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ORIGINAL ARTICLE

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Pre-treatment energy status of primary rat tumours as the best predictor of response to 5-fluorouracil chemotherapy: a magnetic resonance spectroscopy study in vivo

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Abstract *Purpose*: Fluorine-19 magnetic resonance spectroscopy (^{19}F-MRS) studies of the pharmacokinetics of the anticancer drug 5-fluorouracil (FU) in patients at several clinical centres have shown that increased tumour retention of FU is associated with patient response. The mechanism of this increased tumour retention (FU trapping) is unknown. We used a pre-clinical model to investigate whether other MRS-measurable parameters would correlate with the response to FU treatment and, thus, help elucidate the mechanism(s) involved in FU trapping. *Methods*: MRS spectra were obtained using a double-tuned (^{31}P/^{19}F) surface coil from 29 *N*-methyl-*N*-nitrosourea-induced primary rat tumours. ^{31}P-MRS spectra were acquired immediately prior to and at 2.5 h post-treatment with a bolus i.p. injection of FU (100 mg/kg); ^{19}F-MRS spectra were acquired during the intervening 2.5-h period for measurement of the tumour uptake and retention of FU and of its metabolism to the cytotoxic fluoronucleotides (FNuct). From these data, four parameters were measured: tumour pH and energy status (NTP/Pi) before treatment, total FU retention, and FU anabolism to FNuct (expressed as micromoles per gram per 2.5 h). In addition, tumour response was determined at 7 days post-treatment by measurement of the percentage of change in tumour weight and was classified according to standard oncological criteria as follows: progressive (P) for a ≥25% increase, remissive (R) for a ≥50% decrease or stable (S) for values lying between these two. *Results*: Analysis of variance (ANOVA) for statistical assessment revealed that groups P, S and R were not distinguishable using the MRS parameters; although when S and R were combined as one group of non-progressive disease (NPD; $n = 24$), both the NTP/Pi ratio and the total FNuct formed were significantly greater ($P = 0.04$) than those observed in the P group ($n = 5$). Considering all 29 tumours, linear regression showed that there were positive significant correlations between the NTP/Pi ratio and (a) the percentage of response ($P = 0.04$), (b) the pre-treatment pH ($P = 0.002$) and (c) FU retention ($P = 0.02$), but not FNuct formation ($P = 0.66$). Unlike results reported in the clinic, the percentage of response and FU retention were neither significantly correlated ($P = 0.22$) nor associated when groups P and NPD were compared ($P = 0.27$, Fischer's exact test). FNuct, however, was significantly associated with response, as was the NTP/Pi ratio ($P \leq 0.02$). Combination of FNuct with the NTP/Pi ratio increased the significance of the association with response ($P = 0.003$, Fischer's exact test). *Conclusions*: Our results indicate that in this particular model the pre-treatment tumour NTP/Pi ratio was the best predictor of response to a bolus injection of FU, rather than FNuct formation or FU retention. An elevated NTP/Pi ratio could reflect a well-vascularised tumour with an improved capacity for energy-dependent FU uptake and metabolism to FNuct, suggesting that further investigation of this parameter could be an important line of research, which may aid the identification of tumours likely to be sensitive to FU chemotherapy in the clinic.

Key words ^{19}F/^{31}P- NMR spectroscopy · 5-Fluorouracil · Primary tumours

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Introduction

5-Fluorouracil (FU) is an antineoplastic fluoropyrimidine drug used in the treatment of several common

malignancies. The antitumour action of this drug depends on the intracellular uptake and conversion of FU to 5-fluoronucleotides (FNuct) with subsequent binding of 5-fluoro-2′-deoxyuridine monophosphate (FdUMP) to the enzyme thymidylate synthase, incorporation of 5-fluorouridine triphosphate (FUTP) into RNA and/or incorporation of 5-fluoro-2′-deoxyuridine triphosphate (dFUTP) into DNA [1, 2]. This anabolic activation of FU is well documented [1], but the mechanism by which FU enters the tumour cells remains controversial. Evidence for different types of transport, ranging from a purely passive phenomenon to an active process or a facilitated transport has been presented [3, 4]. Studies of rats tumours in vivo [5] and in isolated cells [6, 7] suggest that tumour pH and intracellular ATP may be important factors in the so-called *FU trapping* that has been described in the clinic and in laboratory animals [8].

Fluorine magnetic resonance spectroscopy (^{19}F-MRS) is a non-invasive technique that has successfully been used to monitor the pharmacokinetics of xenobiotics in vivo, with both pre-clinical and clinical applications [9, 10]. FU anabolites are rarely detected in patients, unlike animal models, probably due to the lower does employed and, possibly, also because the low amount of FNuct formed would be bound to the target enzyme and/or incorporated into DNA or RNA and would thus not be MRS-visible [11]. In patients, longer retention of FU by the tumour, i.e. FU trapping, has been significantly associated with patient response [12], an observation that may lead to a clinical test that could allow non-responders to receive more appropriate therapies earlier. Indeed, an ideal test would measure some tumour parameter that would detect non-responders prior to administration of FU.

Although FU retention can predict the therapeutic outcome, the mechanism underlying this drug retention and, thus, the tumour sensitivity or resistance to FU, remains unknown. Of course, other mechanisms for tumour resistance to FU therapy exist, such as up-regulation of target enzymes; however, clinical results have shown a very strong statistical association between response and FU trapping [12]. To look at this phenomenon we used a pre-clinical model of chemically induced primary tumours in rats and studied their response to a single injection of FU. Tumour pH and energy status were measured by ^{31}P-MRS, and FU retention and metabolism were measured by ^{19}F-MRS. These parameters were correlated with each other and with the response to therapy, and the results show that the pre-treatment energy status of the tumours was the best predictor of therapeutic outcome.

