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Original Paper

A Pharmacokinetic and Pharmacodynamic Study *In Vivo* of Human HT29 Tumours using ^{19}F and ^{31}P Magnetic Resonance Spectroscopy

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^{19}F-MRS (magnetic resonance spectroscopy) was used to study the pharmacokinetics of 5-fluorouracil (5-FU) in human (HT29) tumour xenografts, with and without pretreatment of the mice using either thymidine (40 min) or interferon-α (2 and 24 h). A 200 mg/kg i.p. bolus dose of 5-FU was eliminated from control tumours with a $t_{1/2}$ of 25.4 ± 2 min (mean ± SEM, $n = 11$), while both thymidine (500 mg/kg) and interferon (50 000 IU/mouse) significantly increased $t_{1/2}$ to 36.5 ± 6.1 ($n = 5$) and 48.1 ± 13.6 min ($n = 4$), respectively ($P = 0.04$, Gabriel's ANOVA). Thymidine increased 5-FU anabolism to cytotoxic 5-fluoronucleotides, and decreased the amount of tumour catabolites; the latter probably recirculated from liver since isolated HT29 cells did not catabolise 5-FU. These *in vivo* observations were confirmed by ^{19}F-MRS quantification of tumour extracts. Interferon did not significantly affect 5-FU metabolism in the tumour or liver, nor the 5-FU $t_{1/2}$ in liver. Treatment of tumours with 5-FU or interferon had no effect on tumour growth, whereas the combination strongly inhibited growth. ^{31}P-MRS of HT29 tumours showed that 2 and 24 h after i.p. injections of interferon there was a significant increase in the pH_{int} of 0.3 ± 0.04 units ($P = 0.002$), while pH_{ext} and the tumour NTP/Pi ratio were unchanged. The large increase in the negative pH gradient ($-\Delta$ pH) across the tumour plasma membrane caused by interferon suggests the Δ pH may be a factor in tumour retention of 5-FU, as recently shown in isolated tumour cells. © **1997 Elsevier Science Ltd.**

Key words: pharmacokinetics, interferon-α, 5-fluorouracil, human tumours, phosphorus/fluorine magnetic resonance spectroscopy
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INTRODUCTION

THE ANTICANCER drug 5-fluorouracil (5-FU) is the single most active agent in the treatment of colon and pancreatic cancer, and is also used in neo-adjuvant chemotherapy of other tumour types. As a single agent, response rates are low (10–20%), so there has been much research into the use of agents which modify its metabolism and might thereby improve its activity. These biomodulators include methotrexate, interferon-α, *N*-(phosphonacetyl)-L-aspartate and leucovorin [1]. 5-FU is a pro-drug, and a prerequisite for cytotoxicity is intracellular metabolism to the 5-fluoronucleotides, 5-fluoro-2′-deoxy uridine monophosphate (FdUMP) and 5-fluoro-uridine triphosphate (FUTP) (Figure 1). FdUMP inhibits DNA synthesis by forming a ternary complex with the enzyme thymidylate synthase (TS) and methylene-tetrahydrofolate. FUTP becomes incorporated into RNA causing disruption of further RNA and protein

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synthesis. 5-FU can also become mis-incorporated into DNA via FdUTP, causing cytotoxic single- and double-strand DNA breaks. Thus, 5-FU cytotoxicity can be DNA or RNA-directed, or a combination of the two depending on the cell type and the dose schedule used [2]. 5-FU is predominantly deactivated in the liver by catabolism to dihydrofluorouracil (DHFU), and the major catabolites, α-fluoro-β-ureidopropionic acid (FUPA) and α-fluoro-β-alanine (FBAL), are both excreted in the urine. Catabolism also occurs in peripheral blood mononuclear cells and in tumours [3], although tumour catabolism would be unlikely to contribute significantly to the rate of elimination. Inhibition of systemic catabolism of 5-FU can lead to higher and more sustained levels of 5-FU in plasma with corresponding improvements in the therapeutic ratio of 5-FU and 5-FU pro-drugs [4].

The distribution and metabolism of 5-FU that occurs in the body, i.e. 5-FU pharmacokinetics, can be monitored non-invasively in both liver and tumours using ^{19}F-magnetic resonance spectroscopy (^{19}F-MRS) *in situ* [5]. The MRS signal for 5-FU is well resolved from that of the catabolites (FCat), the fluoronucleosides (FNucs) and the fluoronucleotides (FNuct), although *in vivo*, the FNuct are detected as a single peak. The amount of MRS-detectable FNuct formed has been found to be a predictor of tumour cell response in cell lines [6], and in murine [7, 8] and rat tumours [9–11]. ^{19}F-MRS has also been used to monitor 5-FU metabolism in the tumours of patients receiving 5-FU therapy. Although the FNuct peak is rarely detectable, a significant association has been found between patient response and tumour 5-FU uptake [12] or 5-FU retention in the tumour using the half-life ($t_{1/2}$) for 5-FU elimination [13, 14]. The $t_{1/2}$s recorded in tumours are frequently greater than those measured in blood plasma (10–15 min following i.v. administration [15]), suggesting that trapping of 5-FU occurs in tumours [13]. The mechanism of this effect is unknown, but may be related to the pH of tumours. For example, use of ^{31}P-MRS (to measure tumour pH) and ^{19}F-MRS has demonstrated that 5-FU retention is enhanced in a rat fibrosarcoma when the tumour pH is lowered by glucose infusion [16]. Thus, although clinical results and pharmacokinetic studies have already shown that the presence of the pro-drug 5-FU *in situ* in tumours is significantly associated with cytotoxicity and response, further study of this phenomenon is necessary to elucidate the mechanism of why some tumours trap 5-FU, and this ought to aid the prediction of clinical response.

Using MRS, we performed a non-invasive, preclinical study of drug pharmacokinetics and pharmacodynamics in nude mice bearing the human colorectal adenocarcinoma HT29. We tested whether interferon-α has a specific effect on HT29 tumours which would favour 5-FU uptake and retention and/or metabolism. Pharmacokinetic studies used ^{19}F-MRS to study 5-FU metabolism in tumour and liver *in vivo*, and the metabolism was modulated using interferon-α (IFN) or thymidine (Thd). The pharmacodynamic effect of IFN on HT29 tumours was studied using ^{31}P-MRS, and in a growth inhibition study with and without 5-FU treatment.

