Issues of Normal Tissue Toxicity in Patient and Animal Studies

Effect of Carbogen Breathing in Rats after 5-Fluorouracil Treatment

John R. Griffiths, Dominick J.O. McIntyre, Franklyn A. Howe, Paul M.J. McSheehy, Agatha S.E. Ojugo, Loreta M. Rodrigues, Peter Wadsworth, Natasha M. Price, Fiona Lofts, Grainne Nicholson, Kees Smid, Paul Noordhuis, Godefridus J. Peters and Marion Stubbs

From the CRC Biomedical Magnetic Resonance Research Group, Department of Biochemistry and Immunology, St. George's Hospital Medical School, London, UK (J.R. Griffiths, D. McIntyre, F.A. Howe, P.M.J. McSheehy, A.S.E. Ojugo, L.M. Rodrigues, N.M. Price, M. Stubbs), Safety of Medicines Department, AstraZeneca, Macclesfield, Cheshire, UK (P. Wadsworth), the Departments of Medical Oncology (F. Lofts), and Anaesthetics (G. Nicholson), St. George's Hospital Medical School, London, UK and the Department of Medical Oncology, University Hospital VU, Amsterdam, The Netherlands (K. Smid, P. Noordhuis, G.J. Peters)

Correspondence to: Marion Stubbs, D.Phil, CRC Biomedical Magnetic Resonance Research Group, Department of Biochemistry and Immunology, St. George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, UK. Tel: +44 181 725 5852. Fax: +44 181 725 2992. E-mail mstubbs@sghms.ac.uk

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Non-invasive magnetic resonance spectroscopy (MRS) can be used in the clinic to monitor the pharmacokinetics of the chemotherapeutic drug 5-fluorouracil (5-FU) and the effects of modifiers. We report two studies of 5-FU toxicity in normal tissue—one with patients and the other an animal study. 1) ^{19}F MRS signals from fluoronucleotides, cytotoxic anabolites of 5-FU metabolism, were observed in the livers of two patients treated with 5-FU for colorectal cancer, shown by computed tomography (CT) and ultrasound (US) to have no liver metastases. This is the first report of non-invasive monitoring of toxic 5-FU metabolites in normal human tissues. 2) In animals, carbogen breathing enhances tumour uptake and the efficacy of 5-FU, and the method is under trial in patients. This study demonstrates that there were no significant effects of carbogen breathing on the levels of 5-FU and its metabolites in normal rat tissues, or on the histology of the tissues assessed after treatment.

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Magnetic resonance (MR) techniques are of great potential value in medicine because of their non-invasive nature. For instance, ^{19}F magnetic resonance spectroscopy (MRS) can be used in situ to measure the tumour uptake of 5-fluorouracil (5-FU), an anticancer agent in use for more than 40 years (1, 2). Because serial measurements can be taken, this allows pharmacokinetic analyses of the change in drug concentration in the tumour itself (3, 4). In addition to the ^{19}F MRS studies in vivo, other more conventional pharmacokinetic techniques have shown that 5-FU seems to be retained longer in tumours than in normal tissue (5, 6). Enhanced retention has been shown to be significantly associated with response, presumably because higher concentrations of 5-FU sustain their antitumoural effects by favouring the lasting presence of toxic metabolites at the target tissue sites.

Anticancer drugs of all kinds gain access to tumour cells through the vasculature. Many, if not most tumours, contain poorly perfused regions, primarily owing to the chaotic and abnormal blood vessels. The cells within them may resist chemotherapy because they fail to take up enough of the anticancer drug (7). Carbogen ($95\%O_2$/$5\%CO_2$) breathing has been shown to improve the efficacy of radiation (8) and to enhance the uptake of certain chemotherapeutic drugs (e.g. 5-FU and ifosfamide) in rodent tumours (9, 10). The mechanism of action of enhanced drug uptake and efficacy is thought to be due to two effects: (i) CO_2-induced tumour blood vessel vasodilation causing increased blood flow/volume which could enhance drug uptake, and (ii) retention of the drug ('trapping') when the carbogen is replaced by normal air breathing and tumour vasodilation ends. In the case of 5-FU,

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this leads to increased intracellular formation of cytotoxic fluoronucleotides (FNuct) in the tumour (10).

