Effect of Modulators on 5-Fluorouracil Metabolite Patterns in Murine Colon Carcinoma Determined by in Vitro $^{19}$F Nuclear Magnetic Resonance Spectroscopy

Yvonne J. L. Kamm, Ivonne M. C. M. Rietjens, Jacques Vervoort, Arend Heerschap, Gert Rosenbusch, Henny P. Hofs, and D. J. Theo Wagener

Departments of Medical Oncology [Y. J. L. K., H. P. H., D. J. T. W.] and Radiodiagnostics [A. H., G. R.], University Hospital, P. O. Box 9101, 6500 HB Nijmegen, and Department of Biochemistry, Agricultural University, Wageningen [I. M. C. M. R., J. V.], the Netherlands

ABSTRACT

High-resolution $^{19}$F nuclear magnetic resonance spectroscopy at 7 T was used to study the effect of modulators on the metabolism of 5-fluorouracil (5-FUra, 115 mg/kg i.p.) in C38 murine colon tumors grown in C57BL/6 mice. Distinct 5-FUra metabolite patterns were found in perchloric acid extracts of these tumors after treatment.

The $^{19}$F nuclear magnetic resonance spectra exhibited resonances representing 5-FUra, the catabolites $\alpha$-fluoro-$\beta$-ureidopropionic acid and $\alpha$-fluoro-$\beta$-alanine, as well as four distinct fluoronucleotide anabolites. Using this model system the effect of several modulators on 5-FUra tumor metabolite patterns was investigated: methotrexate (300 mg/kg); $\alpha$-interferon (10$^6$ IU/animal); $N$-(phosphonacetyl)-$L$-aspartate (100 and 250 mg/kg); and leucovorin (300 and 750 mg/kg). A significant increase in the anabolite:catabolite ratio was observed for the groups treated with 5-FUra in combination with the modulators methotrexate ($n = 8$), $\alpha$-interferon ($n = 7$), and high-dose leucovorin ($n = 14$), but not for low-dose leucovorin ($n = 7$). Cotreatment with high-dose $N$-(phosphonacetyl)-$L$-aspartate ($n = 8$) resulted in a significant decrease in the anabolite:catabolite ratio compared to treatment with 5-FUra alone ($n = 16$). Possible correlations of metabolite profiles with therapy response are discussed.

INTRODUCTION

5-FUra is the most important drug in the treatment of advanced colorectal carcinoma (1). In the last decade, it was possible to increase the response to 5-FUra from about 20 to 30% by alterations in the mode of administration and the use of modulators (2-4). 5-FUra is metabolized into active anabolites, in particular in tumor cells, and into inactive catabolites, in particular in the liver, but also in extrahepatic tissue (5-7). This theory is supported by the fact that in colon tumor cell lines incubated with 5-FUra only anabolites are formed, but no catabolites. Fig. 1 presents a scheme of 5-FUra metabolism. Only a small percentage of the 5-FUra is taken up in tumor cells and converted to the therapeutically important anabolites: the FNucts and FNucs. The most important FNucts are FdUMP and FUTP, the latter used in order to increase the anabolism of 5-FUra at the cost of its catabolism. Biochemical modulation involves the use of agents, which themselves may be devoid of cytotoxic activity but which enhance the antitumor effect or the selectivity of an active antitumor drug by altering the biochemical pathways (14).

The main objective of the present study was to obtain more insight into the effects of modulators on 5-FUra metabolite profiles in tumor tissue by using $^{19}$F NMR spectroscopy. If changes in metabolism, i.e., enhanced anabolism, can be predicted, it may be possible to rationally design treatment protocols that are likely to have improved response rates. Although many $^{19}$F NMR studies on 5-FUra metabolism have been done in vivo (9, 10, 15-17), we decided to perform an in vitro study, because of the improved resolution of individual metabolite signals and the significantly lower detection threshold. The modulators investigated in the present study were MTX, PALA, IFN, and LV. MTX and LV have already proved to be active in some patient studies (18, 19). IFN has also been used in patients to improve 5-FUra treatment (20). PALA is now used as a new modulator in phase II studies (21). The biochemical effects of these modulators are schematically presented in Fig. 1. For details see Refs. 5 and 22-30.