MATERIALS AND METHODS

Chemicals and animals

Human recombinant interferon-α2a (IFN) was obtained from Hoffman-La Roche (Basel, Switzerland) as a freeze-dried powder and was reconstituted just prior to use by the addition of 0.9% saline to give 500 000 IU/ml. 5-FU was obtained from David Bull Laboratories (Warwick, U.K.) as a saline solution of 25 mg/ml. Thymidine (Thd), 5-fluorotryptophan (FTP) and 3-aminopropylphosphonate (3-APP) were obtained from Sigma (Poole, U.K.). All materials for cell culture were purchased from Gibco (Paisley, U.K.). MF1

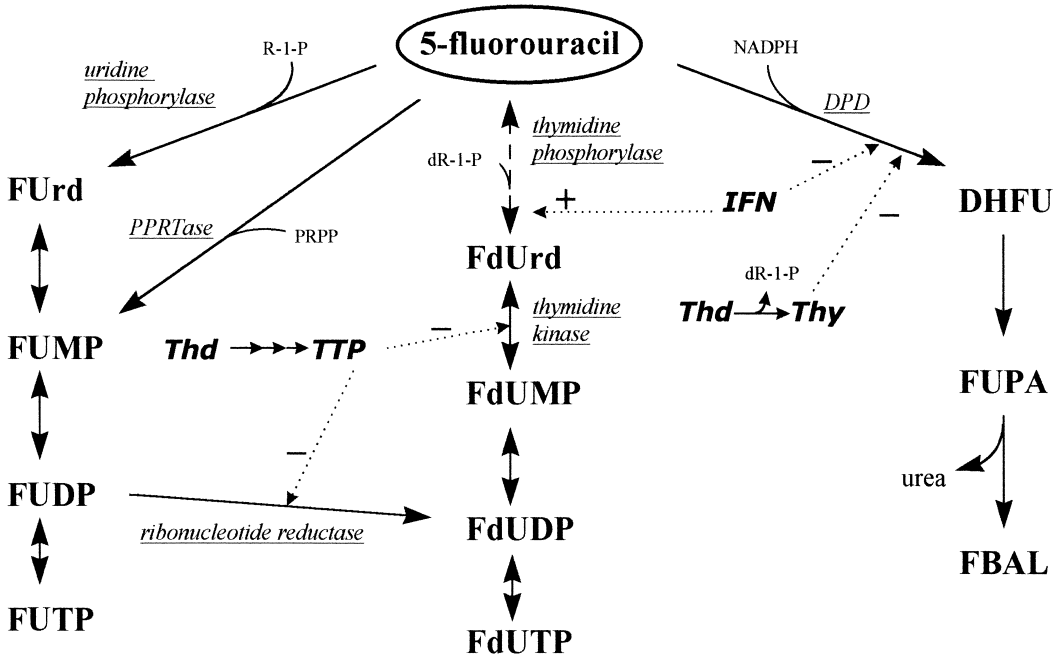

Figure 1. Metabolism of 5-FU showing the key regulatory enzymes and points of interaction with thymidine (Thd) and interferon-α (IFN). 5-FU metabolites are shown in bold, cofactors are shown in Roman type, enzymes are underlined. Abbreviations not defined in the text are as follows: R-1-P and dR-1-P, ribose and deoxyribose-1-phosphate, PRPP, phosphoribosyl pyrophosphate, PPRTase, pyrimidine phosphoribosyl transferase, DPD, dihydropyrimidine dehydrogenase. Broken line indicates that the activity of this pathway is normally reduced compared with that involving uridine phosphorylase. Minus sign indicates inhibition and positive sign stimulation.

nu/nu mice (6–8 weeks) were obtained from Harlan Olac, and maintained in the St. George's Hospital Medical School Biological Research Facilities under standard conditions.

HT29 cells and tumours

Cells were cultured in Ham's F10 or McCoy's medium containing 10% fetal calf serum (FCS) at 37°C in a 5% CO_2 atmosphere. For induction of tumours, cell suspensions (0.2 ml at 2.5×10^7/ml) were injected s.c. (subcutaneously) into anaesthetised nude mice. Tumours were measurable after two weeks and were large enough for MRS studies (> 250 mg) after 4–6 weeks. Tumour diameter was measured using Vernier callipers, and the volume determined by using the formula: length × width × height × (π/6).

For experiments *in vitro*, exponentially growing cells were incubated with and without IFN (50 000 IU/ml) for 18 h, and then removed using 0.01 M EDTA + trypsin and resuspended in the growth medium to a concentration of 1.5×10^8 cells/ml, in a final volume of 2 ml. The cell suspensions were gently shaken at 37°C for 15 min before the addition of 1 mM 5-FU, and the incubation continued for 1 h. A cell viability test using Trypan Blue exclusion indicated that $\geq$ 98% of the cells (± IFN) were viable prior to the addition of 5-FU. Cell viability was also determined by taking 100 µl aliquots for ion-exchange HPLC immediately prior to 5-FU addition and after 1 h incubation, and ATP/ADP ratios were measured as previously described [9]. The remaining cells were extracted for quantitative *in vitro* ^{19}F-MRS analysis by the addition of the cell suspension to 1 ml cold $HClO_4$ (20% v/v). The extract was neutralised using 20% KOH and freeze-dried.

For growth inhibition studies, tumours were measured every 2–3 days. Once a mean size of approximately 50 mg was reached, the animals were divided into groups, each with the same mean tumour size. Treatment was twice weekly by i.p. injection (maximum volume of 0.6 ml), using IFN (50 000 IU per mouse), 5-FU (50–150 mg/kg), Thd (500 mg/kg), or saline, or combinations of Thd + 5-FU, or IFN + 5-FU. In the combinations, the 5-FU injection was preceded by IFN (1 h), and Thd (40 min).

^{19}F-*MRS*

Tumour. Mice (30–35 g) were anaesthetised by i.p. injection of 0.3 ml of a mixture of hypnoval/hypnorm (12.5 mg/kg of midazolam and 25 mg/kg fluanisone, plus 0.8 mg/kg fentanyl citrate) in water, followed 10 min later by i.p. injection of 5-FU (200 mg/kg). Thd-treated groups received Thd (500 mg/kg i.p.) 40 min prior to 5-FU, while IFN-treated groups received IFN (50 000 IU per mouse i.p.) 24 h and a second equal dose 1–2 h prior to 5-FU. The mice were placed on a water-heated pad in a 4.7 T SISCO 200/330 spectrometer, and ^{19}F-spectra were acquired using a 12 mm, two-turn surface coil. Spectra were accumulated in 10 min blocks during the period 15–125 min (IFN studies) or 20–120 min (Thd studies), following 5-FU injection. Each 10 min block consisted of 1712 transients of 0.35 s, using a spectral width of 11 kHz with a 5 µs pulse (90° flip angle at coil centre was 7 µs). These parameters were found to give the optimum signal/noise ratio (SNR) for 5-FU and FNuct. At the end of the experiment, tumours were excised, freeze-clamped and stored at −190°C. Extracts were made as previously described [9].

