In Vivo ^{19}F NMR Comparative Study of 5-Fluorouracil, 1-(2-Tetrahydrofuryl)-5-fluorouracil (FT) and an FT-Uracil Coadministration System in Mouse Tumors

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The suppression of α-fluoro-β-alanine (FBAL) formation from 5-fluorouracil (FU) is an important subject in relation to tumor chemotherapy. This is the first comparative study of FU, 1-(2-tetrahydrofuryl)-5-fluorouracil (FT, a prodrug of FU) and of FT + uracil as a coadministration system (UFT) under an oral dose using the *in vivo* ^{19}F NMR method. The slow release of FU from FT and the suppression of the catabolism of FU to FBAL in mouse livers and tumors by the coadministration of uracil with FT were demonstrated using consecutive NMR measurements. The applicability of the *in vivo* ^{19}F NMR method to the drug evaluation in tumors and livers of small animals was successfully tested.

Keywords 5-fluorouracil; tumor; *in vivo* drug monitor; ^{19}F NMR; mouse

The technique of non-invasive clinical drug monitoring is an ideal method for the administration of pharmaceuticals with a wide range of individual differences in dynamics. The establishment of a non-invasive method for monitoring biochemical reactions will serve for drug evaluation with animals, making continuous data acquisition possible, which increases data reliability, and saves on labor, and saves animal lives. Recently, with the improvement of NMR instrumentation, *in vivo* ^{19}F NMR studies of 5-fluorouracil (FU) and 5′-deoxy-5-fluorouridine have been reported for humans and animals.[1,2] These studies are based on basic works, such as the assignment of ^{19}F NMR signals from the metabolites of FU in blood and urine,[3] and from FU incorporated in RNA.[4] *In vivo* human study[5,6] is a powerful clinical method for the evaluation of individual response to the drugs, although the method is applicable only to the tumors on or near the surface of the body,[5] in positions accessible by a surface coil. The *in vivo* NMR method has reached the level of pharmacokinetics and modulation strategies[6,7] using both patients and animals. Most of the animal studies are reported on rats,[2] using intraperitoneal administration of the drugs. One of the purposes of the present work is to determine whether the *in vivo* NMR study is applicable as a method of routine drug monitoring regarding oral drug administration, and whether the use of smaller animals such as mice is applicable both involving severer conditions for *in vivo* observations. The use of a vertical type superconducting magnet of regular size for obtaining the *in vivo* spectra using mice is also evaluated.

The functions of FU as an anticancer drug are anticipated in its RNA incorporated form or as an inhibitor to thymidilate synthase in the form of 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP). The generation of nucleoside and/or nucleotide forms of FU (F-nuc) is essential. Another pathway of FU reaction is the formation of α-fluoro-β-alanine (FBAL). As shown in Chart 1, FBAL is catabolized further to fluoro-citrate which is considered to cause such side effects as neurotoxicity and cardiotoxicity.[8] Suppression of FBAL without affecting the generation of F-nuc is essential for the use of FU or fluoropyrimidines in cancer chemotherapy. Coadministration of uracil with 1-(2-tetrahydrofuryl)-5-fluorouracil (FT) has been designed for this purpose.[9] The present report is on the first *in vivo* ^{19}F NMR study of FT and of the FT + uracil coadministration system (1 : 4, UFT). Reports on the non-invasive monitoring of fluoropyrimidine under oral administration are also rare, presumably because of differences in the methods of clinical administration among various countries.

Experimental

Five-week-old male ICR mice transplanted with Sarcoma 180 cells (1×10^6) into subepidermal tissue on the back were provided by Taiho Pharmaceutical Co. NMR experiments were performed 7—10 d later when the solid tumor size reached 0.5—1.0 g. One mmol/kg of the drugs, (A) FU (130 mg/kg), (B) FT (200 mg/kg) and (C) UFT (200 mg FT/kg + uracil, 1 : 4 mol ratio) was administered orally as a dispersion in the 0.5% aqueous solution of carboxymethyl cellulose sodium salt.