MATERIALS AND METHODS

Tumor Model. Female C57BL/6 mice 8 to 12 weeks of age were obtained from the Central Animal Laboratory of our university. The C38 murine colon tumor, which is known to be sensitive to 5-FUra, was acquired from Dr. P. Lelieveld of the REPO-INO Institute, Rijswijk, the Netherlands, and is described elsewhere (31). Tumor tissue fragments with a diameter of 3 mm were implanted s.c. in the right flank of the mouse. Treatment was performed after 20 to 21 days, when the tumors reached a weight of 2-4 g. Histological examination of 6 similar tumors was performed and revealed that a constant fraction of 30 ± 5% (SE) consisted of necrosis. The tumor was considered to be an ellipsoid and its volume was estimated from three orthogonal diameter measurements

\[
\text{Tumor volume (mm}^3\text{)} = XYXZ \times 0.5
\]

(31). Mice were divided into groups based on estimated tumor volume and stratified randomization was carried out over the different treatment groups. Animals which were ill after 3 weeks of tumor growth, i.e., the time of the start of the 5-FUra/modulator treatment, were not used for the experiments. This occurred for 6 of 73 mice.

Chemotherapy. The tumor-bearing mice received 5-FUra at a dose of 115 mg/kg. All drugs were administered i.p. as a bolus within 5 seconds. The modulator groups received LV (300 mg/kg/animal together with 5-FUra), 750 mg/kg/h before 5-FUra, or twice 375 mg/kg, once 1 h before 5-FUra treatment and a second time together with the 5-FUra), MTX (300 mg/kg/h before 5-FUra administration), IFN (10$^6$ IU/animal = ± 4 × 10$^6$ IU/kg 24 h before 5-FUra administration), or PALA (100 or 250 mg/kg/24 h before 5-FUra administration).
MODULATION OF 5-FLUOROURACIL METABOLISM

Fig. 1. Interaction between 5-FUra and pyrimidine metabolism. Modulators are shown in boxes; enzymes are shown in circles; +, stimulation; -, inhibition; ±, LV and MTX stimulate (+) FdUMP-CH2THF-TS complex formation resulting in inhibition (-) of the normal thymidylate synthase (TS) catalysis; I, aspartate transcarbamylase (ATC); 2, orotate phosphoribosyltransferase (OPRTase). The ternary complex responsible for thymidylate synthesis inhibition is represented by a heptagona. CP, carbamyl phosphate; OA, orotic acid; CH2THF, N9,N10-methylene tetrahydrofolate; PRPP, 5-phosphoribosyl 1-p-ribofuranosil phosphate; F-, unknown fluor combinations.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV</td>
<td>Stimulation</td>
</tr>
<tr>
<td>MTX</td>
<td>Stimulation</td>
</tr>
<tr>
<td>IFN</td>
<td>Stimulation</td>
</tr>
<tr>
<td>PALA</td>
<td>Stimulation</td>
</tr>
<tr>
<td>MTX</td>
<td>Stimulation</td>
</tr>
<tr>
<td>DHFU</td>
<td>Stimulation</td>
</tr>
<tr>
<td>FUPA</td>
<td>Stimulation</td>
</tr>
<tr>
<td>CFBAL</td>
<td>Stimulation</td>
</tr>
<tr>
<td>FBAL</td>
<td>Stimulation</td>
</tr>
<tr>
<td>F-</td>
<td>Inhibition</td>
</tr>
</tbody>
</table>

Perchloric Acid Extraction. The frozen tissue was pulverized in a liquid nitrogen-chilled stainless steel mortar and ground to a fine powder. Per g of pulverized tissue 5 ml of cold 0.9 m perchloric acid were added, and the sample was stirred with a glass rod and thoroughly mixed (1500 rpm, 20 strokes). After homogenization the sample was incubated for at least 1 h in a cold chamber on a shaker. The sample was centrifuged, the pellet was discarded, and the supernatant was neutralized with KOH and 4% (v/v) of a potassium phosphate buffer (pH 6.5, 1 m; final concentration, 40 mMs) before freeze-drying. After freeze-drying the lyophilisate was dissolved in 0.8 ml demineralized water, the pH of the sample was adjusted to 6.5, and the sample was freeze-dried again.