Whether the novel preclinical findings of enhanced drug uptake with carbogen breathing are applicable to cancer patients can be assessed directly by non-invasive MRS. In the clinic, 5-FU is primarily used for treatment of solid tumours such as colon and breast cancers; it is used both to treat colorectal metastases of the liver and as an adjuvant after surgical resection of the primary tumour. We are conducting a clinical trial, using ^{19}F MRS to measure the pharmacokinetics of 5-FU in the liver metastases of these patients, to determine whether carbogen breathing (i) increases the uptake of native 5-FU, (ii) increases the appearance of toxic FNuct (when detectable), and whether FNuct can act as a measure of potential efficacy (1). A preliminary report of this study is included in this paper.

Using carbogen to enhance anticancer drug uptake into tumours would clearly be of no value if it caused an equivalent increase in uptake by normal tissue. However, in preliminary work on our ^{19}F MRS study on 5-FU metabolism in patients, we recently made a most unexpected finding (reported herein): the appearance of FNuct in the livers of patients being treated with 5-FU as an adjuvant, who had no detectable liver metastases (as determined by CT scans). Although these patients had not breathed carbogen, the presence of substantial quantities of toxic 5-FU metabolites in an apparently normal organ suggested that we should further examine the effect of breathing carbogen on drug uptake by normal tissues. In an earlier study (11) we had measured the uptake of ifosfamide by rat prolactinoma GH3 tumours and normal rat tissues (liver, spleen, kidney, muscle, lung and heart), using high resolution ^{31}PMRS on tissue extracts. Only the tumour tissue took up significantly more drug during carbogen breathing (ca. 3-fold, $p < 0.02$) and there was no enhancement of uptake in any of the normal tissues. However, it was still necessary to demonstrate that carbogen breathing did not enhance 5-FU toxicity in normal tissues, particularly those sensitive to chemotherapeutic agents. In this paper, we therefore describe the effects of 10 min carbogen breathing on the uptake and metabolism of 5-FU in normal rat tissues and histological assessment 1, 2 and 10 days after treatment. Rats were chosen as the experimental animals since they have been used successfully in many studies on 5-FU (12, 13).

MATERIAL

[6-^{3}H]-FdUMP (specific activity 20 Ci/mmol) was purchased from Moravek Biochemicals Inc. (Brea, California, USA) and [5-^{3}H]-dUMP (specific activity 10.9 Ci/mmol) from Amersham International (Buckinghamshire, England). *dl*-Tetrahydrofolate (Sigma Chemicals Co., St Louis, Missouri, USA) was converted into 5,10-methylenetetrahydrofolate by addition of formaldehyde (14).

METHODS

Clinical MRS studies

^{19}F MRS and MR imaging were performed on a clinical 1.5T MR system (Signa: GE, Milwaukee, USA) with a dual-tuned ^{1}H/^{19}F surface coil (20 by 16 cm). The coil was semi-flexible and allowed conformation to the curvature of the chest for optimal coverage of the liver. Coronal and axial gradient echo images were first acquired to ensure correct positioning of the coil over the patient's liver. For patients with liver metastases, a respiratory-gated T_2 weighted axial fast spin-echo sequence was used to localize the metastases. A reference sample situated at the coil centre was used to calibrate the ^{19}F pulse power for each patient and provide a reference signal to allow quantitative comparison of data between different patients. ^{19}F spectra were acquired using a 90° pulse at the coil centre with a 1 s repetition time, 16 kHz bandwidth and 1024 data points. Thirty-two averages with the reference signal on resonance were first acquired. The spectrometer frequency was then offset to observe the 5-FU signal on resonance. Patients were examined twice, typically 48 h apart. On one occasion the patient breathed normally (air) and on the other, air was replaced by carbogen for a total of 5 min, 3 min before the start of the infusion and for a further 2 min after the 5-FU bolus, before returning to air. As the bolus i.v. administration of 5-FU (425 mg/m^2) was started, spectral acquisitions were begun and acquired continuously for 32 min in 1-min blocks. Administration of 5-FU lasted typically 1 min, followed by a bolus of Leucovorin (20 mg/m^2).