Materials and methods

Animal care and experimental procedures

Tumours were chemically induced in 50 day-old female Ludwig/Wistar/Olac rats by 3 s.c. injections of *N*-methyl-*N*-nitrosourea (MNU) at 2-weekly intervals as previously described [13]. An histology study of these MNU-induced tumours showed that 75% of the tumours formed were mammary adenocarcinomas [14]. MNU rats were fed a high-fat diet and the 29 animals used in this study bore tumours weighing between 2 and 8 g. Anaesthesia prior to MRS experiments was performed by a single i.p. injection of 0.5 ml Hypnorm-Hypnovel (10 and 5 mg/kg, respectively). Rats were placed on a water-heated pad in the magnet bore maintained at 37 °C. Injection of FU (David Bull Laboratories, Warwick, UK) was performed via an i.p. catheter at a dose of 100 mg/kg. Tumour size, recorded as the weight, was determined with callipers and calculated as $1 \times w \times h \times (\pi/6)$. Treatment efficacy was assessed at 7 days post-therapy by measurement of the change in tumour weight, and the response was classified according to standard oncological criteria [15] as progressive (P) if an increase of ≥25% was measured, remissive (R) decrease of ≥50% or stable (S) for values lying between these two changes. In addition, groups R and S were combined as a single group of "partial responders", or non-progressive disease (NPD), for comparison with group P.

Magnetic resonance spectroscopy

Spectra were acquired on a SISCO 200/300 spectrometer equipped with a 4.7 T horizontal bore magnet. A double-tuned (^{31}P/^{19}F), 18-mm, 1-turn surface coil was positioned on the top of the tumour along with an external standard of 1.5 μmol 5-fluorotryptophan (FTP) in a capillary (150 μl). After optimisation of MRS parameters, ^{31}P spectra were acquired over a 5-min period (256 transients, spectral width SW = 8 Hz, 90° at the coil centre, repetition time Tr = 1.2 s immediately prior to and at 2.5 h following FU treatment. ^{19}F spectra were acquired in 4-min blocks (160 transients, SW = 25 Hz, 90° at the coil centre, Tr = 1.5 s) for 152 min after the FU injection.

All spectral analyses were performed by the VARPRO time-domain non-linear least-squares methods [16]. The chemical shift of

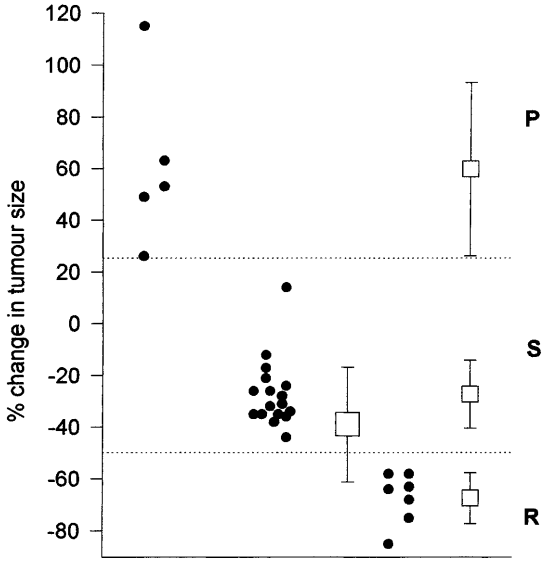

Fig. 1 Response of MNU-rat tumours to FU treatment. Results show the response as a percentage of change in tumour weight at 7 days after a single i.p. injection of FU (100 mg/kg). The *dotted horizontal lines* show the divisions into P (progressive), S (stable) and R (remission) using the standard oncological criteria described in Materials and methods, with the *small boxes* showing the mean values ± SD recorded for these groups, which were significantly different from each other ($P < 0.0001$, Gabriel's ANOVA). The *larger box* shows the mean value ± SD noted for the combined S and R groups (non-progressive disease, NPD, $n = 24$)

peaks in ^{31}P spectra were referenced to phosphocreatine (PCr) at 0 ppm, which was determined to be 8.1 ppm downfield from α-NTP [17]. ^{31}P-MRS data were used to calculate the tumour energy status (ratio of β-nucleotide triphosphate over inorganic phosphate, NTP/Pi) and the tumour pH, which was calculated from the chemical shift difference between Pi and α-NTP as previously described [17]. Providing that the tumour extracellular content remains less than 55%, this pH_{MRS} measurement is considered to reflect the intracellular pH (pHi) rather than the extracellular pH (pHe) [18].

^{19}F peaks were identified according to the literature and were referenced to FU at 0 ppm [11], and peak integrals were measured using VARPRO. Under the experimental conditions used (Tr = 1.2 s, all the peaks – the external standard FTP, FU, FNuct and FU catabolites (FCat), i.e. α-fluoro-β-alanine and α-fluoro-β-ureidopropionic acid – would be partially saturated. To determine the degree of saturation we measured peak integrals in the tumour in vivo under fully relaxed conditions (RT = 30 s) in three animals and compared the results with those obtained under the experimental conditions. This showed that FTP was 36 ± 7% saturated, whereas FU, FNuct and FCat were 59 ± 10%, 69 ± 15% and 56 ± 6% saturated, respectively (mean values ± SD). Assuming that coil loading and the flip-angle were the same for all tumours and the external standard, comparison with the external standard (1.5 μmol) allowed a semi-quantitative determination of the concentration of the intratumoural fluorinated compounds. The spectra acquired were summed in overlapping groups of four blocks (e.g., 0–16 min, 4–20 min, …) and the peak integrals (in micromoles) were plotted against time, allowing calculation of the area under the curve (AUC) using the trapezoidal rule. For comparison of data from different animals the AUC values were divided by the estimated tumour weight to give values expressed in micromoles per gram for the total amount of FU in the tumour and the total amount of FNuct synthesised by the tumour during the experiment, i.e. micromoles per gram per 2.5 h.

