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IN VIVO ^{19}F NMR SPECTROSCOPY OF THE ANTIMETABOLITE 5-FLUOROURACIL AND ITS ANALOGUES

AN ASSESSMENT OF DRUG METABOLISM

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Abstract—The metabolism of 5-fluorouracil (5FU) and its analogues 2′-deoxy-5-fluorouridine (2′FdURD), 5′-deoxy-5-fluorouridine (5′FdURD) and *R,S*-1-(tetrahydro-2-furyl)-5-fluorouracil (Ftorafur) has been studied by ^{19}F NMR in rat liver and the rat S.G. Prolactinoma. In experiments using i.v. bolus injections of 0.46 mmol/kg 5FU was cleared more rapidly from the liver than 5′FdURD ($t_{1/2}$ of 4.7 ± 0.6 vs 15.8 ± 0.8 min, $P < 0.001$). Alphafluoro-beta-alanine (FBALA) production was almost identical after 5FU or 2′FdURD but slower and more sustained after 5′FdURD and still slower after Ftorafur. Both 5FU and 2′FdURD caused formation of toxic fluoronucleotides in S.G. Prolactinomas when administered i.v (0.92 mmol/kg bolus). After i.v. infusion (0.23 mmol/kg/hr for 4 hr) 5FU produced fluoronucleotides whereas 2′FdURD did not; however, both 5FU and 2′FdURD (0.19 mmol/kg daily i.p. for 7 days) suppressed tumour growth. FBALA was observed in tumors following 5FU and 2′FdURD. Infusion of FBALA itself (0.17 mmol/kg/hr for 4 hr i.v.) led to signal in the tumour, so this compound could have been formed in the liver. These data demonstrate that ^{19}F NMR can monitor drug metabolism *in vivo*.

5-Fluorouracil (5FU†) was developed by Heidelberger *et al.* [1] in 1957 as a potential drug for the treatment of cancer. The first study of the metabolism of 5FU monitored by ^{19}F NMR, *in vivo*, was by Stevens *et al.* [2]. Further studies of 5FU and related compounds using ^{19}F NMR have investigated their metabolism in isolated cells [3, 4], perfused mouse liver [5] and laboratory animals [4, 6]. In the clinical setting ^{19}F NMR has been used *in vivo* to study patients receiving 5FU chemotherapy [7, 8] and *in vitro* to examine human plasma and urine [9, 10]. The present study has used ^{19}F NMR with the aim of providing information about some of the factors influencing the action of 5FU and related compounds *in vivo* under experimental conditions.

The clinical effectiveness of 5FU is limited [11] and analogues of 5FU have been synthesized in attempts to increase its therapeutic ratio. These compounds are either intermediates in the activation pathway of 5FU, for example 2′-deoxyfluorouridine (2′FdURD) [12] or nucleoside derivatives with novel substitutions in place of a ribose or deoxyribose. The latter compounds require conversion to 5FU to be effective as cytotoxic agents, and may be regarded as prodrugs of 5FU.

In the present study, we have investigated the metabolism of 5FU, 2′FdURD and two prodrugs of 5FU. The first of these prodrugs is *R,S*-1-(tetrahydro-2-furyl)-5-fluorouracil (Ftorafur) which requires cleavage of the furanyl ring to release 5FU and so be active [13, 14]. The second, 5′-deoxy-5-fluorouridine (5′FdURD) is also cleaved to 5FU [15] but at a greater rate than Ftorafur [16].

The pharmacological action of 5FU is thought to depend on its activation to nucleotide derivatives [12] and these may be detected *in vivo* by ^{19}F NMR [2]. The activation of 5FU by enzymes of uridine triphosphate production will produce 5-fluorouridine mono, di and triphosphates (FUMP,FUDP and FUTP respectively); whereas the enzymes of the thymidylate synthesis and salvage pathways may activate 5FU to 2′-deoxy-5-fluorouridine mono, di and triphosphates (FdUMP,FdUDP and FdUTP respectively) [17]. The metabolism of the drugs used in this study has been summarized in Fig. 1. The fluoronucleotides, FdUMP and FUTP have been shown to have cytotoxic effects; FdUMP via inhibition of thymidylate synthase (5,10-methylenetetrahydrofolate:dUMP C-methyltransferase, EC 2.1.2.45) and FUTP by its incorporation into RNA [12].