For analysis of the ^{19}F data, sequential FIDs (3 × 10 min) were summed as previously described [9] and Fourier transformed using 50 Hz line broadening. All peaks were referenced to 5-FU (0 ppm); however, the 5-FU catabolites, FBAL and FUPA, could only occasionally be resolved *in vivo*, and thus the collective term FCat was used. The peak areas were obtained from a Lorentzian fit to the spectra using the SISCO software (FITSPEC). Graphic representation of the data (peak area versus time) shows the mean time point of the data e.g. a 35 min time point indicates a range of 20–50 min. The area under the curves (AUC) for 5-FU, FNuct and FCat were determined using the trapezoidal rule. While these studies were non-quantitative in the sense that no internal or external standard was used, changes in peak areas would reflect changes in drug and/or metabolite concentrations allowing an estimate of the rate of elimination of 5-FU from the tissue (K_{elim}) (see Pharmacokinetic Analysis below). Secondly, since the coil size and acquisition parameters were always the same (gains, spectral width, pulse width etc.), comparisons could be made of mean peak areas and AUC values between different treatment groups when the mean tumour sizes in each group were the same, so as to minimise the effects of coil loading. Further evidence for the validity of this semiquantitative approach is provided in Results.

Liver. Animals were anaesthetised and injected with 5-FU and/or IFN as described above. The 12 mm coil was positioned over the abdomen above the liver and spectra acquired as for tumour studies for 6 × 10 min. A better SNR was obtained compared with tumour spectra, allowing analysis of single spectra using the FITSPEC software described above.

Quantitation in vitro. Freeze-dried extracts were dissolved in 0.9 ml distilled water with 100 nmole 5-fluorotryptophan (FTP) and the pH was adjusted to 7.0. The glass tube was placed in a three-turn solenoid coil at 4.7 T and shimmed (15–30 Hz). ^{19}F-spectra were acquired ($\geq$ 4000 transients each of 14.2 s) using a 90° pulse (7 µs) with a spectral width of 25 000 Hz. The 14 s delay was sufficient to relax fully the FTP standard and 5-FU metabolites, and give > 90% relaxation for 5-FU. Peak areas were analysed as described above using 40 Hz line broadening.

^{31}P-*MRS and* ^{1}H-*MRS* in vivo

For these experiments, another cohort of tumour-bearing mice was used. The same conditions were used as those described for ^{19}F-MRS *in vivo*, using a 10 or 18 mm surface coil (depending on tumour size). 3-APP dissolved in distilled water (0.3 ml) was injected i.p. (1.4 g/kg) 15 min prior to spectra accumulation. Localised ^{31}P-spectra were acquired on volumes of 0.8 cm^3 within the tumours, using double ^{1}H-image-selected *in vivo* spectroscopy voxel acquisition (DIVA) as previously described [17]. This technique avoids signal contamination from other tissues underlying the tumour, and since the tumours used were 0.8–1 g, this meant that the ^{31}P-signals received reflected the average of 90–100% of the whole tumour. Mice received 0.2 ml IFN (50 000 IU per mouse) i.p. 24 h after the first ^{31}P-spectra were acquired, and a second equal dose approximately 24 h later, at 1–2 h prior to acquisition of a second ^{31}P-DIVA spectrum. Control mice were injected with 0.2 ml of 0.9% saline. ^{31}P-spectra were analysed using the VARPRO method to obtain the γ-NTP/Pi ratios and the chemical shifts between Pi and α-NTP and 3-APP to allow determination of extracellular pH (pH_{ext}) and intracellular pH (pH_{int}) as previously described [17, 18].

Pharmacokinetic analysis

The rate of signal loss of 5-FU fitted a one-compartment model of first-order elimination from the tissues, and curve fitting of the data using commercially available software (PHARMKIT) [19] was used to estimate the $t_{1/2}$ for absorption and the K_{elim} from the liver for 5-FU. Very similar results for K_{elim} could be obtained using a simple semi-log plot of the peak area against time, and so for comparison between liver and tumour this method was used throughout. For liver data, six time points were used, while for tumours, at least eight time points were used. Fits were good, giving P values always < 0.01 for the correlation coefficient r.

Statistical analysis

Significant differences between means were assessed using Student's t-test, a paired t-test, or where appropriate, Gabriel's one-way analysis of variance (ANOVA). Correlations between parameters were made using linear correlation to give the coefficient, r. A P value of < 0.05 was considered statistically significant.

RESULTS

^{19}F-MRS of HT29 tumours

The pro-drug was visible in all tumours receiving 5-FU alone, with the maximum signal intensity observed in the first acquisitions at 30–35 min postinjection, decreasing by at least 70% over the following 70 min (Figures 2(a) and 3(a)). The rate of elimination of 5-FU (K_{elim}) was reproducible, with an average of $2.98 \pm 0.41\%$ min^{-1} in one cohort of 5 tumours (451 ± 71 mg) (Figure 2), and $2.52 \pm 0.2\%$ min^{-1} in another cohort of 6 larger tumours (821 ± 145 mg) (Figure 3; Table 1). The mean K_{elim} values were not significantly different between these two groups, and indeed there was no significant correlation between tumour size (g) and K_{elim} ($r = -0.25$, $P = 0.46$). A small peak detectable at about 4.5–5 ppm downfield from 5-FU, and consistent with the