19F NMR Spectroscopy. 19F NMR spectra were measured on a Bruker AMX 300 using a dedicated 19F probe as described in detail elsewhere (36). Freeze-dried sample residues were dissolved in 1.6 ml (0.4 m) potassium phosphate, pH 6.5, and placed in a 10-mm NMR tube together with a coaxial insert containing 3H2O as lock substance, as well as parafluorobenzoic acid as an internal standard. Between 3,000 and 50,000 transients were accumulated, depending on the signal-to-noise ratio required and the concentration of the fluorine-containing compounds in the sample. Data acquisition parameters were: spectral width 60,000 Hz; flip angle 30 degrees with a pulse width of 5 μs; repetition time 0.75 s; temperature 7°C. Continuous WALTZ-16 composite-pulse 1H decoupling was used at a power level of about 0.5 W (90-degree pulse = 100 μs). The available fluoropyrimidine reference compounds were purchased from Sigma (Bornem, Belgium).

Analysis of 19F NMR Spectra. The 19F NMR resonance frequency of 5-FUra was set to a chemical shift value of 0 ppm. The integrals of the signals of 5-FUra (F), the 5-FUra anabolites (A) (FNuct plus any FNucs together), and the 5-FUra catabolites (C) (FUPA and FBAL) were calculated by comparison of the integrals of their signals to the integral of the 19F NMR resonance of the internal standard p-fluorobenzoic acid (59.20 ppm). Because the measurements were performed at relatively low temperature and, thus, high viscosity, saturation of the resonances of A, C, and F was not observed. This could be derived from an experiment in which the repetition time was set to 9.15 s instead of 0.75 s but in which all other parameters were unchanged. The relative comparison of the two spectra showed that no increase in signal intensity of the A, C, and F peaks was observed with a repetition time of 9.15 s compared to 0.75 s. Only for the signal of the internal standard was a few percent (5–10%) increase in intensity observed. This implies that under the conditions of the present measurements, the influences of T1 effects on signal intensities are lower than the effects of the inaccuracy of the integration and the intersample variation. The detection threshold corresponds to 2 μM in the extract solution. Integrals were determined using the Bruker UXNMR software.
Statistics. Group means have been represented with their SE. Differences in group means were analyzed by Student's t test (two sided, α = 0.05) after log transformation (log normal distribution). Due to the large number of treatment groups and parameters compared to the number of animals, the analysis was kept explorative.

RESULTS

19F NMR Detection of Tumor 5-FUra Metabolite Patterns. In the 19F NMR spectrum of the extract from a tumor treated with 5-FUra, shown in Fig. 2, signals from 5-FUra (Peak 3 at 0 ppm) and various anabolites and catabolites can be observed. In the spectra presented in Fig. 2 Peak 2 at 3.44 ppm is observed, representing the fluoronucleosides, i.e., FUrd and FdUrd. Identification of the resonances of the catabolites DHFU (Peak 6), FUPA (Peak 4) and FBAL (Peak 5), respectively, at −33.20, −17.84, and −19.30 ppm, could be performed on the basis of literature data (37). The FNucs (Peak 2) and DHFU (Peak 6) resonances are relatively weak and are observed only in spectra with the high signal:noise ratios obtained with long accumulation times (50,000 transients). Various FNuct peaks (group 1) are observed between 4 and 5 ppm. In most spectra four different peaks can be distinguished namely at 4.64 (Peak a), 4.48 (Peak b), 4.26 (Peak c), and 4.15 (Peak d) ±0.04 ppm (Fig. 2, insets). More detailed identification of the various nucleotide peaks might be possible on the basis of comparison with literature data (37–40). However, values reported appear to vary from one paper to another due to different conditions. Addition of the commercially available compound FdUMP to a tumor extract resulted in a significant increase of the resonance at 4.26 ppm (Peak c), proving the fact that the important anabolite FdUMP underlies this peak region, but probably also other FNuct. Addition of FUrd and FdUrd both resulted in an increase of the peak at 3.44 ppm (Peak 2). Therefore specific identification of the anabolite peaks requires the synthesis of appropriate reference compounds.