The route of breakdown of 5FU (see Fig. 1) to alpha fluoro-beta-alanine (FBALA) is thought to be analogous to the breakdown of thymidine [18], though the ultimate step may release F^+ ions. Breakdown of 5FU occurs in most tissues; the major site is in the liver, but the pathway is thought to be reduced in tumours compared with normal tissue [17] which may account for some chemotherapeutic selectivity.

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† Abbreviations: 5FU, 5-fluorouracil; 2′FdURD, 2′-deoxy-5-fluorouridine; 5′FdURD, 5′-deoxy-5-fluorouridine; Ftorafur, *R,S*-1-(tetrahydro-2-furyl)-5-fluorouracil; FUMP, FUDP and FUTP, 5-fluorouridine mono, di and triphosphates, respectively; FdUMP, FdUDP and FdUTP, 2′-deoxy-5-fluorouridine mono, di and triphosphates, respectively; FNUC, any or all fluoronucleotides; FBALA, alpha-fluoro-beta-alanine.

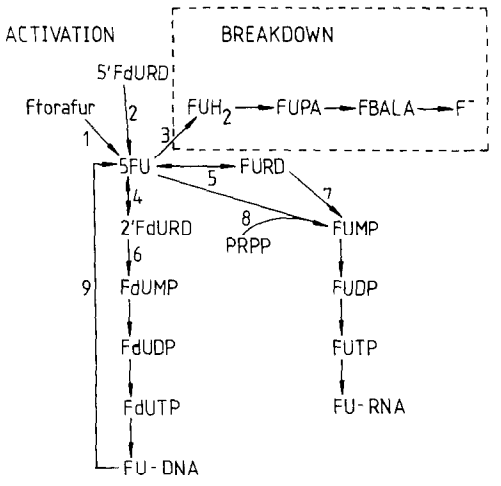

Fig. 1. Diagram to illustrate the metabolism of 5-fluorouracil and related compounds. Abbreviations used are: 5'FdURD, 5'-deoxy-5-fluorouridine; Ftorafur, *R,S*-1-(tetrahydro-2-furyl)-5-fluorouracil; 5FU, 5-fluorouracil; FUH_2, dihydrofluorouracil; FUPA, fluoroureido propionoic acid; FBALA, fluoro-beta-alanine; F^-, fluoride ion; FURD, 5-fluorouridine and its 5'-monophosphate, FUMP; 5'-diphosphate, FUDP; and 5'-triphosphate, FUTP; 2'FdURD, 2'-deoxy-5-fluorouridine; and its 5'-monophosphate, FdUMP; 5'-diphosphate, FUDP; and 5'-triphosphate, FdUTP; FU-DNA, 5-fluorouracil incorporated deoxyribose nucleic acid; FU-RNA, 5-fluorouracil incorporated ribose nucleic acid; PRPP phosphoribosyl pyrophosphate. Enzymes are numbered as follows: (1) pyrimidine nucleotide phosphorylase; (2) dihydrothymidine dehydrogenase; (3) soluble and microsomal oxidative enzymes; (4) thymidine phosphorylase; (5) uridine phosphorylase; (6) thymidine kinase; (7) uridine kinase; (8) pyrimidine phosphoribosyl transferase; (9) uracil DNA glycosidase.

In summary, it can be said that the fluoropyrimidine chemotherapeutic agents all require the production of fluoronucleotides (FNUC) for their cytotoxic action; the ^{19}F NMR signals arising from these metabolites cannot easily be resolved *in vivo*, and they appear as a single peak under the experimental conditions used in this investigation (see Materials and Methods). Furthermore, the breakdown of this group of compounds results in the production of FBALA which may also be detected *in vivo* by ^{19}F NMR, as may 5FU [2]. The aim of this paper is to use information provided *in vivo* by ^{19}F NMR, to determine speed of uptake, activation and breakdown of the fluoropyrimidines in the rat. Metabolism in both liver and tumour were investigated and the effects of bolus administrations and infusion studied.