Statistical analysis

Differences between group mean values recorded for the MRS-determined parameters were assessed using Gabriel's analysis of variance (ANOVA). The strength of a relationship between two or more parameters was measured using linear regression or multiple regression (allowing for interaction) to obtain the correlation coefficient R and the associated P value for significance (< 0.05) as described by Bland [19] with, in some cases, a Bonferroni correction [19]. For determination of the significance of the association of a parameter(s) with response, Fischer's exact test was applied to 2×2 contingency tables (NPD versus P), since the criteria for chi-square tests was not attained when tumours were split into the three groups of P, S and R [19]. Combination of predictor variables in Fischer's test was achieved by use of the NTP/Pi ratio as the primary determinant, followed by the secondary determinant of FNuct, FU or pH.

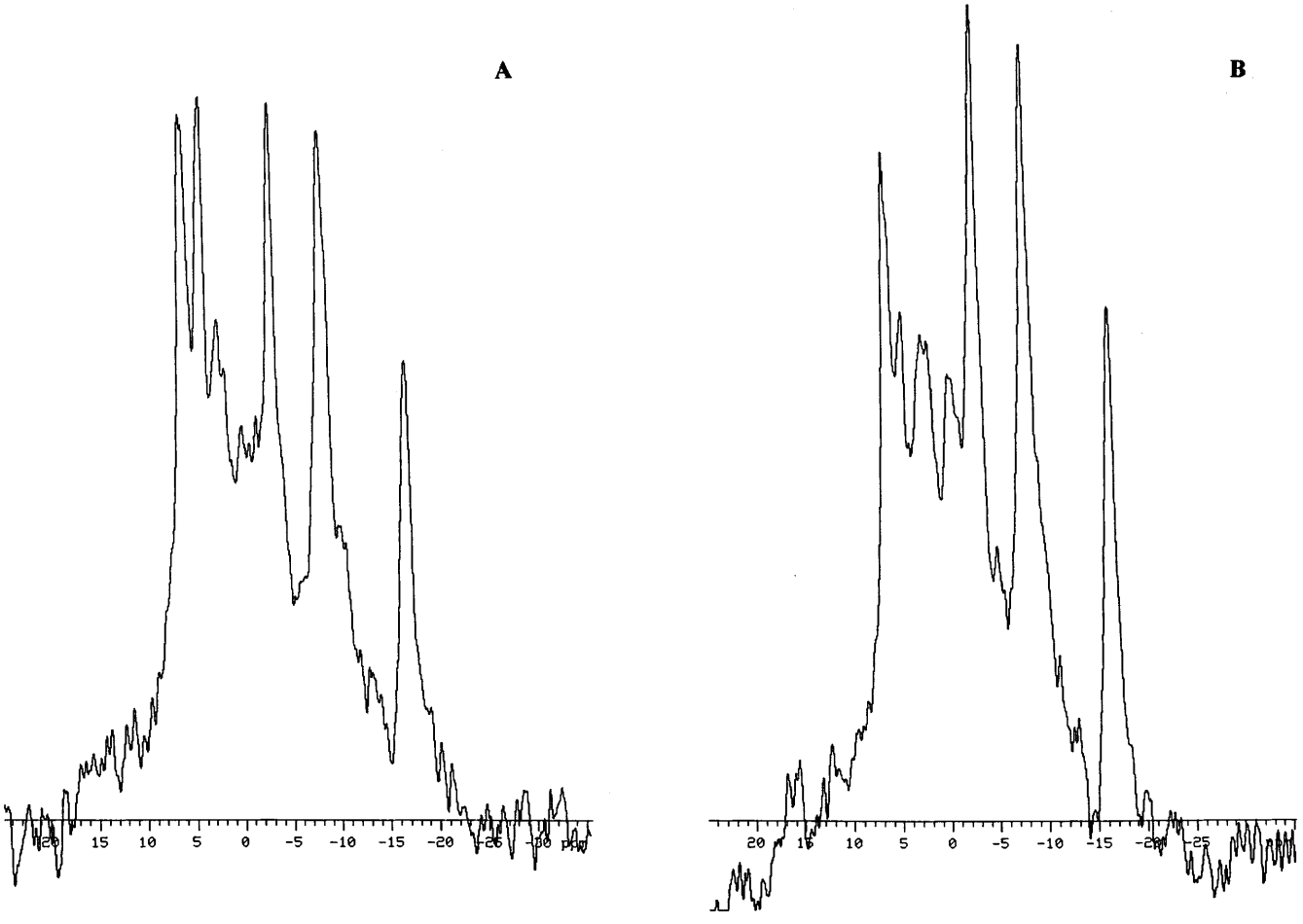

Fig. 2A,B ^{31}P spectra of an MNU tumour as obtained **A** prior to and **B** at 2.5 h after FU treatment (100 mg/kg i.p.). Peaks are assigned as follows: phosphomonoesters (PME; 6.3 ppm), inorganic phosphate (Pi; 4.2 ppm), phosphodiesters (PDE; 2.5 and 1.8 ppm) and 4–6 γ,α,β-nucleotide di- and triphosphates (NTP), respectively (−3.0, −8.1 and −16.9 ppm)

Results

Tumour response to treatment

Untreated MNU tumours grew with a doubling time of ca. 30 days, similar to that previously reported [20]. The tumour response to the single bolus injection of FU varied between a decrease in size of 85% to an increase of 115% (Fig. 1). The 29 tumours were divided into the 3 groups progressive (P), remissive (R) and stable (S) according to the standard oncological criteria for assessment of response described in Materials and methods. Figure 1 demonstrates that the two groups S and R showed a significant mean decrease in tumour weight, and they were thus also assimilated into one group of non-progressive disease (NPD).

^{31}P magnetic resonance spectroscopy

Figure 2 shows typical ^{31}P spectra made prior to and at 2.5 h post-FU treatment. The spectra are similar to those previously described for this tumour and show clear signal resonances for the PME, PDE, Pi and γ, α, β-NTP (Fig. 2). PCr was low or undetectable, consistent with the scanty contribution of phosphorus signals from the body wall or skin [17]. Following FU treatment, all tumours showed a significant increase in the NTP/Pi ratio and intracellular pH, whereas other ratios did not markedly alter. Table 1 shows the mean changes observed in the NTP/Pi ratio and pH in the three groups, which were similar and not significantly different; thus, changes in (Δ) NTP/Pi and pH were parameters that could not be used to distinguish the types of response.