Protocol for preclinical studies

The carbogen-breathing protocol was based on that previously published (9, 10). Normal tissue from Wistar-Furth rats (5 per group, mean weight 166 ± 2.3 g, $n = 40$) was sampled at three time points 2, 24 and 240 h (3 separate cohorts) post-5-FU treatment. In Group 1, the animals were given 5-FU (50 mg/kg) i.p. during 10 min air breathing, supplied by a nozzle. Group 2 breathed carbogen (2 L/min) for 1 min prior to 5-FU (50 mg/kg i.p.) and continued for a further 9 min (total = 10 min) before returning to air breathing. In Group 3, the animals were given 10 min of carbogen breathing only (no 5-FU). In all Groups, 100 µl of blood was taken from the aorta for white blood cell (WBC) counting after the animal was given a terminal dose of anaesthetic. In Groups 1 and 2, five animals were killed at 2, 24 and 240 h after treatment, and bone marrow, small intestine and liver samples were taken for 5-FU analyses and histology. In Group 3, WBC and samples of bone marrow, small intestine and liver were taken after 24 and 240 h for histology only (no 5-FU present), and the 2 h time point was omitted since no histological changes or changes in white blood cells would be expected after only 2 h. The WBC counts were per-

formed at St. George's Hospital Medical School and the histology at AstraZeneca by Dr Peter Wadsworth.

Sample treatment

Samples of bone marrow from rat femurs and from liver and small intestine were a) frozen in liquid N_2 (for 5-FU analyses), and b) placed in formal saline from where sections were cut for histological analyses after haematoxylin and eosin (H&E) staining.

Analysis of FdUMP concentration, 5-FU tissue levels and 5-FU incorporation into RNA

Before analysis, frozen samples were pulverized (14) and suspended with 3 volumes of the appropriate assay buffer. This suspension was immediately prepared for the FdUMP and 5-FU assay. Tissues were extracted with trichloroacetic acid for assay of 5-FU and FdUMP; the acid-insoluble precipitate was used for isolation of RNA and measurement of 5-FU incorporation into RNA. FdUMP concentrations were measured as previously described (15) by means of a dilution assay based on the competition between FdUMP in the tumours and radiolabelled FdUMP to bind to *L. casei* thymidylate synthase.

5-FU tissue levels were measured as described previously (5). Incorporation into RNA was performed in RNA isolated from the first precipitation step, as already described (16): the assay is based on the degradation of RNA containing FUTP to 5-FU by incubation with RNAse and alkaline phosphatase and uridine phosphorylase. 5-FU was then extracted, derivatized and measured by gas-chromatography coupled with mass spectrometry (5, 16).

RESULTS

Clinical data

This research was carried out with informed consent from the patients and with full ethics committee approval. FNuct signals were detected in the livers of two patients with colorectal cancer who were not thought to have malignant deposits in their livers (see below for case reports). The spectrum from patient 1 (see Fig. 1) is the sum of the signal acquired over 16 min, since the signal/noise (7.6; based on ratio of peak signal to root mean square noise) is not high enough for FNuct to be seen in a single block (2 min). Studies on normal livers of volunteers (without administration of a 5-FU bolus) and on phantoms have demonstrated that there are no RF noise artefacts, indicating this peak is from a real anabolite. Overall, these results imply that marked 5-FU activation to form FNuct can occur in normal liver. This is in line with ex vivo incubation of rat hepatocytes, in which exposure to 5-FU resulted in the formation of FNuct which was incorporated into RNA (2).

In view of these unexpected findings, the two patients were followed-up carefully; one is alive and well 2 years later and with no evidence of recurrence; the other patient went on to develop liver metastases 7 months later and has subsequently died.