MATERIALS AND METHODS

Chemicals. 5FU was obtained in 10-mL ampules containing 250 mg (0.19 M) 5FU, as the sodium salt in water for injection (B.P.). Ftorafur was purchased from the Sigma Chemical Co. (Poole, U.K.). 2'FdURD and 5'FdURD were a generous gift of Roche (Welwyn Garden City, U.K.). FBALA was purchased from Koch-Light Laboratories Ltd (Colnbrook, U.K.).

NMR machine and protocols. Experiments were carried out using a 14-mm diameter, 2 turn surface coil tuned to 75.5 MHz in a 1.9T Oxford Research Systems TMR 32 spectrometer. The magnet was shimmed on water proton signals. Water linewidths were between 15 and 35 Hz for tumour, and between 35 and 55 Hz for liver. Data was acquired over a 4 KHz spectral width using 180° pulse at the coil centre at 1 sec intervals for 2, 4, 8 and 16 min periods. Twenty Hz exponential line-broadening was imposed on the data prior to Fourier transformation. The areas of the spectral peaks were then integrated using a computer programme which allows one base-line to be chosen for a series of spectra in the same time course.

Rats and animal protocols. Two studies of drug metabolism were performed. Firstly, 200–240 g female Wistar rats were used for studies on liver metabolism. In the second study 180–220 g female Wistar–Furth rats were used for tumour studies. S.G. Prolactinoma tumours were passaged by the method of Prysor-Jones and Jenkins [19]. Tumours for NMR spectroscopy were grown on the flanks of the rats by subcutaneous injection of 5×10^7 tumour cells in 1 mL. Tumours were used when their smallest diameter was greater than the diameter of the coil (14 mm) but the largest diameter was less than 30 mm.

Preparation for NMR studies. For short term experiments involving bolus injections of drugs the rats were anaesthetized with sodium pentobarbitone (60 mg/kg) (Sagatal, May and Baker) by i.p. injection. For long term anaesthesia rats were anaesthetized with 14% (w/v) urethane (10 mL/kg).

All animals were cannulated via the jugular vein for i.v. injection of experimental drugs within 30 min of the administration of the anaesthetic. For bolus injections, 5FU was used as 0.19 mol/L of the sodium salt in water; 2'FdURD and 5'FdURD were made up to 0.19 mol/L in 0.9% saline. Bolus injections were administered between 45 and 60 min after anaesthesia over a period not exceeding 15 sec. These drug doses, either 0.46 mmol/kg (low dose) or 0.92 mmol/kg (high dose), are similar to clinically used doses in terms of g/m^2 of body surface area. For infusions, 5FU, 2'FdURD and FBALA were made up in a total of 6 mL in 0.9% saline and infused for 4 hr. The total infused dose of 5FU or 2'FdURD was 0.92 mmol/kg whereas 0.69 mmol/kg of FBALA was infused. The animals were placed in the centre of the magnet and the coil was placed over the liver or tumor as required. In experiments where spectra were obtained from the liver it was exposed by incision. NMR data collection began at the start of drug administration.

The effects of 5FU and 2'FdURD on growth of the S.G. prolactinoma. A total of 30 rats was used in a test of the ability of 5FU and 2'FdURD to inhibit tumour growth when given by i.p. bolus injection as 0.19 mmol/kg (the minimal dose which gives an observable ^{19}F NMR signal *in vivo*) each day for 7 days. Tumour cells were injected (section 3) on day 1). From day 2 5FU, 2'FdURd or 0.9% saline were administered daily to 10 rats each. Measurements of

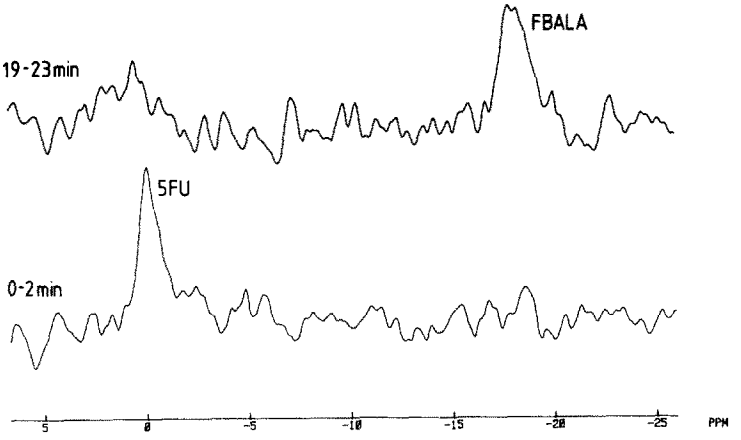

Fig. 2. Representative ^{19}F spectra (N = 3) of the rat liver taken 0–2 min and 19–23 min after the administration of 0.46 mmol/kg 5FU i.v. showing 5-fluorouracil (5FU) and fluoro-beta-alanine (FBALA).

