. At this time (MRS 2) the 5-FU peak had disappeared and the catabolites had reduced. IFN-α was started the day after the scan and 1 week later he had MRS 3 followed after a further week by MRS 4. At this stage the patient had showed evidence of a further response. Absolute frequencies are arbitrary. Reproduced with permission.

these two widely used modulators further strengthens the position of ^{19}F MRS in clinical research.

Specific issues in *in vivo* ^{19}F MRS

The quality of *in vivo* MRS measurements are affected by a range of instrumental and methodological considerations. Despite the intrinsic measurement sensitivity of the ^{19}F nucleus, the concentration of the metabolites of interest is low, leading to limitations in the signal that can be measured in a clinically practicable examination time. This is particularly the case with low dose continuous infusion 5-FU. Sensitivity can be enhanced by (i) using optimized coils, and surface coils for small lesions, to enhance the signal to noise ratio (currently manufacturers do not provide optimized ^{19}F coils); (ii) measuring large volumes, and thus increasing the total signal observed, at the cost of tissue or tumor specificity; (iii) improving spectral resolution by improving the field uniformity over the region of interest (provided line width is not limited by metabolite T_2 decay or by variations in sample susceptibility); and (iv) increasing the magnetic field strength, which enhances the signal to noise ratio and the chemical shift dispersion. The latter approach is adopted in *in vivo* animal studies where fields of about 4.7 Tesla are often used. In clinical studies most measurements are performed at 1.5 Tesla, and few instruments are available at higher field strengths, where the costs are very high.

Localization to a specific tissue, tumor or region is necessary to identify metabolic function within that tissue. Due to the low signal with ^{19}F MRS, many of the recent advances in spectroscopic localization have yet to be applied to this isotope. The majority of measurements to date have been localized only with surface coils, with tissue selectivity obtained by selecting those patients who have large tumors such that signal from the tumor will dominate the response of the surface coil, Brechner *et al.*[86] and Walach *et al.*[87] have utilized one-dimensional CSI, a technique that stratifies the signal into parallel planes or slices, but where lateral extent of sensitivity is defined by the surface coil response. We have also implemented this technique, and have further applied ISIS, which is a three-dimensional technique, selecting a three-dimensional voxel. Both of these techniques demonstrate localization, but lead to a reduced signal due to the smaller tissue volume examined. The methods have been successfully applied to bolus studies, but further developments require improvements in coils and methodology to provide increased sensitivity. Localization techniques and related issues are discussed in more detail elsewhere.[88,89]

Quantification of ^{19}F signals is of long-term importance in studies of drug metabolism. Without a volume selected signal, such quantification is difficult due to the poor definition of surface coil response. Dependent upon the measurement technique selected, corrections for T_1 and T_2 relaxation

are also required. If a pulse and acquire technique, or a method that uses a localization scheme such as ISIS, which detects the FID, is employed, T_2 decay can be neglected. If a coil that provides a uniform irradiation is used, T_1 effects can be avoided by choice of a long repetition time, although at the cost of sensitivity. Alternatively separate measurements with a range of repetition times can be employed to calculate T_1. With a surface coil more sophisticated approaches are required. The major current difficulty in performing quantitative measurements *in vivo* is the need to balance the time taken to obtain this additional information against the time required to obtain useful spectral information. Schlemmer *et al.*[90] have reported an *in vivo* assessment of FBAL levels in the livers of patients with liver metastases from colorectal cancer. FBAL was measured to have a mean maximum level of 1.45 ± 0.69 mmol/g with a mean level of 0.98 ± 0.37 mmol/g over the first 50 min following rapid infusion (10 min) administration of 5-FU. Despite methodological difficulties (localization was via a large surface coil and T_1s were measured for solutions), an increase in uptake of 5-FU (presumed to be predominantly in tumor) was seen with i.v. doses (600 mg/m^2) and i.a. doses (100 mg/m^2). No difference was seen for FBAL.

One interesting approach that provides increased information about metabolite compartmentalization, and which may provide enhanced sensitivity, is the use of gadolinium labeled chelates (Gd-DTPA) and other MR contrast agents. Gd-DTPA is routinely used for MR imaging to provide enhanced signal in areas of blood–brain barrier breakdown, by enhancing T_1 relaxation rates. Rowland *et al.*[91,92] have applied this technique to drug metabolism studies by injecting a bolus of Gd-DTPA at a short period after injection of a drug such as 5-FU. In rats, enhanced 5-FU signal was seen in normal liver and in a methylnitrosourea induced mammary tumor. The catabolite signal (FUPA and FBAL) was not affected. This indicated that a part of the 5-FU signal was in the extracellular space and that the catabolites were in a different space to the Gd-DTPA, presumably intracellular. This technique provides a way of enhancing signals, increasing sensitivity and interrogating the distribution of the drug together with its metabolites. There is potential to use a range of agents that are intra-cellular or extra-cellular and which enhance or destroy signal in the compartment of interest.

Whilst this review has focused on studies of drug pharmacokinetics and metabolism, MRS can provide direct measurements of cellular metabolism, allowing information on the pharmacodynamics of drugs (their effects on normal tissue metabolism) to be assessed. ^{31}P MRS has been employed in many animal studies and in an increasing number of clinical studies. The spectra provide information on energy metabolism and phospholipid metabolism, the latter providing information that reflects and may predict response.[93] ^{1}H spectroscopy can also provide information on cellular metabolism, although the significance of the changes observed is less clearly understood. This latter technique has the advantage of greater sensitivity, allowing volumes of a few milliliters to be assessed. It is becoming practicable to simultaneously observe two nuclei, opening the prospect of simultaneously monitoring a drug with ^{19}F spectroscopy and its effects with ^{31}P spectroscopy.[59,61] Advances in techniques of ^{13}C spectroscopy and increased availability of ^{13}C would provide the opportunity to extend these methods to a wider range of drugs.

Summary and conclusions

^{19}F MRS is at present able to monitor the tissue pharmacology and metabolism of 5-FU and its analogs in patients receiving this form of chemotherapy. This information can be derived non-invasively and in real time, making it applicable to repeated measurements. The increasing availability of clinical MRI systems, makes MRS potentially more accessable to a population of patients than other non-invasive methods such as PET, which also relies on close access to a facility that produces short-lived nuclides. Conversely, PET is sensitive enough to detect tracer amounts of labeled drug whereas ^{19}F MRS requires μM to mM levels of drug or metabolite. Despite MRS having the advantage of sensitivity, allowing identification of broad groups of metabolites of 5-FU *in vivo*, this technique cannot provide accurate identification of individual active metabolites. Further development is required to address the problems of sensitivity, in addition to the issues of volume localization and absolute quantification of the spectral data.

Current and potential future applications of ^{19}F MRS in monitoring fluoropyrimidine chemotherapy include investigating the distribution and metabolism of these drugs, in order to predict patient toxicity, facilitating stategies to prevent these adverse effects. Direct monitoring of intratumoral metabolism of these drugs will improve the understanding of issues such as uptake, regional distribution, modulation and salvage, and their impact on tumor out-

come. Ultimately a knowledge of the effects of drug schedule on these parameters will enable the investigator to optimize therapy for an individual patient's needs—an important consideration in the use of these drugs in both curative or palliative treatments. Successful application, coupled with further development of this technique, will act as a model by which other fluorinated and non-fluorinated drugs can be investigated.

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