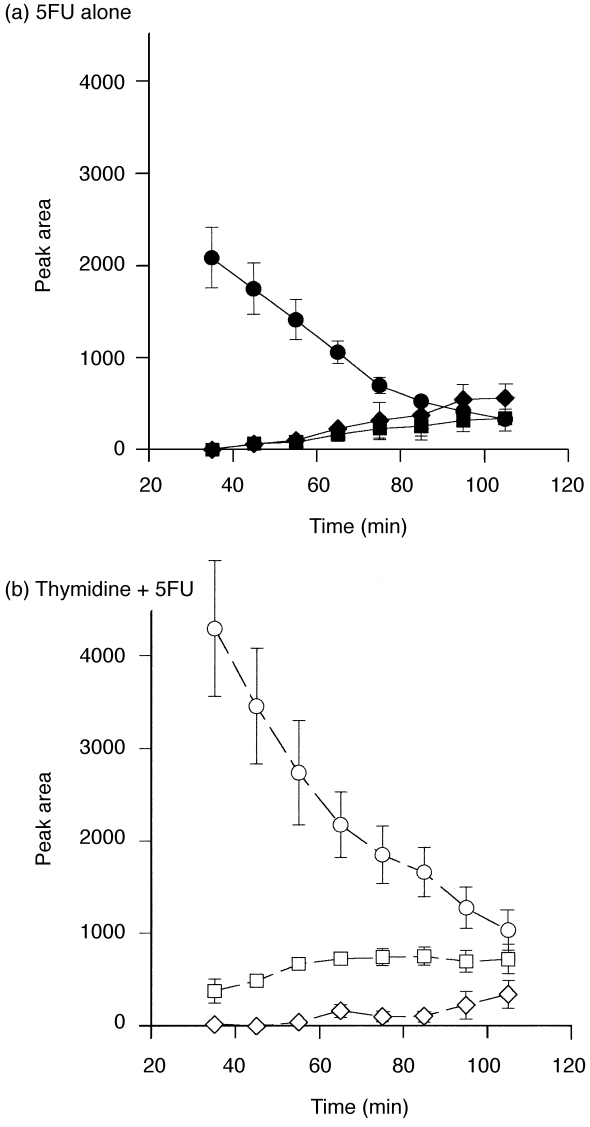

Figure 2. 5-FU pharmacokinetics in HT29 tumours without (a) and with (b) Thd pretreatment. Results show the mean peak area ± SEM of 5-FU (●, ○), FNuct (■, □) and FCat (◆, ◇) (FBal and FUPA) in five tumours at 35–105 min postinjection of 5-FU (200 mg/kg i.p.).

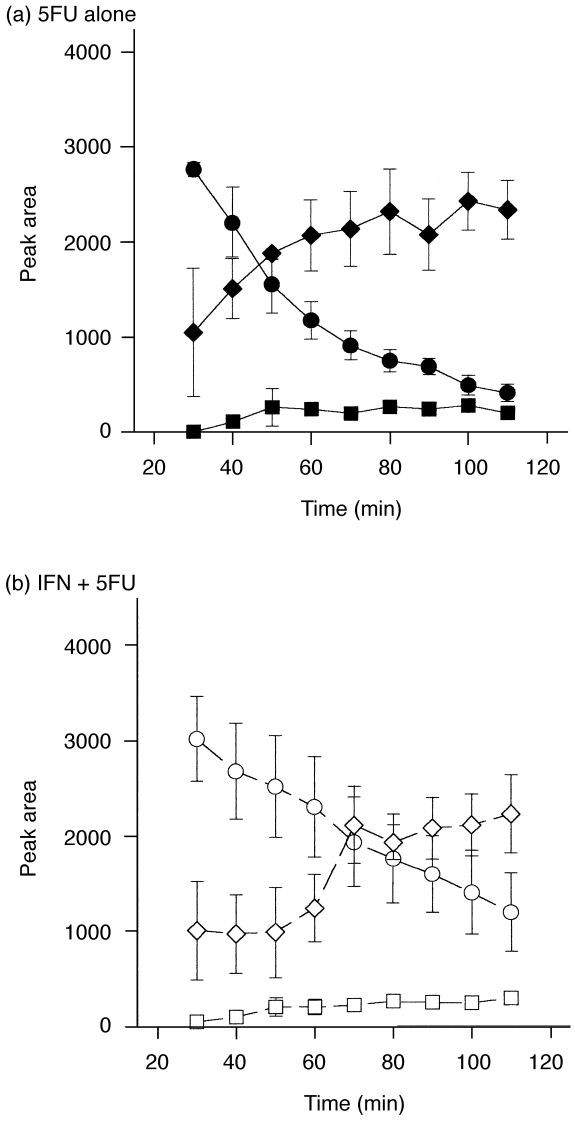

Figure 3. 5-FU pharmacokinetics in HT29 tumours with and without IFN pretreatment. Results show the mean peak area ± SEM of 5-FU (●, ○), FNuct (■, □) and FCat (◆, ◇) (FBal and FUPA) in six tumours (5-FU alone) or four tumours (IFN + 5-FU) at 30–110 min postinjection of 5-FU (200 mg/kg i.p.).

Table 1. *Effect of IFN and Thd on 5-FU pharmacokinetics in liver and tumour*

Group	Tissue size (mg)	Treatment	K_{elim} (%/min) 5-FU	$t_{1/2}$ (min) 5-FU	5-FU Area under curve (AUC × 10^{-2})	FNuct Area under curve (AUC × 10^{-2})	FCat Area under curve (AUC × 10^{-2})
1	HT29: 821 ± 145 (6)	5-FU	2.52 ± 0.20	26.5 ± 1.9	921 ± 154	166 ± 28	1602 ± 274
2	HT29: 960 ± 320 (4)	IFN + 5-FU	1.45 ± 0.41**	48.1 ±13.6	1625 ± 377	163 ± 28	1303 ± 242
					NS	NS	NS
3	HT29: 451 ± 71 (5)	5-FU	2.98 ± 0.41	23.2 ± 3.2	705 ± 98	130 ± 51	190 ± 85
4	HT29: 471 ± 82 (5)	Thd + 5-FU	1.90 ± 0.32*	36.5 ± 6.1	1580 ± 261	462 ± 53	82 ± 42
					$P = 0.014$	$P = 0.002$	NS
5	Liver (5)	5-FU	4.13 ± 0.39	16.8 ± 1.6	961 ± 154	—	1055 ± 481
6	Liver (4)	IFN ± 5-FU	3.28 ± 0.19	21.1 ± 1.2	1128 ± 250	—	1427 ± 316
					NS		NS

Tumour and liver pharmacokinetic data were obtained from the results shown in Figures 2–4, and show the mean ± SEM from (n) animals. $t_{1/2}$ is directly related to K_{elim} by the function $t = (0.693/k)$. Mean K_{elim} values were compared using Gabriel's one-way ANOVA for groups 1–4. **$P = 0.05$ group 2 versus groups 1 and 3, *$P = 0.05$ group 4 versus size-matched control group 3. When using a two-sample t-test to compare group means for K_{elim}, $P = 0.029$ group 1 versus 2, $P = 0.074$ group 3 versus 4, and $P = 0.073$, group 5 versus 6. AUC values were compared using a two-sample t-test in size-matched groups to give the P values. NS, non-significant.

presence of the cytotoxic FNuct anabolites, increased only very slightly, or in some cases not at all, giving an average FNuct/5FU ratio at 105–110 min (the last time point, Ltp) of 0.5–1 (calculated from data in Table 2). This low anabolic rate of HT29 tumours could have been due to either the absence of the necessary anabolic enzymes, or a low energy state of the cells. Histology revealed quite significant necrosis (33 ± 5%, mean ± SEM, $n = 6$) in tumours of 0.3–0.7 g, suggesting the tumours overall had a poor blood supply, and/or low pO_2 content. Indeed, HPLC analyses of freeze-clamped tissue from the cohort of five smaller tumours (mean size 0.5 g) gave low ATP/ADP ratios of 0.2–0.7 (results not shown), consistent with a low anabolic capacity in these HT29 tumours.