tumour diameters were taken as the geometric mean (i.e. $\sqrt{}$(length × width).

Data treatment. Graphs were plotted using Sigma Plot software (Version 3.10, The scientific Graph program, Jandel Scientific.) which allowed linear regression lines to be fitted to the kinetic data.

RESULTS

5FU, 2'FdURD, 5'FdURD and Ftorafur uptake, elimination and metabolism in the liver monitored by ^{19}F NMR

Time course for uptake and elimination. A bolus injection of 5FU resulted in the appearance of peaks corresponding to 5FU and FBALA in the ^{19}F NMR spectra of the liver. When a bolus injection of 5'FdURD was administered signals corresponding to 5'FdURD and FBALA, only, were observed. When 2'FdURD was given by bolus injection its signal was undetectable after the first 2 min block of data collection and only a peak corresponding to FBALA was seen in subsequent spectra. A time course for the elimination of 2'FdURD is, therefore, not plotted.

Signals from 5FU in the rat liver following a bolus injection (0.46 mmol/kg) were detected at maximum intensity in the first accumulation (0–2 min) and fell to the limit of detection within the first 20 min (see Fig. 2). 5'FdURD signals from a 0.46 mmol/kg bolus injection (Fig. 3B) were detected for longer than 5FU (Fig. 3A).

Measurement of drug kinetics. The first order rate constant (kl) for the elimination of 5FU from the liver was calculated for doses of 0.46 and 0.92 mmol/kg from the graphs in Fig. 3A and C, respectively, thus allowing the drug half-life ($t_{1/2}$) in the liver to be obtained. The $t_{1/2}$ of 5FU in the liver was found to be 4.7 ± 0.6 min for 0.46 mmol/kg and 9.5 ± 1.6 min for 0.92 mmol/kg, significantly different ($P < 0.001$). The $t_{1/2}$ for 5'FdURD in the liver was calculated from the graph in Fig. 3B. Following a dose of 0.46 mmol/kg the $t_{1/2}$ of 5'FdURD in the liver is 15.5 ± 0.8 min which is significantly longer

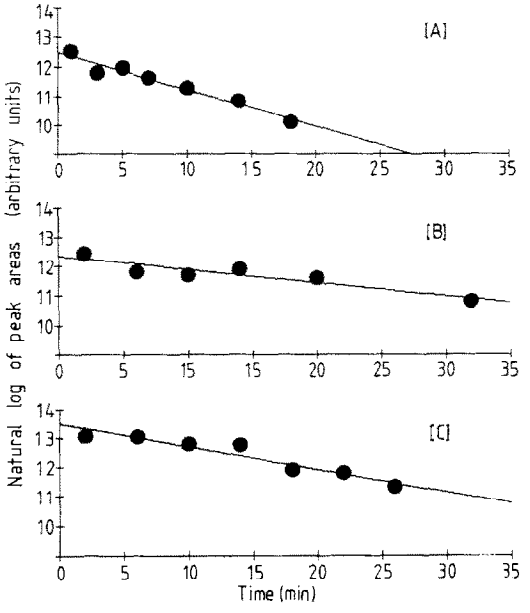

Fig. 3. Graph of $\log_e$ peak area against time for the metabolism of a bolus injection of (A) 0.46 mmol/kg 5-fluorouracil (5FU), (B) 0.46 mmol/kg 5'-deoxy-5-fluorouridine (5'FdURD) and (C) 0.92 mmol/kg 5FU. Results are from representative experiments and lines are fitted by linear regression.