Case reports

Patient 1, a 54-year-old female, underwent a sigmoid colectomy for Dukes' B carcinoma in August 1998. This was locally advanced with infiltration to the right pelvic side wall. Staging investigations were negative. The patient underwent radiotherapy to the left pelvic wall, completed in October 1998. 5-FU/Leucovorin chemotherapy was commenced in November and the patient's MRS scan was taken during the first course. The patient proceeded to course three with a 25% dose reduction and finished six courses of chemotherapy in March 1999. Her CEA remained normal throughout and in September 1999 a repeat liver ultrasound showed no evidence of metastases. An ultrasound in January 2000 demonstrated no liver disease, which was confirmed by CT. This patient is alive and free from disease.

Patient 2, a 48-year-old female, was diagnosed in November 1998 with a poorly differentiated adenocarcinoma (Dukes' C–9/21 nodes involved) of the transverse colon. The patient had a medical history of cervical dysplasia requiring cone biopsy and was also under regular surveillance for non-invasive transitional cell carcinoma of the bladder. The patient proceeded to right hemicolectomy. Laparotomy was normal and a CT showed that the liver was free of disease. 5-FU/Leucovorin chemotherapy was started in January 1999 and the MRS scan was conducted during this first course. The patient completed six courses of chemotherapy in June 1999 after a 20% reduction in dose, with a CEA of between 5 and 8. In August 1999, a routine ultrasound showed liver metas-

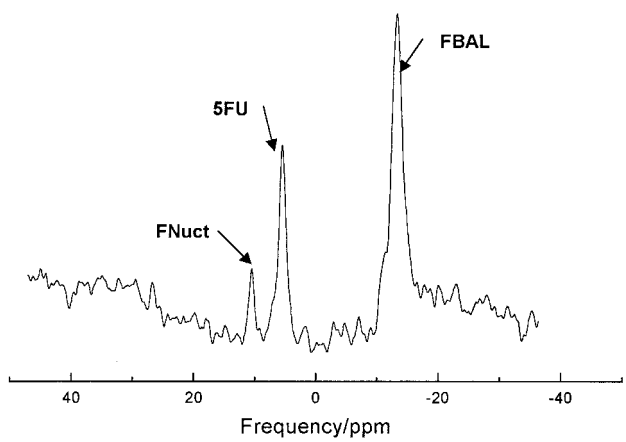

Fig. 1. ^{19}F spectrum acquired (over 16 mins) concurrently with a bolus dose of 5-FU (425 mg/m^2) from the liver of a patient (Patient 1) subsequently shown to be free of metastases. Abbreviations: FNuct = fluoronucleotides; 5-FU = 5-fluorouracil; FBAL = fluoroβalanine, conjugated FBAL (see (21)).

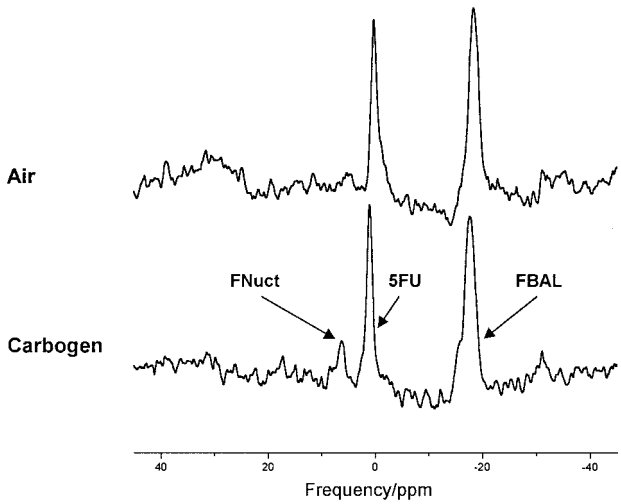

Fig. 2. ^{19}F spectra averaged over 8–15 mins from the start of the bolus of 5-FU in a patient with colorectal metastases while breathing air (top) and carbogen (bottom). Note that the peak areas of 5-FU and FBAL are similar but that the FNuct peak is significantly larger with carbogen breathing.

tases. The patient refused chemotherapy and died of advanced disease in October 2000.