Time Dependence of the Tumor 5-FUra Metabolite Pattern. In order to determine the optimal time interval between 5-FUra administration and tumor excision as well as the best parameter for characterization of the modulator effects, tumor extracts obtained at various time intervals after 5-FUra administration were analyzed by 19F NMR. The results of these experiments are presented in Fig. 3. Possible parameters that may reflect the effect of modulators on 5-FUra metabolism are the absolute concentration of anabolites (A) representing cytotoxicity (8); of catabolites (C) representing detoxification; of total fluorine-containing compounds (T) including A, C, and F representing drug uptake (38); the ratio of anabolites to 5-FUra (A/F) (11); of anabolites to catabolites (A/C); and of anabolites to total fluorine-containing compounds (A/T) (41). Fig. 3a shows the absolute concentrations of A, C, and F as observed at the various time intervals. The results demonstrate that A and C increase in a similar parallel way up from 0 (time of 5-FUra injection) to about 70 min. F increases rapidly within the first 15 min to decrease thereafter. Fig. 3b depicts the values of ratios obtained at increasing time intervals between 5-FUra administration and section. From the data presented it can be concluded that the A/C and A/T parameters become relatively stable for time intervals greater than 40 min. The A/F ratio does not show a "steady state" period, because F goes to zero. Based on these findings it was decided to excise tumors 50 min after 5-FUra treatment and to take the absolute concentration of A and the A/C and A/T ratios as important parameters with which to study the effects of modulators on 5-FUra metabolism, although other parameters are also presented.

Effect of Modulators on Tumor 5-FUra Metabolite Pattern. Fig. 2b shows a typical extract spectrum for a mouse treated with MTX and 5-FUra. With respect to Fig. 2a the anabolite peaks are more intense, also in relation to the 5-FUra and the catabolite peaks. The signals of the FNucts are well resolved. However, the distinct FNuct peaks did not show a consistent intensity pattern for animals in the same group. Furthermore, no reproducible differences were found.
between various treatment groups. Comparison of the catabolite peaks in the spectra of Fig. 2 demonstrates that in the MTX cotreatment sample (Fig. 2b) the FUPA peak (Peak 4) is more intense than the FBAL peak (Peak 5) in contrast to the results of 5-FUra treatment alone (Fig. 2a). This results in a significantly lower FBAL:catabolites (FBAL/C) ratio (0.19 ± 0.05 compared to 0.51 ± 0.09 for 5-FUra alone, P < 0.05), indicating an effect of MTX cotreatment on catabolism of 5-FUra.

In two series of experiments the effects of the four modulators on the 5-FUra metabolite pattern of the tumor were determined. The results of the experiments are presented in Table 1. There was no difference in tumor volume between the different treatment groups. Due to relatively large interindividual variation, the SE is relatively high for all parameters.

For MTX (300 mg/kg) a significant effect on the tumor 5-FUra metabolite pattern was observed. Modulation with MTX resulted in a significant increase of the absolute concentration of A (62 ± 4 compared to 46 ± 5 nmol/g, P < 0.01) and of the A/C ratio observed in the tumor extract (0.90 ± 0.17 compared to 0.57 ± 0.06, P < 0.05). The total uptake of drug as measured by the parameter T = A + C + F was not significantly altered by MTX.

Combination therapy of 5-FUra with IFN (105 IU/animal) also led to a significant increase of the A/C ratio (0.92 ± 0.17 compared to 0.57 ± 0.06, P < 0.05) and gave the highest A levels observed (70 ± 15 compared to 46 ± 5 nmol/g for 5-FUra alone, P = 0.06). The total concentration of fluorine-containing compounds showed a tendency to be increased due to higher levels of unmetabolized 5-FUra, but this effect was not significant.