($P < 0.001$) than the $t_{1/2}$ measured for the molar equivalent dose of 5FU. Au *et al.* [20] give values for the $t_{1/2}$ of 5FU (0.19 mmol/kg) in the rat plasma as 15.3 ± 2.5 min and the $t_{1/2}$ for 5'FdURD (1.15 mmol/kg) in the plasma as 128.0 ± 49.0 min.

FBALA formation. The signals observed from FBALA after the administration of a bolus injection (0.46 mmol/kg) of 5FU or 2'FdURD followed similar patterns, as shown in Fig. 4A. However, the signals received from FBALA following 5'FdURD injection (0.46 mmol/kg) were more sustained than

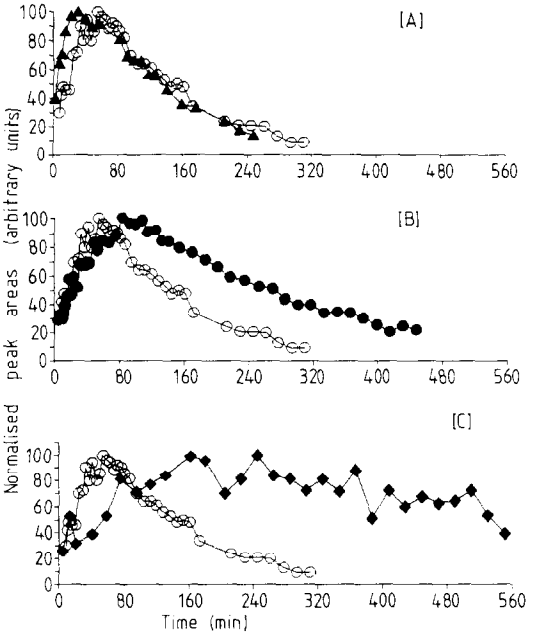

Fig. 4. Comparison of fluoro-beta-alanine (FBALA) production in liver from a 0.46 mmol/kg bolus injection of 5-fluorouracil (5FU) compared to FBALA production from some analogues of 5FU. (A) FBALA production from 5FU (○—○) compared to FBALA from 2'-deoxy-5-fluorouridine (2'FdURD) (▲—▲). (B) FBALA production from 5FU (○—○) compared to FBALA from 5'-deoxy-5-fluorouridine (5'FdURD) (●—●). (C) FBALA production from 5FU (○—○) compared to FBALA from 0.46 mmol/kg *R,S*-1-(tetrahydro-2-furyl)-5-fluorouracil (Ftorafur) (◆—◆). Results are from representative experiments; data are normalized to highest peak area in each experiment (maximum = 100).

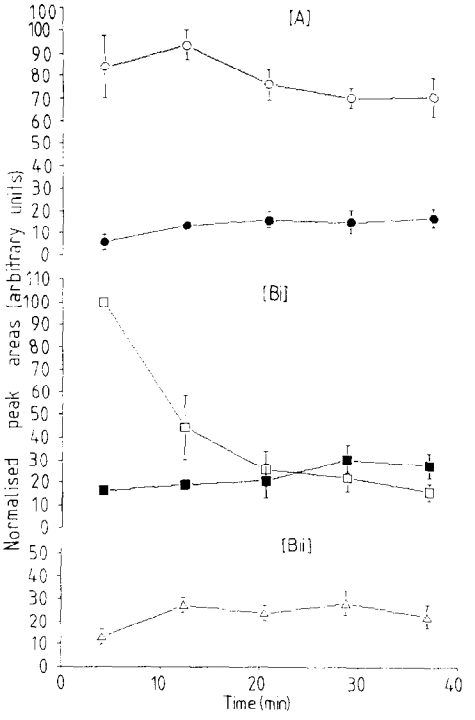

Fig. 5. Graphs representing the metabolism of a bolus injection of 0.92 mmol/kg 5-fluorouracil (5FU) or 2'deoxy-5-fluorouridine (2'FdURD) in the S.G. Prolactinoma. (A) shows the activation of 5FU (○—○) to 5-fluoronucleotides (FNUC) (●—●). (Bi) shows the activation of 2'FdURD (□—□) to FNUC (■—■); (Bii) shows the level of 5FU released from the same dose of 2'FdURD (△—△). All values were normalized to highest peak area in each experiment (maximum = 100) and values displayed are the mean (±SE) value at each time point for three experiments.

those of FBALA from 5FU or 2'FdURD (see Fig. 4B). A bolus injection of Ftorafur (0.46 mmol/kg) resulted in a more sustained FBALA signal, shown in Fig. 4C, compared to that from an equimolar quantity of 5FU or 5'FdURD. The level of FBALA produced from a bolus injection of Ftorafur, however, was detected with a much lower signal to noise ratio than the FBALA produced from a bolus injection of 5FU. Errors in the integration of this weak signal are reflected in the variation in the signal intensity shown in Fig. 4C.