Table 1 shows the pre-treatment NTP/Pi and pH values recorded for the three groups. Analysis by ANOVA showed that groups S and R were not significantly different from each other or from group P in terms of these parameters. However, combination of groups S and R into an NPD group demonstrated a 2-fold greater NTP/Pi ratio relative to group P ($P = 0.04$). Although the mean tumour weight of group S was significantly smaller than that of groups R and P, this parameter did not correlate with the percentage of change in tumour weight ($R = 0.15$, $P = 0.45$), i.e. response was not related to tumour weight at the time of treatment.

Tumour uptake and metabolism of FU

Prior to FU injection, no endogenous fluorinated compound was detected. FU was visible in all tumours, persisting for most of the 2.5-h time course; however, the peak integral maximum and the time at which it occurred were very variable. Figure 3 shows the peaks detectable during the 2.5-h period from a tumour representative of group R and the concentration-time profiles obtained for FU, FNuct (5.1 ppm) and FCat (−16.9 and −18.7 ppm) in that tumour. Typically, the FNuct AUC was ca. 40% of the FU AUC (mean% ± SEM 0.42 ± 0.04, $n = 29$). A $t_{1/2}$ value for FU elimination could not be reliably determined from these data, since drug disappearance was non-linear and adequate modelling was not possible. FCat signals were even more variable (results not shown), but since FCat detected in the tumour is likely to originate from liver catabolism [11], this parameter was not considered in subsequent analyses.

Table 1 shows that the tumour retention and metabolism of FU was lowest in the P group (in which there was an increase in tumour weight) as compared with the other two groups, although these differences were not significant (ANOVA). However, combination

Table 1 Summary of parameters measured in the different tumour groups[a] (*P* Progressive, *S* stable, *R* remission, *NPD* non-progressive disease – groups R and S combined)

	P	S	R	NPD (S + R)
FNuct	1.61 ± 1.18	4.13 ± 2.15	3.03 ± 2.11	3.81 ± 2.15
P		0.058		0.037#
FU	5.29 ± 2.99	11.15 ± 6.14	8.02 ± 5.54	10.23 ± 6.03
P		0.11		0.088
NTP/Pi	0.88 ± 0.52	1.66 ± 0.85	1.80 ± 0.77	1.70 ± 0.81
P		0.12		0.04#
pH	7.08 ± 0.13	7.18 ± 0.17	7.20 ± 0.17	7.19 ± 0.16
P		0.42		0.16
ΔNTP/Pi	+0.97 ± 0.6	+1.02 ± 1.82	+1.23 ± 1.81	+1.12 ± 1.75
ΔpH	+0.17 ± 0.11	+0.10 ± 0.26	+0.20 ± 0.23	+0.12 ± 0.25
Tumour weight	5.6 ± 1.2	4.0 ± 1.1	5.4 ± 1.7	4.4 ± 1.4
P		0.017*		0.087
n	5	17	7	24

[a] Results show the mean values ±SD recorded for NTP/Pi, pH and tumour weight (g) prior to treatment and for FU and FNuct (expressed as micromoles per gram per 2.5 h). Δ signifies the change in the NTP/Pi ratio and pH after FU treatment as compared with the pre-treatment values. The *P* values shown are for a Gabriel's ANOVA comparing groups P, S and R, where *signifies group S as being significantly different from groups P and R, and for comparison of groups P and NPD, where # signifies group NPD as being significantly different from group P