The FCat peak, upfield from 5-FU (approximately −19 ppm), was also visible in the first acquisitions, and increased with time, with a plateau at around 90 min postinjection. The source of this FCat was probably recirculation from 5-FU catabolised in the liver, and not catabolite formation within the tumour cells. This was confirmed by incubating a high concentration of 5-FU (1 mM) with cultured HT29 cells for 1 h, followed by extraction and analysis by quantitative MRS *in vitro*. The only metabolites detectable were FNuct, at a concentration of 29 nmole/10^8 cells, suggesting that the 5-FU catabolic pathway was inactive in these cells. A much greater level of FCat was detected in the cohort of larger tumours (Figure 3) than in the smaller tumours (Figure 2) (and comparing groups 1 and 3 in Table 1). Indeed, a plot of FCat AUC/5-FU AUC (to allow for the fact that larger tumours would have greater ^{19}F-signals) against tumour size showed a significant correlation ($r = 0.72$, $P = 0.012$), suggesting that larger tumours accumulated FCat more readily in the tumour intra- or extracellular space.

Modulation of tumour metabolism

Pretreatment of mice with Thd or IFN significantly altered 5-FU pharmokinetics in the tumour (Figures 2 and 3, respectively), and combining the control groups 1 and 3 (see Table 1) gave a mean K_{elim} of 2.73 ± 0.48 (95% CI) which was significantly different from the K_{elim} of both the IFN and Thd treated groups ($P = 0.04$, Gabriel's ANOVA).

Over the period studied, Thd doubled the total amount of ^{19}F-signal in the tumour, as judged by the overall increases in the AUC (Table 1). There were marked, significant increases in the total 5-FU, and particularly FNuct AUC, reflecting a more rapid rate of appearance of FNuct compared with controls (Figure 2). The total FCat levels were very variable, and while on average Thd treatment reduced FCat AUC by approximately 50%, this was not significant (Table 1). The 5-FU K_{elim} was reduced 1.6-fold, and the mean 5-FU and FNuct peak areas at the Ltp of 105 min were increased 2–3-fold (Table 2). Consistent with these findings *in vivo*, quantitative MRS analysis *in vitro* of these tumours freeze-clamped 2 h after 5-FU administration showed that FNuct and 5-FU

Table 2. *Effect of IFN and Thd on* 5*-FU pharmacokinetics in liver and tumour at the last time point (Ltp)*

Group	Tissue size (mg)	Treatment	5-FU Peak areas at the last time point (Ltp)	FNuct Peak areas at the last time point (Ltp)	FCat Peak areas at the last time point (Ltp)	5-FU nmoles in tumour extract	FNuct nmoles in tumour extract	FCat nmoles in tumour extract
1	HT29: 821 ± 145 (6)	5-FU	402 ± 94	194 ± 47	2327 ± 772	—	—	—
2	HT29: 960 ± 320 (4)	IFN ± 5-FU	1186 ± 418	289 ± 44	2224 ± 409	—	—	—
			NS	NS	NS			
3	HT29: 451 ± 71 (5)	5-FU	327 ± 109	347 ± 132	562 ± 123	10 ± 7	30 ± 8	42 ± 6
4	HT29: 471 ± 82 (5)	Thd + 5-FU	1028 ± 213	756 ± 130	341 ± 151	41 ± 7	50 ± 2	26 ± 5
			$P = 0.019$	NS	NS	$P = 0.011$	$P = 0.039$	$P = 0.07$
5	Liver (5)	5-FU	577 ± 130	—	2890 ± 1137	—	—	—
6	Liver (4)	IFN ± 5-FU	726 ± 191	—	3622 ± 909	—	—	—
			NS		NS			

All results show the mean ± SEM of (n) tissues. The last time points are from the graphs in Figures 2 (105 min), 3 (110 min) and 4 (65 min). Tumour extracts were made at the end of the *in vivo* measurements (120 min postinjection of 200 mg/kg) and analysed as described in Materials and Methods. Mean values of sized match groups were compared using a two-sample t-test to give the P values. NS, non-significant.

were significantly increased, while FCat was slightly lower compared with control tumours (Table 2).

These quantitative *in vitro* findings were important since they demonstrated that the peak areas determined *in vivo* were proportional to the concentrations of the administered pro-drug, 5-FU, and its metabolites in the tumour. Figure 4 shows that there was a significant correlation between the FNuct Ltp and the amount of FNuct found in the freeze-clamped extracts ($r = 0.64$, $P = 0.046$). However, as would be expected, no significant correlation was found for 5-FU ($r = 0.5$, $P = 0.16$), reflecting the fact that the Ltp represented a period of 30 min and the 5-FU concentration was changing rapidly in the tumour ($t_{1/2}$ of 21–30 min), while the FNuct value was fairly stable during the last 60 min of the experiment.

IFN did not significantly affect the initial amount of 5-FU or the formation of FNuct in the tumours (Figure 3, groups 1 and 2 in Tables 1 and 2). This was confirmed in a single isolated cell experiment where 5-FU metabolism was unaffected by an 18 h pre-incubation of the cells with IFN (FNuct reduced from 29 to 26 nmole/10^8 cells). However, IFN did significantly reduce the K_{elim} by approximately 2-fold, thus increasing the 5-FU AUC and the final mean peak area at the Ltp, albeit non-significantly. The appearance of FCat in the tumour appeared to be slightly delayed by IFN (Figure 3), but this also did not significantly affect the FCat AUC or Ltp (Tables 1 and 2).

^{19}F-*MRS of mouse liver*

5-FU and FCat peaks were visible in host liver following injection of the same dose of 5-FU (200 mg/kg), although again, the FNuct peak intensity where visible, was very low. The 5-FU K_{elim} of 4.13 ± 0.39% was greater than that in the tumour (Table 1), and the 5-FU signal had, in most cases, disappeared 70 min after injection, while FCat signals tended to plateau at approximately 60 min (Figure 5). IFN caused a 21% decrease in the 5-FU K_{elim}, which was not significantly different from controls; FCat levels were unaffected (Table 1). Curve-fitting of the data allowed a $t_{1/2}$ of absorption into the liver to be inferred, which gave 6.4 ± 1.9 min for controls and 8.0 ± 2.5 min for IFN-treated animals (mean ± 95% confidence limits), values which were not significantly different. Thd modulation of liver 5-FU pharmacokinetics was not studied.