The effects of 5FU and 2'FdURD on growth of the S.G. Prolactinoma

Fourteen days after inoculation of the rats 7 out of 10 of the control tumours had grown with a mean (±SE) diameter of 11 ± 3 mm (N = 7). One of the 2'FdURD treated rats had a tumour at this time, which was 14 mm, the remaining rats in the 2'FdURD treated group and the 5FU treated group had no tumours. As both 5FU and 2'FdURD caused similar growth inhibition the pattern of drug metabolism in the tumour was investigated.

Metabolism of 5FU and 2'FdURD in the S.G. Prolactinoma monitored by ^{19}F NMR

Administration by bolus injection. The administration of 5FU by i.v. bolus injection at the high dose (0.92 mmol/kg) to rats bearing the S.G. Prolactinoma resulted in the appearance of 5FU and FNUC in the ^{19}F NMR spectra (see Fig. 5A). A 0.92 mmol/kg dose was necessary for a clear amount of FNUC to be seen in the tumour spectra. Following the administration of 0.92 mmol/kg 2'FdURD by i.v. bolus injection the original drug and FNUC were observed in the spectra (see Fig. 5Bi); 5FU signals were also observed (see Fig. 5Bii). The administration of 5FU or 2'FdURD produced FNUC in an apparently similar amount and rate in the S.G. Prolactinoma.

No significant signal from FBALA was detected in the tumours within 40 min of the administration of either 5FU or 2'FdURD.

Administration by continuous infusion. ^{19}F NMR has been used to assess the effect of the drug administration regime upon drug metabolism in the tumor by following the uptake and fate of 5FU or 2'FdURD given by infusion (0.23 mmol/kg/hr i.v. for 4 hr). The infusion of 5FU into a rat bearing the S.G. Prolactinoma led to the formation of FNUC and FBALA which are clearly visible in the spectra along with 5FU (see Fig. 6A). After the infusion of 2'FdURD, however, peaks for 5FU and FBALA are seen but no FNUC is visible (see Fig. 6B). This result