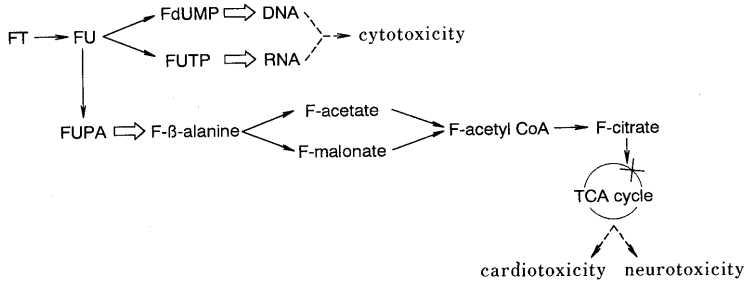

Chart 1

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Before drug administration, a ^{31}P NMR spectrum was taken under anesthesia by intra-peritoneal pentobarbital injection (65 mg/kg) to monitor energy metabolism and tissue pH in tumors. Following this measurement, the drug was given and *in vivo* ^{19}F NMR spectra were taken. Animal body was wrapped with styrofoam sheets in order to maintain the body temperature during NMR observations. The tumor tissue and organs were removed immediately after the *in vivo* NMR observations for quantitative analysis.

In vivo NMR spectra were obtained by a JEOL GSX-270WB spectrometer (6.3 T, 89 mm vertical bore). ^{31}P NMR was taken at 109 MHz with a 15 mm diameter 5 turn surface coil with an 18 μs single pulse and 2 s pulse repetition time (T_R). ^{19}F *in vivo* NMR spectra were taken at 254 MHz with a 12 mm diameter 1 turn surface coil for tumors or livers. A single hard pulse with a width of 18 μs (23 degree pulse at 4 mm depth) and a pulse repetition time of 0.06 s or 0.2 s was used. A delay time of 20—60 min between drug administration and the start of the first NMR data acquisition was allowed for the anesthesia, animal positioning, rf tuning and shimming. Typical observation times were 15 min for livers and 30 min for tumors.

For the quantitative analyses of metabolites, ^{19}F NMR spectra of excised organs or tumors were taken (*ex vivo* measurements) by a JEOL FX-100 spectrometer (2.3 T, 23 °C) at 93.7 MHz. The data were collected as follows: (1) 4—8 mice for each drug were sacrificed immediately after the *in vivo* observations to obtain matched data of quantification, and (2) 5—8 mice kept in the cage without anesthesia until the programmed time of 3 h after the drug administration. The excised tissue was immersed in boiling water for 3 min in a 10 mm NMR tube to deactivate enzymes. Final concentrations of drugs and metabolites in the tissue were determined from signal areas, with a correction for relaxation times (T_1) as reported previously.[10] Either FU (internal) or hexafluorobenzene (external) was used as the chemical shift standard.

Results

^{19}F NMR spectra of urine, water extracts of a liver and of a tumor are shown in Fig. 1. The chemical shift and coupling constants were in good agreement with the reported data[3]: The compounds in the urine of FT administered mouse were assigned to FBAL (−19 ppm), α-fluoro-β-ureidopropionic acid (FUPA, −17 ppm), FU (0 ppm), and FT (2 ppm). The signals near 5 ppm downfield from FU in the liver and tumor extracts were F-nuc. The shift differences in FT signal in three samples were due presumably to the pH differences between these solutions.

^{19}F relaxation times determined for the tube-packed samples after the enzyme deactivation were as follows. T_1 (±10%) was 3 s for FBAL, 1.6 s for FU, 1.4 s for FT at both observation frequencies (94 and 254 MHz), and 1.1 and 1.4 s at 94 and 254 MHz, respectively, for F-nuc, suggesting a contribution from the restricted motion of this species. T_2 values were shorter than 5 ms for F-nuc and FT at 254 MHz. The evaluated depths of optimum NMR sensitivity under the conditions of this *in vivo* observation of $T_R = 0.2$ s were 3 mm for F-nuc, FT, and FU with T_1 around 1.5 s, and 4 mm for FBAL with T_1 of 3 s at 254 MHz.