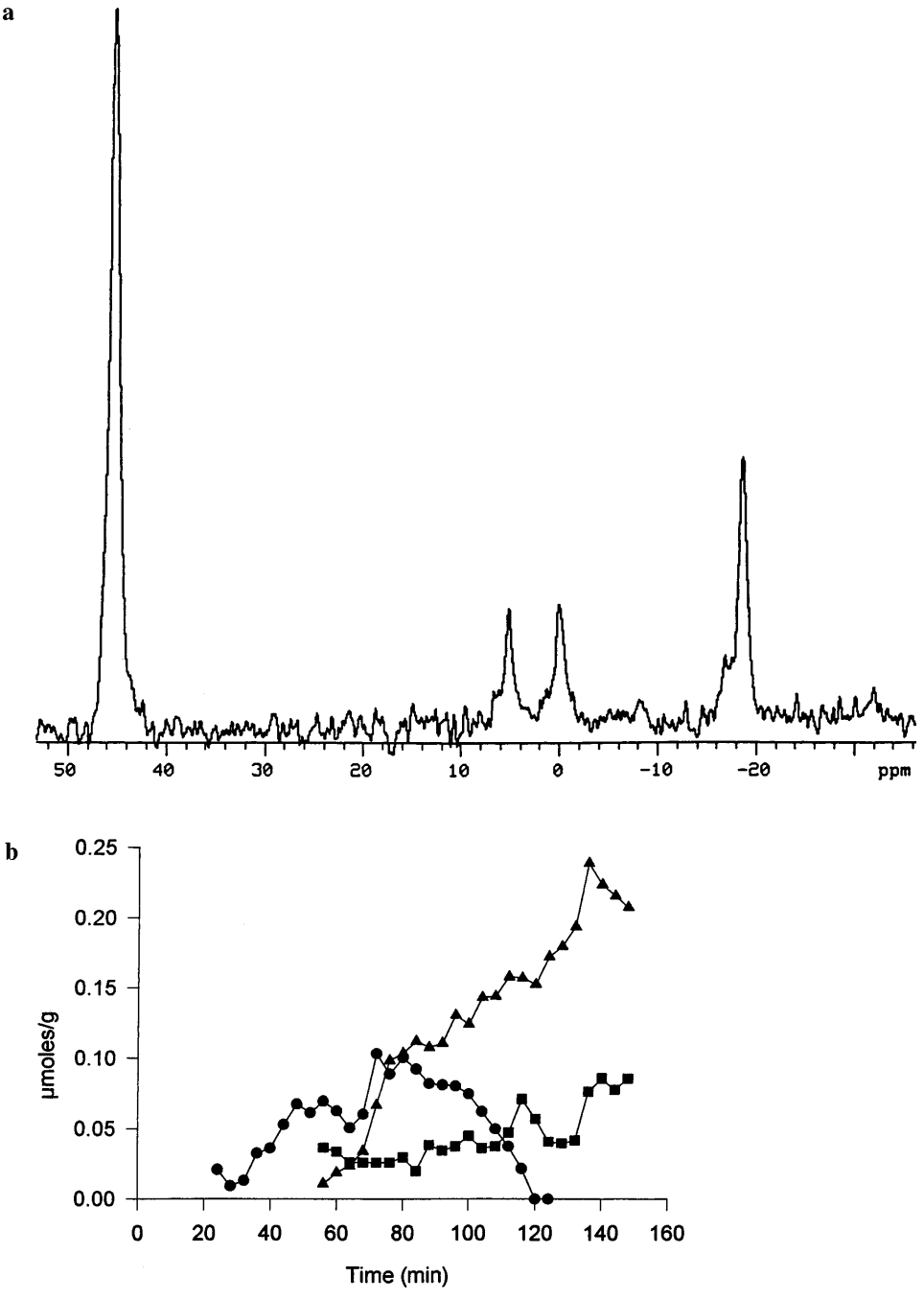

Fig. 3a,b FU uptake and metabolism by an MNU tumour. **A** ^{19}F spectrum of the same MNU tumour shown in Fig. 2, showing the sum of all spectra made between 0 and 152 min. Peaks are identified as follows: FTP (45.1 ppm), FNuct (5.1 ppm), FU (0 ppm) and FCat (−16.9 and −18.7 ppm). **B** Individual concentration-time courses determined for FU (●), FNuct (■) and FCat (▲) in this tumour as described in Materials and methods

of groups S and R as the NPD group showed a ca. 2-fold increase in FNuct formation ($P = 0.037$) and FU retention ($P = 0.088$) relative to group P.

Correlation of change in tumour weight with MRS-measured parameters

Figure 4 shows the four main parameters measured (pH and NTP/Pi prior to treatment, total FNuct and FU micromoles per gram per 2.5 h) correlated with the percentage of change in tumour weight. All the correlations were weak, and only one was significant, that for NTP/Pi ($R = -0.38$, $P = 0.041$), although not after a Bonferroni correction ($P = 0.16$). Notwithstanding the poor correlations, closer inspection showed that there was by no means random scatter, all the P group had low FU, FNuct, pH and NTP/Pi values, whereas all the tumours with high values for these parameters showed a decrease in tumour weight. The correlations were made weaker by two out-lying tumours in the R group (inverse symbols in the plots in Fig. 4). These two tumours had NTP/Pi ratios much lower than the mean of the R group (at about 1 SD), and the pH values were also lower

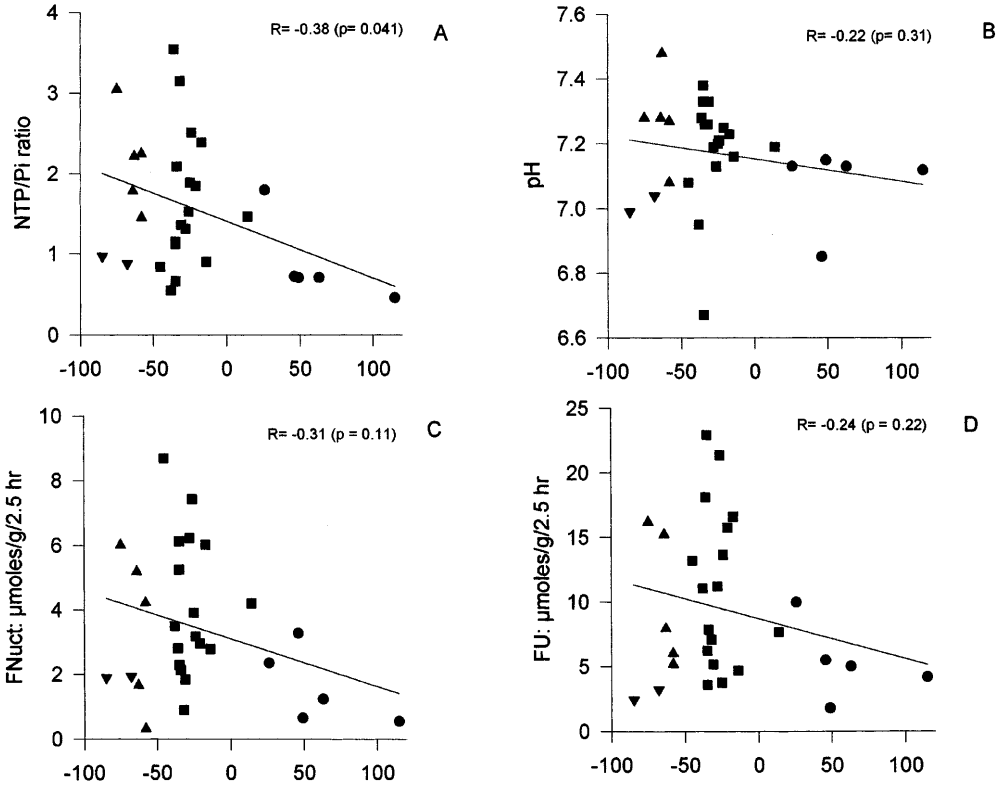

Fig. 4A–D Correlation of the percentage of change in tumour weight (response to FU treatment) with pre-treatment **A** NTP/Pi, **B** pH, and total **C** FNuct and **D** FU. Symbols are as follows: ● P, ■ S, ▲ R, with ▼ indicating the two "out-lying" tumours

(Fig. 4). We did not perform histology on these tumours, and it is possible that they were necrotic, which can markedly lower the formation of FNuct [21]. Removal of these two tumours from the plots in Fig. 4 improved the correlations – FNuct ($R = -0.4$, $P = 0.038$), FU ($R = -0.38$, $P = 0.051$), NTP/Pi ($R = -0.49$, $P = 0.009$), pH ($R = -0.31$, $P = 0.12$) – such that only pre-treatment pH would have been non-significantly correlated with a decrease in tumour weight. On the other hand, application of a Bonferroni correction to these data ($n = 27$) to allow for some correlations being by chance again yielded only the NTP/Pi ratio as being significant ($P = 0.036$).