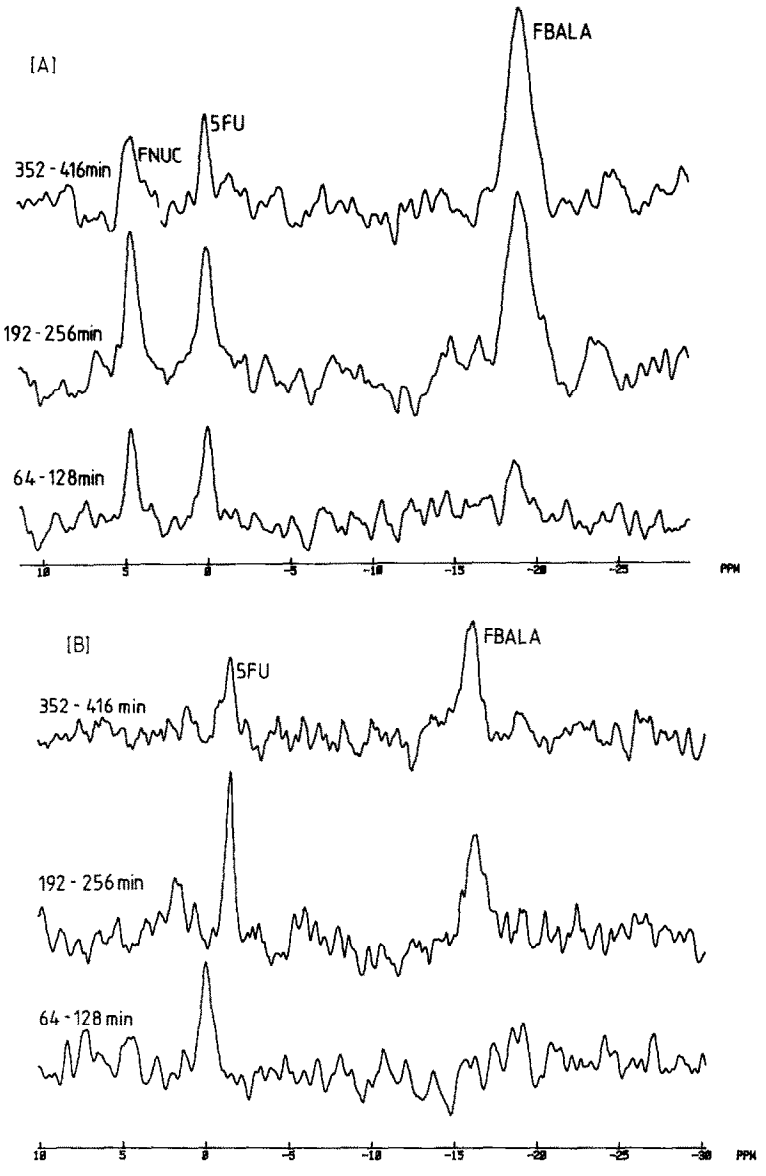

Fig. 6. Representative ^{19}F NMR spectra from the S.G. Prolactinoma during and following the infusion of (A) 5-fluorouracil and (B) 2'deoxy-5-fluorouridine at 0.23 mmol/kg/hr for 4 hr. Spectra are from 3840 scans taken over a 64 min period; 64–128, 192–256 and 352–416 min after the beginning of the infusion. Peaks are labelled as follows; 5FU, 5-fluorouracil; 2'FdURD, 2'-deoxy-5-fluorouridine; FNUC, 5-fluoronucleotides; FBALA, fluoro-beta-alanine.

suggests there might be a difference between the ability of 2'FdURD and 5FU to inhibit tumour growth when the drugs are given by continuous infusion, but this could not be tested as the cell line was lost.

The i.v. infusion of 0.17 mmol/kg/hr FBALA for 4 hr to a tumour bearing rat resulted in its appearance in ^{19}F spectra acquired from the tumour (results not shown). The dose of FBALA infused was estimated to be the minimum amount of FBALA that would be produced from 5FU, infused at 0.23 mmol/kg/hr i.v. for 4 hr, calculated from data on the renal clearance of 5FU [20]. The amount of FBALA produced from 2'FdURD was assumed to be similar to the amount produced from 5FU.

DISCUSSION

The results of these experiments with fluoropyrimidines show that ^{19}F NMR can measure both pharmacokinetics and drug metabolism, *in vivo*. The $t_{1/2}$ of 5'FdURD in the liver is about three-fold longer than that of 5FU which suggests that the rate of FBALA production from 5'FdURD may be limited by the rate of formation of 5FU; this also explains why the amount of FBALA produced from a bolus injection of 5'FdURD increases more slowly and is more sustained than the amount of FBALA from 5FU. This means that the host cellular concentration of 5FU could be less from 5'FdURD administration than from an equimolar dose of 5FU. The lower 5FU

levels suggest that the toxicity of 5'FdURD should be less than the toxicity of 5FU. Indeed, Au *et al.* [20] found that the host toxicity of 5FU infusion (35–45 mg/kg/day) was considerably greater than an infusion of 5'FdURD (500 mg/kg/day) in normal rats.

The greater values for the $t_{1/2}$ of 5FU in the liver for the 0.92 mmol/kg dose over the 0.46 mmol/kg dose is probably due to enzyme saturation at the higher dose. Saturation of catabolism may mean that higher amounts of 5FU are present in the host tissue which may lead to the formation of FNUC and consequently may reflect an increase of general toxicity.

The rapid disappearance of 2'FdURD signals following its administration suggests a faster conversion rate of 2'FdURD than 5'FdURD to 5FU, although, no 5FU was observed in the spectra following 2'FdURD or 5'FdURD. The lack of detectable signals from 2'FdURD or its intermediates in the pathway of FBALA production are probably because of the rapidity with which it was converted into intermediates in the pathway of breakdown, each of which is below the detection threshold. However, the fact that 5FU and 2'FdURD produce FBALA in such a similar pattern indicates that the availability of 5FU produced from 2'FdURD is not a limiting factor in the breakdown of 2'FdURD to FBALA; also the $t_{1/2}$ for 2'FdURD appears to be much less than that of 5FU so that the rate limiting step in the production of FBALA from 2'FdURD would, therefore, be after the production of 5FU.