In vivo ^{19}F NMR spectra of a mouse liver after FU administration (130 mg/kg) is shown in Fig. 2. The rate of catabolism of FU to FBAL was rapid. The spectrum of excised liver after the *in vivo* measurements (2.5 h) indicated the presence F-nuc with the dominant signal of FBAL. These results are comparable to the data obtained from human livers.[6]

In vivo spectra at the position of epidermal tumors are shown in Fig. 3a—5a with *ex vivo* spectra of the same tumors (b). The metabolism of FU in a tumor (Fig. 3) was slower than in liver. Because of the low signal to noise ratio of the *in vivo* measurements, F-nuc was confirmed only in the *ex vivo* measurement under the dose of 1 mmol/kg. Doubling the dose made the detection much easier (data not shown). The sharp strong signal of FT at 2 ppm downfield from FU in the tumor of FT administered mouse was retained during the *in vivo* observation as shown in Fig. 4a. Although a slow FU release from FT was demonstrated, the signal of F-nuc from FT was not detected either the *in vivo* or *ex vivo* measurement. *In vivo* spectra of mouse tumor following the administration of FT + uracil are shown in Fig. 5a. F-nuc was detected along

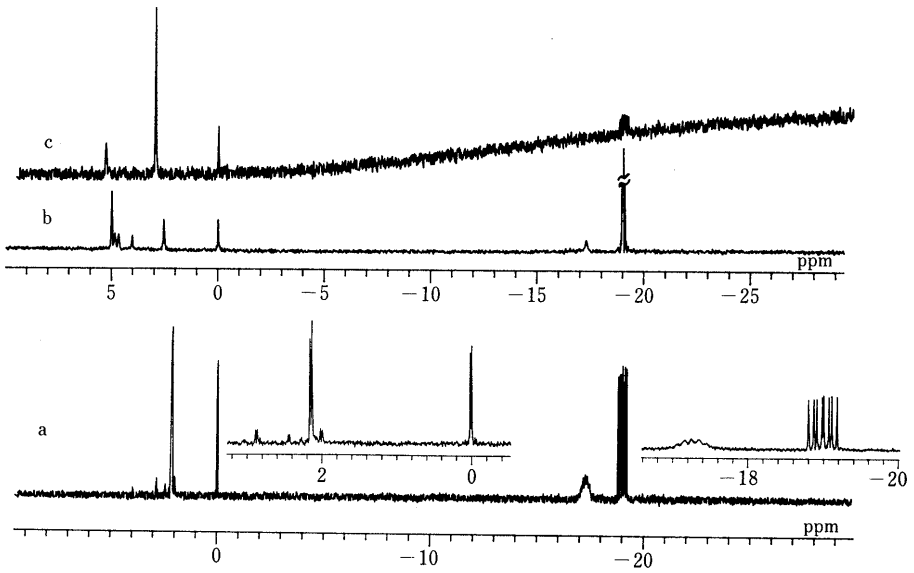

Fig. 1. ^{19}F NMR Spectra of Urine, Water Extracts of a Liver and a Tumor after Drug Administration

a. Urine of a FT administered mouse attached with the expanded spectra of 3—0 ppm and −16——20 ppm regions to show the F–H spin couplings. The signal of FU was set as the shift standard. The main metabolites were FBAL (−19 ppm), FUPA (−17), FU, and FT (2) from right to left. Small signals between 2—5 ppm are not assigned. b. Water extracts of a liver excised at 4 h after UFT administration, observed under proton decoupling. The signal at 5 ppm is from F-nuc. The signal of FBAL is overscaled. c. Water extracts of a tumor excised at 3 h after UFT administration, observed without proton irradiation. Assignments are FBAL, FU, FT and F-nuc from right to left.

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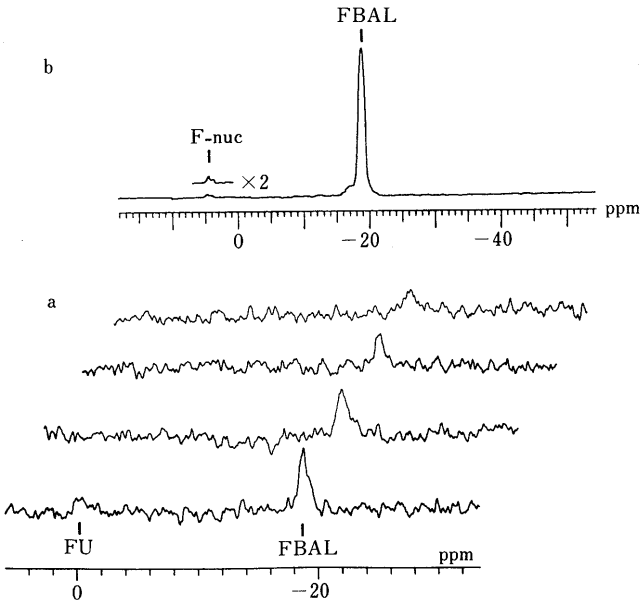