Cotreatment of the mice with LV (300 mg/kg together with 5-FUra) did not result in any significant change in the tumor 5-FUra metabolite pattern. In further experiments LV was given either 1 h before 5-FUra at a dose of 750 mg/kg or as a split dose of 375 mg/kg 1 h before 5-FUra treatment and 375 mg/kg together with the 5-FUra. Table 1 also presents the metabolic profiles for these experiments. The results show a decrease in A, C, T, and an increase in F, while the A/C ratios for both high-dose LV cotreatments are comparable to those obtained with MTX or IFN, i.e., significantly higher compared to 5-FUra alone (0.90 ± 0.12, P < 0.05; and 0.94 ± 0.08, P < 0.01, compared to 0.57 ± 0.06).

The low PALA dose (100 mg/kg 24 h before 5-FUra) did not result in changes in the 5-FUra metabolite pattern. With the high PALA dose (250 mg/kg) A, F, and T levels are reduced, the FBAL/C ratio is significantly increased (1.19 ± 0.27 compared to 0.51 ± 0.09, P < 0.05) indicating an alteration in relative rates of individual catabolic steps. Also, a significant reduction in the A/C ratio is seen (0.33 ± 0.06 compared to 0.57 ± 0.06, P < 0.05).

**DISCUSSION**

In the present study 19F NMR analysis of extracts of 5-FUra-treated tumors was demonstrated to be a useful model system for studies of...
measurements of intratumoral metabolism of 5-FUra modulated by some modulators have been tested by 19F NMR analysis in cell NMR extract studies of 5-FUra-treated tumors have been reported (8, dependent on the applied exposure regimen. This phenomenon has not been studied yet by 19F NMR. Thus, to our knowledge, the been described before in clinical studies on 5-FUra therapy (18) and (see "Materials and Methods" for details).

In the MTX group the absolute A concentration was significantly higher than that obtained with 5-FUra alone, while F and C remained unchanged. This result shows that MTX cotreatment improves the A/C ratio by increasing the tumor concentration of therapeutically active 5-FUra-derived nucleotides. This positive effect of MTX has been described before in clinical studies on 5-FUra therapy (18) and in vivo 19F NMR studies (12, 32, 44). Cotreatment with MTX is presently used in the clinic for improved 5-FUra therapy response. It is the only combined treatment modality with a significant effect on survival. Thus our tumor extract model supports this positive modulator effect of MTX. Positive effects of IFN (20) and LV (19) on 5-FUra therapy have also been reported in clinical trials. The increase in A/C ratio for IFN was due to an increase in mean A concentration, although this increase did not meet the significance criterion because of a large SE. In the high-dose leucovorin group of the present study, an increase in the A/C ratio was observed due to a large decrease in C and a smaller decrease in A. This treatment group had low T and high F levels, indicating reduced drug uptake and reduced anabolism and catabolism.

Finally, the effect of cotreatment with a high dose of PALA is remarkable. This finding may be compatible with reported resistance of 5-FUra treatment to PALA modulation (47, 48). However, other studies have mentioned an improved therapeutic index with this combination (18). In theory the observed decline in A concentration and A/C ratio observed in the present study might be explained by a reduction in the concentration of UTP as a result of the PALA-induced depletion of its precursor orotic acid. Reduced levels of UTP may then enhance FUTP incorporation into RNA with an increased therapeutic effect accompanied by a decrease in the observed concentration of free FUTP. To solve this matter is an important topic for future research including measurements of the fluorinated products in the RNA, thymidylate synthase, and DNA fractions.

ACKNOWLEDGMENTS

The authors thank the Central Animal Laboratory (head: Dr. J. Koopman) for providing facilities for animal experiments, H. van Rennes from the Department of Medical Oncology for her help with the preparation of the tumor extracts, and W. H. Doesburg for the statistical analysis.

REFERENCES

MODULATION OF 5-FLUOROURACIL METABOLISM


Effect of Modulators on 5-Fluorouracil Metabolite Patterns in Murine Colon Carcinoma Determined by in Vitro $^{19}$F Nuclear Magnetic Resonance Spectroscopy

Yvonne J. L. Kamm, Ivonne M. C. M. Rietjens, Jacques Vervoort, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/54/16/4321

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.