In the examination of the metabolism of Ftorafur, which slowly releases 5FU [16], there was a sustained production of FBALA for at least 500 min (see Fig. 5). These results suggest that Ftorafur may provide a sustained but low level of 5FU. Ftorafur was reported to have a similar toxicity in clinical use to a slow infusion of 5FU; a method of 5FU administration designed to give reduced host toxicity with little or no loss of anti-tumour activity over bolus injections [21].

To detection of FBALA in the tumour following i.v. infusion demonstrates the possibility of FBALA uptake into tumours from the plasma (e.g. following 5FU catabolism in the liver) and suggests that the appearance of FBALA signals in tumours after 5FU administration does not require 5FU breakdown in the tumour itself.

The presence of 5FU in the spectra from the tumour following 5FU administration by i.v. bolus was more sustained than might be expected if 5FU may freely cross the cell membrane. From the graph in Fig. 4 we may calculate that the decrease in the level of 5FU detected in the tumour over 40 min is less than the expected decrease of 5FU in the blood calculated from the plasma $t_{1/2}$ of 5FU (15.3 + 2.5 min) [20] for a similar period. This slow decrease is probably a reflection of a slow rate of diffusion of 5FU out of the tumour and a lower rate of 5FU catabolism in the neoplastic tissue compared to liver [17].

The presence of 5FU signals following a bolus injection of 2'FdURD shows how the route of activation can be followed. Birnie *et al.* [22] originally demonstrated the rapid conversion of 2'FdURD to 5FU *in vitro*. We have demonstrated, *in vivo*, that a substantial proportion of 2'FdURD is not converted directly to FdUMP by the action of thymidine kinase in the tumour. The possibility of contamination of the spectra by 5FU released by the liver is unlikely due to the speed at which 2'FdURD is converted to FBALA in the liver.

The similarity in the amount of FNUC produced from 5FU or 2'FdURD given by bolus injection to the tumour is interesting, because both drugs produced almost 100% inhibition in tumour growth. The absence of FNUC signals after 2'FdURD infusion, though, is surprising. The size of the 5FU peak in these experiments appears to be at least as large as that obtained after 5FU infusion (compare Fig. 5A and B), and a bolus injection of 2'FdURD does cause FNUC formation (see Fig. 4). Furthermore, bolus injection of 2'FdURD causes tumour growth inhibition. We have no explanation for these discrepancies. It would have been interesting to have tested the effects of drug infusions on tumour growth, but unfortunately the S.G. Prolactinoma line was lost before this could be accomplished.

The rates of metabolism of 5FU and 5'FdURD have been measured *in vivo* in the rat liver. Similarly, the relative rates of metabolism in the liver of the 5FU analogues 2'FdURD and Ftorafur can be deduced from the pattern of production of FBALA observed by NMR. FBALA and 5FU have been detected by ^{19}F NMR in patients following the administration of 5FU [7] after 10-min infusion of 5FU. In further studies, however, data may be obtained, *in vivo*, in human studies which may allow the determination of relative rates of metabolism of 5FU, 2'FdURD and 5'FdURD in the liver of individual patients. This could assist the design of a drug regime specific to each patient.

The rates of metabolism of 5FU and 2'FdURD, measured by ^{19}F NMR, appear to be less in tumour than liver which is consistent with the general observations of Heidelberger *et al.* [17]. The S.G. Prolactinoma is sensitive to both 5FU and 2'FdURD and forms FNUC in apparently similar amounts when these drugs are given by bolus injection. Previous work [4, 23] suggests that there is a link between the level of FNUC detected by ^{19}F NMR in the Walker carcinosarcoma and the tumour inhibitory properties of 5FU. This suggests a role for ^{19}F NMR in the assessment of tumour activation of the fluoropyrimidines which will give a quick, non-invasive method to determine the effectiveness of drug dose.

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