Fig. 2. ^{19}F NMR Spectra of the Mouse Liver after FU Administration

a. *In vivo* observation at 254 MHz, starting at 26 min after FU administration with 30 min data acquisition for each spectrum, from bottom to top. Spectrum bandwidth 40 kHz, pulse width 18 μs, pulse repetition time T_R = 60 ms. b. Excised liver after the *in vivo* measurement (155 min). 94 MHz. The concentrations of metabolites determined from the signal area were 0.10 mmol/kg (FBAL) and 0.007 mmol/kg (F-nuc).

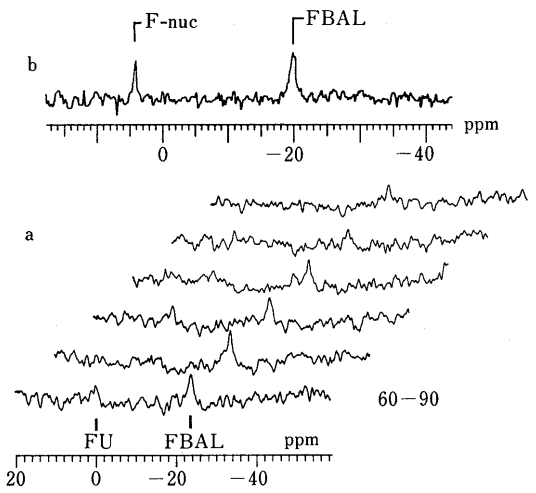

Fig. 3. ^{19}F NMR Spectra of the Mouse Tumor after FU Administration

a. *In vivo* observation at 254 MHz. 30 min data acquisition for each from bottom to top. Spectral band width 40 kHz, pulse width 18 μs, T_R = 200 ms. b. Excised tumor after the *in vivo* observation (245 min). 94 MHz. The concentrations were 0.25 mmol/kg (FBAL) and 0.06 (F-nuc).

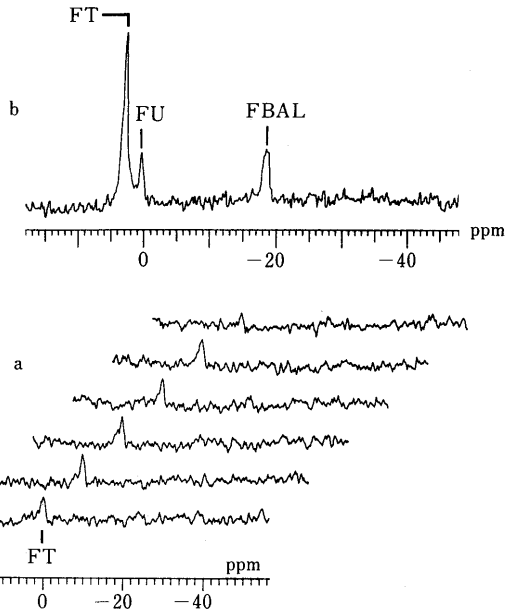

Fig. 4. ^{19}F NMR Spectra of the Mouse Tumor after FT Administration

a. *In vivo* observation at 254 MHz starting at 55 min after the FT administration with 30 min acquisition from bottom to top. Spectral band width 40 kHz, pulse width 18 μs, T_R = 200 ms. b. Excised tumor after the *in vivo* observation (265 min). 94 MHz. Concentrations of metabolites were 0.17 mmol/kg (FBAL), 0.07 (FU) and 0.27 (FT).