^{31}P-*MRS of HT29 tumours*

The pharmacodynamic effect of IFN treatment on HT29 tumours was studied using DIVA ^{31}P-MRS. Figure 6 shows typical ^{31}P-spectra of HT29 tumours, before and after IFN treatment, with the transmitter frequency set at 3-APP (FID 1) for measurement of pH_{ext}, and on α-NTP for pH_{int} (FID 2). In this tumour there was a downfield shift of the Pi peak of 0.18 ppm consistent with an increase in pH_{int} of 0.17 units, while the pH_{ext} was unchanged. In all IFN-treated tumours there was an increase in the pH_{int}, giving a highly significant mean increase of 0.30, while the pH_{ext} was unaffected (Table 3). Pretreated HT29 tumours had a negative pH gradient across the cell membrane ($-\Delta$ pH) of between 0.14 and 0.2, typical of solid tumours where the pH_{ext} can be substantially lower than the pH_{int} [20]. In the 14 pretreated tumours, tumours with a higher pH_{int} had a greater $-\Delta$ pH. IFN caused a significant increase in this $-\Delta$ pH of 0.30, 24 h after treatment (Table 3). The energy state of the tumours, as judged by the γ-NTP/Pi ratio, was not significantly affected: 1.27 ± 0.12 pre- and 1.29 ± 0.15 post-IFN treatment, which are values in the normal range for animal tumours [17]. Injection of the vehicle saline did not significantly affect any of the parameters measured.

Growth inhibition of HT29 tumours

Once tumours had reached a mean size of > 200 mg, growth proceeded exponentially, with a doubling time of approximately 5.4 ± 1.7 (mean ± 95% CI) days. The tumours were resistant to 5-FU, even when high doses of 150 mg/kg were used twice weekly. Treatment with IFN alone also had no effect on tumour growth, while the combination of IFN and 5-FU blocked growth completely during the treatment period (Figure 7). The final mean tumour size was significantly

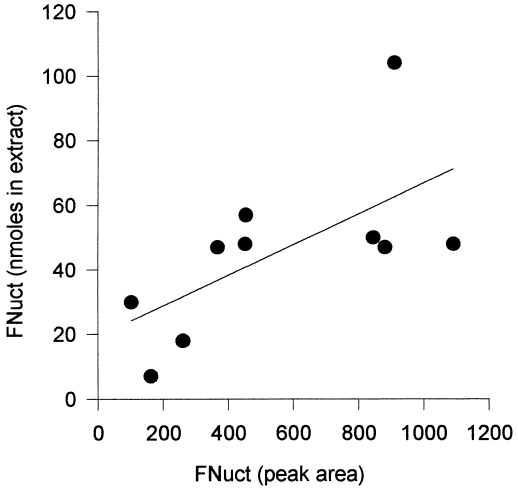

Figure 4. Correlation of FNuct peak area with quantitated FNuct value in tumour extracts. Results from 10 tumours ± Thd treatment. Equation for the regression line is $Y = 19.4 + 0.047X$ ($r = 0.64$, $P = 0.046$). FNuct peak area *in vivo* and FNuct value in extracts (nmoles) were determined as described in Materials and Methods.

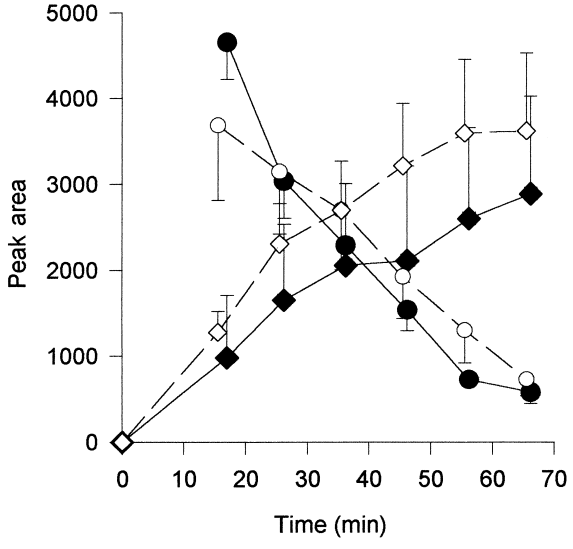

Figure 5. 5-FU pharmacokinetics in mouse liver with and without IFN pretreatment. Results show the mean peak area ± SEM of 5-FU (●, ○) and FCat (◆, ◇) in five livers (5-FU alone, closed symbols) or four livers (IFN + 5-FU, open symbols), at 15–65 min postinjection of 5-FU (200 mg/kg i.p.).

different to that of the saline group using Student's *t*-test ($P = 0.008$), but in an ANOVA it did not quite reach significance compared to all groups ($P = 0.055$) (Figure 7). The study was discontinued at 25 days postinduction due to significant toxicity in the groups receiving 5-FU: 3 died in each group, and there was cyanosis and laboured breathing. However, up until that point there had been no evidence of significant toxicity in any of the three treated groups (no change in body weight compared to controls), and post mortem examinations did not reveal evidence of peritonitis or internal haemorrhage. Thus, IFN did not increase 5-FU toxicity in this model. Experiments conducted with a lower concentration of 5-FU (50 mg/kg), which was not toxic, failed to elicit any change in the growth of tumours independent of the combined use of Thd or IFN (results not shown).

DISCUSSION

We have shown that 5-FU pharmacokinetics can be monitored non-invasively by ^{19}F-MRS in human HT29 xenografts in mice, and modulated by pretreatment with thymidine or interferon-α. ^{31}P-MRS demonstrated that IFN increased tumour pH_{int} and the negative Δ pH across the plasma membrane, which may have been directly responsible for the reduced 5-FU K_{elim} observed in the HT29 tumours. Unlike many animal tumour cell lines [5–11], these tumours essentially did not anabolise 5-FU to FNuct. This is similar to the clinical setting in which tumour or liver FNuct signals are rarely detected, while 5-FU and its catabolites are readily visible [21]. Nevertheless, in patients, the 5-FU signal is significantly associated with response [12], and a $t_{1/2}$ for 5-FU retention of ≥ 20 min can predict an objective response