Using two or more of the four predictor variables and correlating with the percentage of response ($n = 29$) did not increase the significance. For example, after allowance for interaction, the best predictor combination was FNuct plus the NTP/Pi ($R = 0.5$, $P = 0.062$). FU plus NTP/Pi and pH plus NTP/Pi gave $R = 0.45$, $P = 0.12$ and $R = 0.4$, $P = 0.23$, respectively. Again, increasing the number of combined variables did not improve the significance.

Plotting of the four parameters against each other ($n = 29$, plots not shown) showed significant positive correlations between NTP/Pi and pH ($R = 0.56$, $P = 0.002$), FU and FNuct ($R = 0.67$, $P = 0.00008$) and NTP/Pi and FU ($R = 0.43$, $P = 0.02$) and a weaker positive correlation between pH and FU ($R = 0.34$, $P = 0.077$). FNuct did not correlate with pH or with the NTP/Pi ratio ($R < 0.2$). These results suggested that more "energised tumours" tended to have a higher pH and a greater uptake and/or retention of FU, which was likely to lead to increased synthesis of FNuct.

Prediction of the response to FU

Figure 5 shows a scatter plot for the pre-treatment NTP/Pi ratio against the percentage of response as divided into the groups of P, S, R and NPD. Using the single SD about the mean of the NPD group as an arbitrary cut-off, it was possible to allocate tumours as being of high energy (NTP/Pi ratio ≥ 0.9) or low energy (< 0.9). Table 2 demonstrates a significant association between high-energy tumours and response ($P = 0.01$), i.e. it was possible to "predict retrospectively" 20 of the 24 NPD tumours (83% correct) and 4/5 of the P group (80% correct). Note that four tumours bordered this 0.9 ratio (Fig. 5), but even when a cut-off of 1.0 was used there remained a significant association with response ($P = 0.036$). Via a similar approach, FNuct was also significantly associated with response (FNuct, $P = 0.024$), but the other two parameters were not FU, $P = 0.27$; pH, $P = 0.55$ – (contingency tables not shown). Table 2 shows that response was highly significantly associated ($P = 0.0027$) with the NTP/Pi ratio in combination with FNuct such that 100% of the NPD group could be predicted. Indeed, in this combination the contingency table was unaltered even if the NTP/Pi ratio cut-off was 1.0. A similar approach using other combinations of FU or pH with the NTP/Pi ratio did

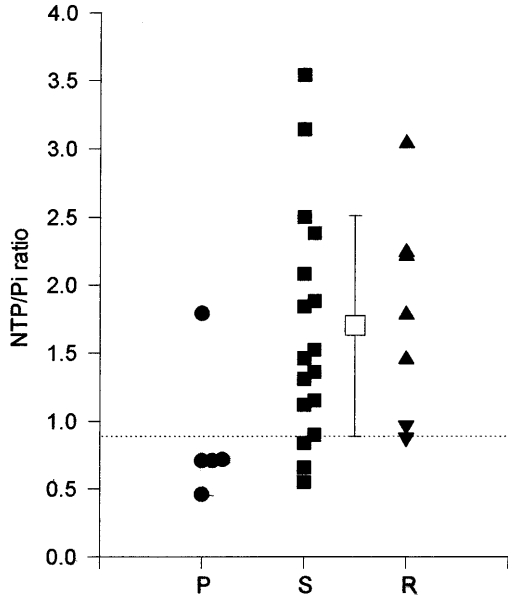

Fig. 5 Tumour NTP/Pi ratio prior to FU treatment. Results show the pre-treatment NTP/Pi value recorded for each tumour studied as divided into the 3 groups of P, S and R. The *box* shows the mean value ± SD noted for the 24 tumours in the combined S + R group (NPD). The *dotted horizontal line* shows the limit of the single SD

not improve on the association obtained with the NTP/Pi ratio (0.9) alone.

Discussion

Retention of FU by tumours has been significantly associated with patient response at a number of different clinical centres [12, 22, 23], allowing a prediction of the likely response to chemotherapy. The mechanism of this so-called "FU trapping", i.e. the reason why some tumours have a larger $t_{1/2}$ value for FU elimination, is unknown but may be related to tumour pH and the energy status of the tumour cells [5, 6]. Indeed, in isolated tumour cells, the pH gradient across the tumour cell membrane, i.e. the difference between the intracellular pH (pHi) and the extracellular pH (pHe), has been shown to be correlated with the intracellular retention of FU, an effect that may be partially dependent on the intracellular ATP concentration [7]. We carried out an exploratory study to determine in a pre-clinical model which MRS-determined parameters correlate with and, therefore, potentially predict the response to FU treatment. Even though many of the correlations found in this relatively small study only bordered on significance, there was a clear indication that the tumour energy status was the best predictor of response, a result of potential clinical value.