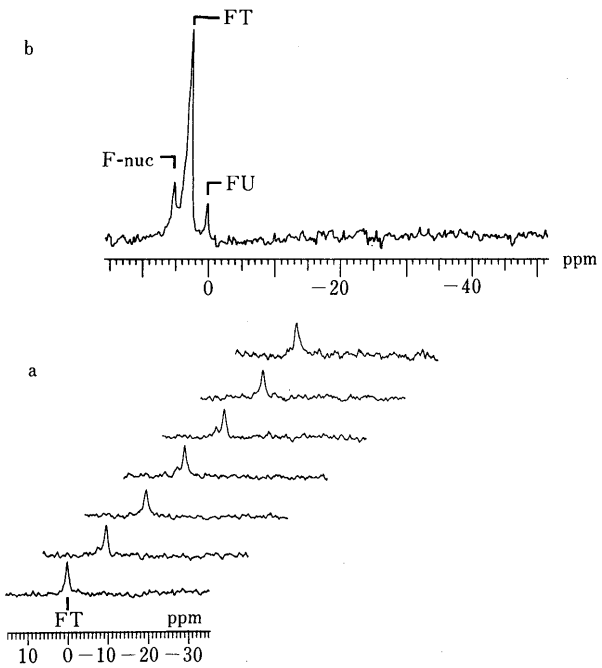

Fig. 5. ^{19}F NMR Spectra of the Mouse Tumor after FT+uracil (UFT) Administration

a. *In vivo* observation at 254 MHz starting at 51 min after the FT administration with 30 min acquisition, from bottom to top. Spectral band width 40 kHz, pulse width 18 μs, T_R = 200 ms. b. Excised tumor after the *in vivo* observation (270 min). 94 MHz. The concentrations were 0.06 mmol/kg (FU), 0.43 (FT) and 0.09 (F-nuc).

with FT and FU. Figure 5b is an *ex vivo* spectrum of the same tumor excised immediately after this *in vivo* observation (270 min). The concentrations of metabolites obtained from the *ex vivo* spectrum were as follows: F-nuc 90 μmol/kg, FT 430, FU 60, and FBAL was under the detection limit of the *ex vivo* measurements of 20—30 μmol/kg. From these experiments, the rate of catabolism of a given drug to FBAL was found to be in the order FU > FT > FT + uracil. The efficiency of F-nuc formation was FT + uracil > FU > FT. It was also shown that metabolites above 200 μmol/kg in tumors and 100 μmol/kg in livers were detected *in vivo* under the time resolution of 30 min.

The concentrations of metabolites in tumors and livers found 3 h after the drug administration are summarized in Figs. 6 and 7. *In vivo* and *ex vivo* determination of

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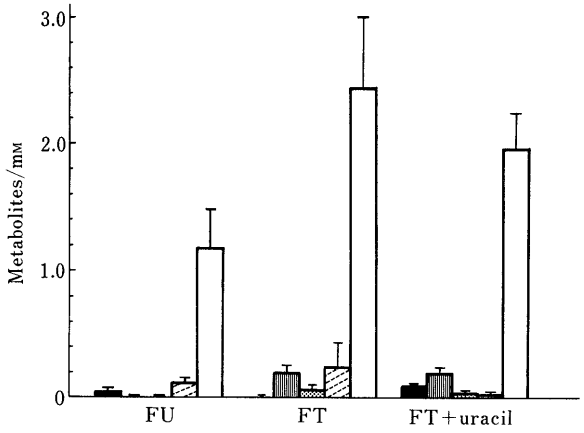

Fig. 6. The Metabolites in Livers Excised at 3 h from FU, FT and UFT Administered Mice (Each 5—8 Mice) Determined by ^{19}F NMR Spectra at 94 MHz

■, F-nuc; ▥, FT; ▩, FU; ▨, FUPA; □, FBAL.