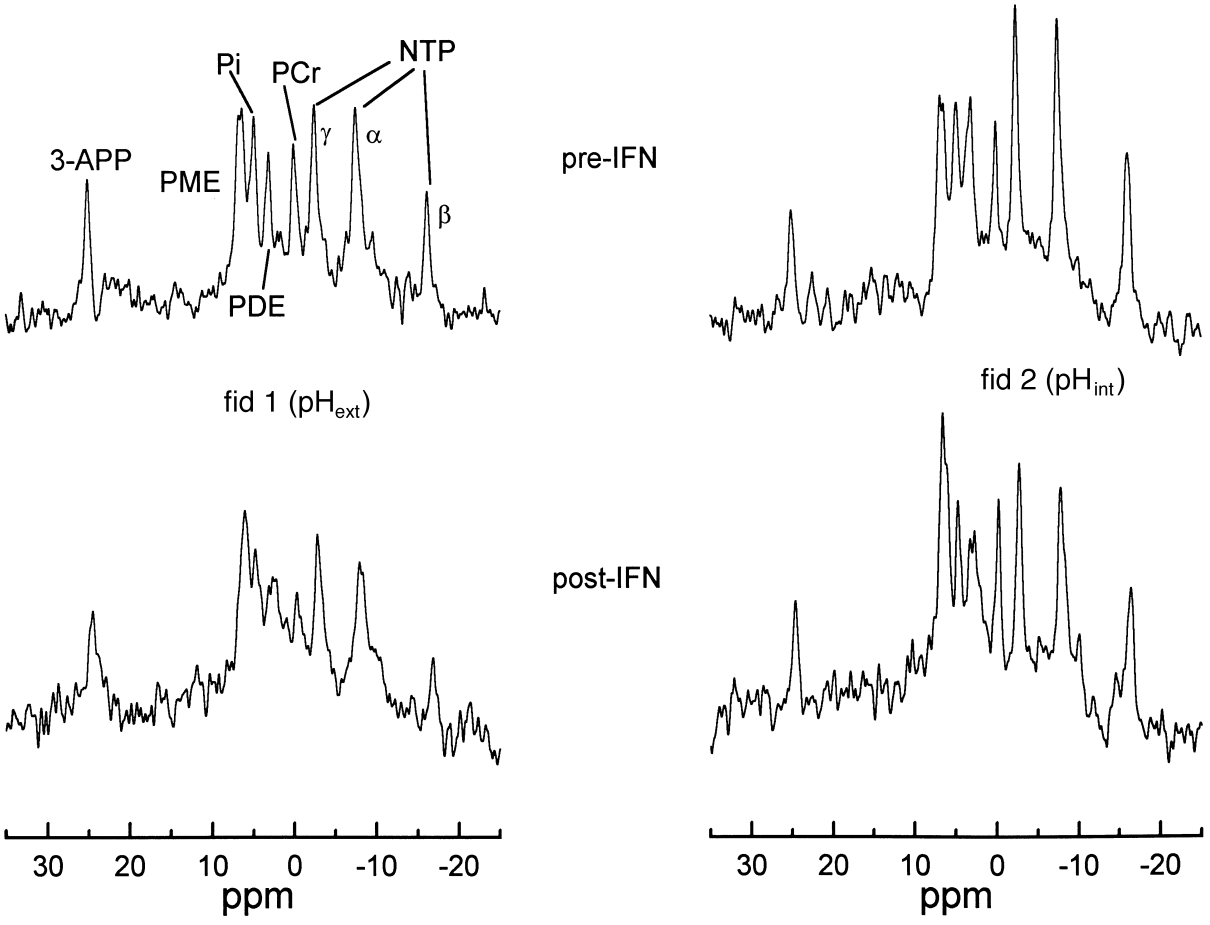

Figure 6. DISIS-localised ^{31}P-spectra of an HT29 tumour (1.1 g) pre- and post-IFN treatment. Peaks are labelled using the following abbreviations: 3-APP, 3-aminopropyl phosphonate, PME, phosphomonoesters, Pi, inorganic phosphate, PDE, phosphodiesters, PCr, phosphocreatine, NTP, nucleoside triphosphates (mostly ATP). FID 1 and 2 were used to determine pH_{ext} and pH_{int}, respectively, which were 6.87 and 7.08 for pretreated and 6.86 and 7.25 for post-treated.

Table 3. Effect of interferon (IFN) on the pH of HT29 tumours

Treatment	Tumour size (g)	pH_{int}	Mean change pH_{int}	pH_{ext}	Mean change pH_{ext}	Δ pH	Mean change Δ pH
None	0.88 ± 0.13	7.02 ± 0.06 (6)	—	6.82 ± 0.06 (6)	—	−0.20 ± 0.03 (6)	—
Saline	0.96 ± 0.13	7.04 ± 0.06 (6)	0.02 ± 0.05†	6.90 ± 0.07 (6)	+0.08 ± 0.06‡	−0.14 ± 0.03 (6)	+0.06 ± 0.04§
None	0.90 ± 0.11	6.98 ± 0.05 (8)	—	6.84 ± 0.04 (8)	—	−0.14 ± 0.05 (8)	—
IFN	1.01 ± 0.11	7.27 ± 0.05 (8)	+0.30 ± 0.04⋆	6.85 ± 0.04 (7)	+0.01 ± 0.04†	−0.41 ± 0.08 (7)	−0.30 ± 0.04⋆

Results show the mean ± SEM pH values from (*n*) tumours. The significance of the mean changes in pH following treatment with IFN or saline were tested using a paired *t*-test which gave the following *P* values: ⋆ = 0.0002, † ≥ 0.75, ‡ = 0.26, § = 0.16.

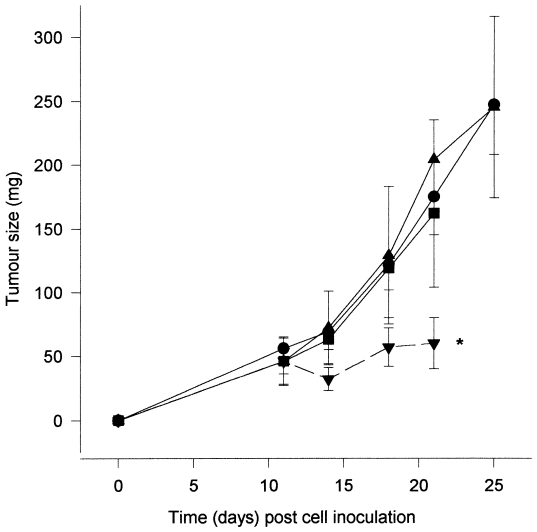

Figure 7. Growth inhibition of the HT29 tumour by combination of IFN with 5-FU. Results show mean ± SEM of nine tumours per group, where *$P = 0.055$ compared to groups receiving saline or IFN (Gabriel's one-way ANOVA). Saline (●), IFN (▲), 5-FU (■), IFN + 5-FU(▼).

[13, 14]. Since unmetabolised 5-FU is not cytotoxic, one assumes that FNuct is formed, but at a concentration below the detection level of MRS, which is approximately 0.1 μM in animal tumours at 2 T [22]. These observations suggest that modulation of the tumour environment designed to increase retention of the pro-drug 5-FU would improve clinical response.