Of the four MRS-determined parameters, only the pre-treatment tumour energy status (NTP/Pi ratio) correlated significantly with response ($P = 0.04$). A high energy status could reflect a well-vascularised tumour, allowing FU to enter the extracellular tumour space more easily; indeed, NTP/Pi and FU were significantly correlated ($P = 0.02$). However, the FU detected by MRS is both intra- and extracellular, and the extracellular space in these tumours is ca. 50% [18]. This could explain why the FU signal was poorly correlated with response ($P = 0.22$). For cytotoxicity, FU must be transported into the cell, an event probably favoured by a large negative pH gradient ($-\Delta pH$) [7], and then actively metabolised to the intracellular species FNuct. MRS experiments in our laboratories measuring both pHi and pHe in five different tumour types have shown that a higher pHi is associated with a larger $-\Delta pH$ across the tumour cell membrane [24]. In this study we measured only pHi, and this was strongly correlated with NTP/Pi ($P = 0.002$), suggesting that chemical energy may be necessary for the maintenance of pH and a large $-\Delta pH$. Thus, energised tumours may be (a) better vascularised; (b) more likely to retain FU, through having a larger $-\Delta pH$; and (c) better capable of anabolising FNuct from FU. Similar observations have been made in a murine model, where the pre-treatment energy status of RIF-1 tumours was correlated with FNuct formation and the subsequent response to FU treatment [25]. This discussion suggests that treatments designed to raise transiently the NPT/Pi ratio and/or the $-\Delta pH$ of

Table 2 Contingency table for the association of response with the NTP/Pi ratio or with the NTP/Pi ratio plus FNuct

	P group	NPD group	Total	*P* value
NTP/Pi ratio:				
≥0.9	1	20	21	
<0.9	4	4	8	
Total	5	24	29	0.0124[a]
NTP/Pi ratio plus FNuct:				
High	2	24	26	
Low	3	0	3	
Total	5	24	29	0.0027[b]

[a] Fischer's test for difference between tumours of low energy (NTP/Pi ratio <0.9) and high energy (*top*)
[b] Fischer's test for difference between tumours of low energy or low FNuct (FNuct formation <1.6 μmol/g per 2.5 h) and those of high energy or high FNuct (*bottom*). Cut-offs for NTP/Pi and FNuct were determined from the SD of the mean values shown in Table 1

tumours prior to FU treatment may augment FU uptake and activation and, consequently, the tumour response.

In the light of the clinical reports, it was surprising that in the complete data set presented herein ($n = 29$), neither FU nor FNuct correlated significantly with the percentage of response ($P = 0.22$ and $P = 0.11$, respectively). The poor correlation was in part due to two tumours in group R that had a lower than average pH, NTP/Pi ratio, FU retention and FNuct formation. Removal of these two tumours led to significant correlations between the percentage of response and FNuct, FU, and NTP/Pi (all $P < 0.05$). However, application of a Bonferroni correction to these correlations would remove all significance in the $n = 29$ group and leave only NTP/Pi significant in the $n = 27$ group. This, however, is a very conservative test, and we consider that the non-corrected correlations are indicative of important relationships between some of the MRS-determined parameters and the tumour response to treatment. It is interesting to speculate that the two ''outlying'' tumours may have represented a sub-set of the MNU-induced tumours that were more sensitive to low levels of FNuct. For example, they might have lower levels of the enzyme thymidylate synthase, rendering them more sensitive to inhibition of DNA synthesis by the fluoronucleotide FdUMP. This heterogeneity of FU metabolism by different tumours, as well as the composite nature of the FNuct peak, underlines the potential drawbacks of using FNuct levels to predict tumour response in the clinic. Indeed, this was highlighted in a pre-clinical model using nude mice in which thymidine-induced increases in FNuct levels detected by ^{19}F-MRS led to increased cytotoxicity in one human xenograft but not in another [26].

Notwithstanding these considerations, there was a significant association between FNuct and response ($P = 0.02$, Fischer's exact test). This is the test has been used in the clinic to show an association between the FU $t_{1/2}$ in the tumour and the patient response, and as such it is more relevant in a comparison of this experimental model with the situation in the clinic. We could not accurately measure a $t_{1/2}$ for FU but instead used the total FU detected during the experiment (micromoles per gram per 2.5 h). However, FU was not associated with response ($P = 0.27$) despite the strong correlation observed between FU retention and FNuct formation ($P = 0.00008$). NTP/Pi (tumour energy status), however, was associated with response ($P = 0.01$), and when it was combined with FNuct there was a highly significant association ($P = 0.003$), allowing 100% of the NPD group, i.e. partial responders, to be predicted. These results suggest that ^{31}P-MRS in combination with ^{19}F-MRS might improve the detection of tumours likely to respond to FU treatment.

In summary, we used a pre-clinical, primary rat tumour model to determine (a) if other MRS-determined parameters besides ''FU trapping'' may be used to predict the response to FU treatment and (b) what factors may determine this ''FU trapping''. Due to the relatively small data set used, the observed correlations indicate further areas of research rather than clearly unambiguous relationships. Nevertheless, our results are not inconsistent with observations in the clinic that increased tumour retention of FU and/or formation of FNuct are associated with patient response. More importantly, we found the tumour energy status to be the best predictor of response, perhaps because it reflects factors that lead to increased FU access, uptake and metabolism by the tumour. In combination with another MRS-determined parameter, FNuct, the selectivity was increased at the expense of sensitivity, allowing 100% prediction of partial responders. These observations indicate that further research in this area would be beneficial and suggest that the use of double-tuned (^{31}P/^{19}F)-MRS coils in the clinic could further aid the prediction of FU response.