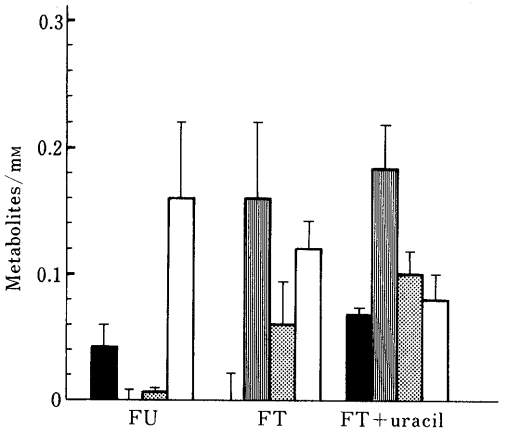

Fig. 7. The Metabolites in Tumors Excised at 3 h from FU, FT and UFT Administered Mice (Each 5—8 mice) Determined by ^{19}F NMR Spectra at 94 MHz

■, F-nuc; ▥, FT; ▩, FU; □, FBAL.

metabolites were in very good agreement. As anticipated from the literature data,[9] FT had the effect of creating a slow release of the effective component FU, but the presence of F-nuc was not confirmed in the case of a single dose of FT. Coadministration of uracil with FT was effective in F-nuc formation and FBAL suppression. These results are in good agreement with the clinical evaluation of FT and UFT systems in practice.[11]

Discussion

The *in vivo* ^{19}F NMR method of monitoring fluoropyrimidine under the condition of oral administration in mice was shown to be a powerful tool. The slow release of FU from FT and the selective formation of F-nuc in tumors by the coadministration of uracil with FT was successfully demonstrated. The NMR data from 4—8 each of *in vivo* and 5—8 *ex vivo* observations showed a good consistency, which indicated that the present instrumental condition was appropriate for the purpose: the difference in the depths of optimum sensitivity for different metabolites (with different T_1) due to the use of a surface coil for *in vivo* detection was not a serious problem for small objects such as mice. Anesthesia often causes the suppression of body temperature, which may affect the rate of metabolism. Also, the recommended animal setting for *in vivo* measurements is on its stomach rather than in an upright position. The method of this study, without intended animal heating and using an upright position in the vertical bore magnet, seemed to be appropriate for the purpose since no difference was observed in the composition of metabolites between the mice after the *in vivo* study and those kept in the cage. The reason may be that the measurements were completed within 3 h and/or the reactions did not involve the steps of large temperature coefficients.

In vitro NMR experiments showed that the ^{19}F chemical shift of FT had a strong pH dependence of 2.5 ppm between pH 6 and 10 (0.1 to 2.6 ppm measured from the external standard hexafluorobenzene), while that of FU shifted less than 0.2 ppm (−2.2 to −2.1 ppm) in this region. The change in the signal position of FT in the 3 spectra of Fig. 2 is an example. A pH shift dependence tends to cause a spectral misassignment in the *in vivo* NMR measurements, especially in tumors where a wide pH variation is anticipated. The pH values in the tumor tissues in the present experiment, which were determined from the ^{31}P chemical shift of inorganic phosphate from that of phosphocreatine, however, fell within a small range of 6.6 and 7.2. At these pH values, the chemical shift of FT measured from FU is expected to be 2.3 and 2.6 ppm, giving a negligible difference in the *in vivo* measurements.

The metabolites of 0.2 mmol/kg for tumors and 0.1 mmol/kg for livers were detectable *in vivo* within 30 min. A better sensitivity in livers compared with tumors was due to the volume of the object. The problem in the NMR study of either the time resolution or the high drug dose may be solved by using larger animals such as rats: the dose may be reduced to 1/10 of the present experimental value (from 200 mg FT/kg for mice to 20 mg FT/kg for rats), which is close to the clinical value (*e.g.* 600 mg FT/d = 10 mg/kg/d). The measurement in human liver will be attained with much ease where a larger detection coil can be applied. The dose of 15—30 mg FU/kg is used for the recent human *in vivo* NMR measurement of tumors and livers.[12] In order to evaluate the *in vivo* NMR detectability in rigor, it is desirable to have both analytical and *in vivo* NMR data from the same object. To the best of our knowledge, however, such matched data is scarce. The present results of *in vivo* and *ex vivo* NMR data should serve for this purpose. Because of the rapid technical improvement in this field, improved time resolution or a lower drug dose can be expected for the *in vivo* NMR study on a small animal of 30 g in the near future. The regular use of vertical type NMR spectrometers will open a further area of application of the method in advanced pharmacokinetics.

Acknowledgements The authors acknowledge the generous gift of drugs and tumor bearing mice from Taiho Pharmaceutical Co., Ltd.

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