Modulation by thymidine

Thymidine (Thd) can affect the overall pharmacokinetics of 5-FU by at least three different mechanisms (Figure 1), but the major effect is likely to be on systemic levels of circulating 5-FU, via a competitive inhibition of the catabolic enzyme dihydropyrimidine dehydrogenase (DPD) in liver and other tissues [15, 23]. ^{19}F-MRS has confirmed that this leads to a decrease in the catabolite/anabolite ratio and an increase in the retention of 5-FU in both murine liver [5, 8, 24], and mouse [8] and human tumour xenografts [25]. In our study, Thd increased the initial 5-FU level in the tumour by approximately 2-fold, and decreased the K_{elim} leading to an increase in the AUC and the final 5-FU level at 105 min. This increased retention of 5-FU was confirmed in the freeze-clamped tumour extracts (taken at 115 min post-drug administration): the small residual 5-FU remaining at this point was increased 4-fold. These results are consistent with the systemic effect of Thd to increase plasma levels of 5-FU and reduce whole body elimination. The secondary role of Thd to reduce tumour catabolism is probably not relevant in these tumours, since isolated HT29 cells did not produce detectable levels of FCat, and the FCat detected *in vivo* and in the extracts probably recirculated from the deactivation of 5-FU in the liver. The initial rate of FNuct formation was trebled, which led to a 3.5-fold increase in the AUC value, although the Ltp and the quantified-extracts showed that the FNuct was doubled. More than 50% of the MRS-detectable FNuct in animal tumours is FUTP [6, 9, 10] which is incorporated into RNA, and thus the 2-fold increase in FNuct was consistent with isolated cell experiments in which Thd increased FU-RNA in HT29 cells [26].

Modulation by interferon

There are numerous reports of synergistic interactions between IFN and 5-FU in tumour models involving several different mechanisms [1]. Observations in tumour cell lines treated concurrently with 5-FU and IFNα or γ include increased activity of Thd phosphorylase and increased levels of FdUMP [27, 28], abrogation of TS upregulation [29, 30] and increased DNA damage [31]. A possible additional interaction, which may contribute *in vivo*, is the effect of IFN on the pharmacokinetics of 5-FU, with decreased activity of DPD [32], leading to reduced 5-FU clearance and increased plasma levels in some [33, 34] but not all clinical studies [35].

In this study, no alteration in MRS-detectable FNuct was detected following IFN treatment, although increases in tumour FdUMP concentrations, or the ternary complex of FdUMP-TS, would not be detectable by MRS *in vivo* [22]. We did, however, detect a reduction in the rate of elimination of 5-FU from tumours. This change was not a result of alterations in plasma 5-FU pharmacokinetics: the human IFN would not be expected to affect 5-FU metabolism in murine tissues, and this was confirmed by its lack of effect on 5-FU pharmacokinetics in mouse liver. It therefore represented an effect of IFN on the uptake or retention of 5-FU in the human xenograft tissue. Of course, increased intracellular retention of 5-FU *per se* could lead to increased levels of FNuct in the form of FUTP which is formed at much higher concentrations than FdUMP. However, if the rate of FUTP formation in HT29 cells were already maximal, then increased tumour 5-FU levels may eventually only lead to more sustained levels of FU-RNA, which would also be undetectable *in vivo* [22].

Tumour uptake and retention of 5-FU

The mechanism by which IFN can decrease the tumour 5-FU K_{elim} is intriguing. It is possible that the magnitude of the negative pH gradient across the cell plasma membrane ($-\Delta$ pH) may play a role in the relatively long retention of 5-FU by tumours compared with other tissues. Solid tumours have a slightly alkaline pH_{int}, similar to normal tissues, but a lower pH_{ext}, and thus have a $-\Delta$ pH [20]. An ^{19}F-MRS study of rat fibrosarcomas showed that 5-FU elimination was 2.5-fold slower at a glucose-induced lower pH of < 6.9 than at pH 7.3 [16]. pH_{int} and pH_{ext} were not distinguished in this study, although the pH measured was most likely to represent pH_{int} [36]. A glucose-induced reduction of pH_{int} leads to an even greater decrease in pH_{ext} [37], and thus it is possible that the increased retention or 'trapping' of 5-FU observed in the rat fibrosarcoma was related to the difference between pH_{int} and pH_{ext} across the tumour cell membrane. This hypothesis is consistent with observations made on isolated Lettre and HT29 cells, in which the $-\Delta$ pH correlated significantly with 5-FU uptake over the pH range 6–8 [38]. IFN treatment markedly increased the pH_{int} of HT29 tumours, while pH_{ext} was unaltered, leading to a large increase in the $-\Delta$ pH from a mean of 0.14 to a mean of 0.41. Our hypothesis suggests that the larger $-\Delta$ pH would tend to increase the intra/extracellular 5-FU ratio, and thus presumably reduce the return of 5-FU to the plasma. Pharmacokinetically, this would be reflected as a decrease in the K_{elim} of 5-FU from the tumour. Indeed, experiments using isolated human lymphoma cell lines have shown that both IFN-α [39] and IFN-γ [40, 41] can permanently increase the pH_{int} of cells, and a similar effect induced by IFN-α has been reported in murine

tumours *in vivo* [42]. The mechanism is thought to involve increased synthesis or functional expression of the Na^+/H^+ antiporter in the plasma membrane. Increased expression resets the pH_{int} control to a higher value, so that pH_{int} remains elevated [43].

Prolonged retention of 5-FU in human and mouse tumours relative to plasma has been demonstrated [44]. These low levels of tissue 5-FU, detected by HPLC of biopsies, may have resulted from the breakdown of FU-RNA. 5-FU formed in this way would provide substrate for conversion to FdUMP, and thus maintain long-term inhibition of TS. These results provide a mechanism by which longer retention of a pro-drug can be correlated with the responses observed in the clinic [13, 14]. In fact, in the present study on the HT29 tumour model, the IFN–5-FU combination completely inhibited tumour growth, while either drug alone was without effect. Since IFN has been shown to decrease the TS levels of these same HT29 cells *in vitro* [30], the pharmacokinetic effect that we have observed is probably not the only factor in this marked synergism. However, these non-invasive studies have elucidated a new mechanism by which IFN may augment 5-FU cytotoxicity.

In conclusion, the current study using a human colon xenograft model indicates a novel mechanism by which IFN may interact with 5-FU, and suggests that altering the tumour pH environment to promote tumour retention of 5-FU, or indeed other chemotherapeutic drugs, could be important for therapeutic gain. In addition, inhibition of 5-FU catabolism by Thd can also promote tumour retention of 5-FU, consistent with recent preclinical studies investigating the efficacy of 5-FU pro-drugs in combination with catabolic inhibitors [4]. Non-invasive monitoring of anticancer drugs to give pharmacokinetic and pharmacodynamic information may aid rational drug development and applications in the clinic.

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