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References

1. Heidelberger C, Danenberg PV, Moran G (1983) Fluorinated pyrimidines and their nucleosides. Adv Enzymol 54: 58–119
2. Danenberg PV, Langenbach RJ, Heidelberger C (1974) Structures of reversible and irreversible complexes of thymidylate synthase and fluorinated pyrimidine nucleotides. Biochemistry 13: 926–933
3. Jacquez JA (1962) Permeability of Ehrlich cells to uracil, thymidine and fluorouracil. Proc Soc Exp Biol Med 109: 132–135
4. Wolhueter RM, McIvor RS, Plagemann PGW (1980) Facilitated transport of uracil and 5-fluorouracil, and permeation of orotic acid into cultured mammalian cells. J Cell Physiol 104: 309–319
5. Guerquin-Kern JL, Leteurtre F, Croisy A, Lhoste JM (1991) pH dependence of 5-fluorouracil uptake observed by in vivo ^{31}P and ^{19}F nuclear magnetic resonance spectroscopy. Cancer Res 51: 5770–5773
6. Yamamoto S-I, Kawasaki T (1981) Active transport of 5-fluorouracil and its energy coupling in Ehrlich ascites tumour cells. J Biochem 90: 635–642
7. Ojugo ASE, McSheehy PMJ, Stubbs M, Alder G, Bashford CL, Maxwell RJ, Leach MO, Judson IR, Griffiths JR (1998) Influence of pH on the uptake of 5-fluorouracil into isolated tumour cells. Br J Cancer 77: 873–879
8. Wolf W, Presant CA, Servis KL, El-Tahtawy A, Albright MJ, Barker PB, Ring R III, Atkinson D, Ong R, King M, Singh M, Ray M, Wiseman C, Blayney D, Shani J (1990) Tumour trapping of 5-fluorouracil: in vivo ^{19}F NMR spectroscopic pharmacokinetics in tumour-bearing humans and rabbits. Proc Natl Acad Sci USA 87: 492–496
9. Newell DR, Maxwell RJ, Golding BT (1992) In vivo and ex vivo magnetic resonance spectroscopy as applied to pharmacokinetic studies with anticancer agents: a review. Nucl Magn Reson Biomed 5: 273–278
10. Findlay M, Leach MO (1994) In vivo monitoring of fluoropyrimidine metabolites: magnetic resonance spectoscopy in evaluation of 5-fluorouracil. Anticancer Drugs 5: 260–280
11. McSheehy PMJ, Griffiths JR (1989) ^{19}F MRS studies of fluoropyrimidine chemotherapy. A review. Nucl Magn Reson Biomed 2: 133–141

12. Presant CA, Wolf W, Waluch V, Wiseman C, Kennedy P, Blayney D, Brechner RR (1994) Association of intratumoral pharmacokinetics of fluorouracil with clinical response. Lancet 343: 1184–1187
13. Gullino PM, Pettigrew HM, Grantham FH (1975) *N*-Nitrosomethylurea as mammary gland carcinogen in rats. J Natl Cancer Inst 54: 401–414
14. Stubbs M, Coombes RC, Griffiths JR, Maxwell RJ, Rodrigues LM, Gusterton BA (1990) ^{31}P-NMR spectroscopy and histological studies of the response of rat mammary tumours to endocrine therapy. Br J Cancer 61: 258–262
15. Cleton FJ (1995) Chemotherapy: general aspects. In: Peckham M, Pinedo HM, Veronesi U (eds) Oxford textbook of oncology, vol I. Oxford University, Oxford, pp 445–453
16. Van der Veen JWC, De Beer R, Luyten PR, Van Ormondt D (1988) Accurate quantification of in vivo ^{31}P-NMR signals using the variable projection method and prior knowledge. Magn Reson Med 6: 92–98
17. Howe FA, Stubbs M, Rodrigues LM, Griffiths JR (1993) An assessment of artefacts in localised and non-localised ^{31}P MRS studies of phosphate metabolites and pH in rat tumours. Nucl Magn Reson Biomed 6: 43–52
18. Stubbs M, Bhujwalla ZM, Tozer GM, Rodrigues LM, Maxwell RJ, Morgan R, Howe FA, Griffiths JR (1992) An assessment of ^{31}P MRS as a method of measuring pH in rat tumours. Nucl Magn Reson Biomed 5: 351–359
19. Bland M (1995) An introduction to medical statistics, 2nd edn. Oxford Medical Publications, Oxford
20. Baluch S, (1993) Studies using magnetic resonance spectroscopy for the assessment of tumour response. PhD thesis (Univ. of London) Ch. 2, p 45
21. Kamm YJL, Heerschap A, Rosenbusch G, Rietjens IMCM, Vervoort J, Wagener DJT (1996) 5-Fluorouracil metabolite patterns in viable and necrotic tumour areas of murine colon carcinoma determined by ^{19}F NMR spectroscopy Magn Reson Med 36: 445–450
22. Findlay MPN, Leach MO, Cunningham D, Collins DJ, Payne GS, Glaholm J, Mansi JL, McCready VR (1993) The non-invasive monitoring of low dose, infusional 5-fluorouracil and its modulation by interferon-a using ^{19}F magnetic resonance spectroscopy in patients with colorectal cancer: a pilot study. Ann Oncol 4: 597–602
23. Schlemmer HP, Bachert P, Semmler W, Hoenberger P, Schlag P, Lorenz WJ, Kaick G van (1994) Drug monitoring of 5-fluorouracil: in vivo ^{19}F NMR study of 5-FU chemotherapy in patients with metastases of colorectal adenocarcinoma. Magn Reson Imaging 12: 497–511
24. McSheehy PMJ, Stubbs M, Ojugo ASE, Box C, McCoy CL, Leach MO, Judson IR, Griffiths JR (1997) The relationship between intra- and extracellular pH in solid tumours in vivo (abstract). Proc Int Soc Magn Reson Med 5: 1095
25. Sijens PE, Baldwin NJ, Ng TC (1991) Multinuclear NMR investigation of the metabolic response of the murine RIF-1 tumour to 5-fluorouracil chemotherapy. Magn Reson Med 19: 373–385
26. Tausch-Treml R, Baumgart F, Ziessow D, Köpf-Maier P (1996) 5-Fluorouracil metabolism and cytotoxicity after pretreatment with methotrexate or thymidine in human hypopharynx and colon carcinoma xenografts: a ^{19}F nuclear magnetic resonance spectroscopy study in vivo. Cancer Chemother Pharmacol 37: 259–265