These two patients were studied while we were optimizing our ^{19}F MR method for detecting 5-FU and its metabolites. Since they had no evidence of liver metastases, we expected to see only 5-FU and its catabolic products (FBAL). For comparison, we have included the ^{19}F spectra of colorectal metastases in the liver of a patient whilst breathing air and subsequently carbogen (Fig. 2) as a preliminary example of the results from the study which is currently in progress. This shows the increase in the FNuct signal when the patient is breathing carbogen.

PRE-CLINICAL DATA

White blood cell counts

The effect of carbogen breathing on the toxicity of 5-FU on WBC in rats is shown in Fig. 3. No statistically significant differences between any of the groups were apparent.

Histology

Group 1 (5-FU only): At 24 h, there was a moderate reduction of bone marrow cellularity in the rat and a mild necrosis of small intestinal crypt epithelial cells. There were no treatment-related changes at 2 h and 240 h. Group 2 (5-FU and carbogen): At 24 h, there was a moderate reduction of bone marrow cellularity and a mild necrosis of small intestinal crypt epithelial cells. There were no treatment-related changes at 2 h and 240 h. Group 3 (carbogen only): There were no treatment-related histopathological changes at 24 and 240 h. All other histopathological changes observed were considered to be incidental in nature.

5-FU analyses

There were increases in 5-FU and FdUMP in the rat liver at 2 and 24 h after 5-FU treatment when carbogen breathing (Group 2) was compared with air breathing (Group 1). However, these differences were only statistically significant when using Student's t-test ($p < 0.05$) for the 2 h FdUMP measurement (see Table 1). No such changes were seen in the small intestine. At 10 days after treatment the 5-FU and FdUMP were not detectable in either liver or small intestine. The concentrations are lower than expected based on previous data in mice (5), which suggest that the retention in tissues is shorter in rats than in mice. However, the incorporation into RNA was higher than expected and 5-FU was still present 10 days after treatment.

DISCUSSION

Finding substantial concentrations of FNuct in the livers of patients receiving 5-FU treatment for colorectal tumours was most unexpected. This result is surprising for two reasons. First, because it was previously thought that FNuct concentrations are not formed in large amounts in normal tissues—certainly not in large enough amounts to be detectable with MRS, which is a relatively insensitive technique. Secondly, because FNuct concentrations have previously only once been observed by ^{19}F MRS and reported in human tumours (and only then after interferon was given with 5-FU (1)) and, to our knowledge, have never been observed in normal liver.

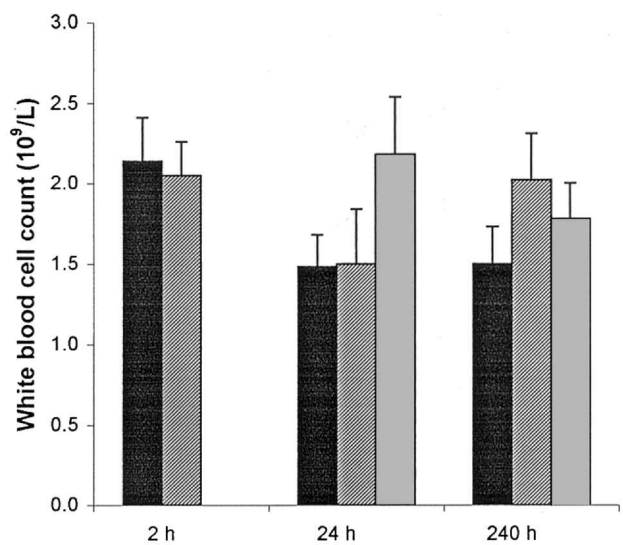

Fig. 3. Levels of rat white blood cells at various time points following the three different treatment protocols. Results show mean ± SEM at 2, 24 and 240 h (10 days) post-treatment with 5-FU (Group 1), 5-FU + carbogen (Group 2), and carbogen alone (Group 3). NB: no sample was taken for Group 3 at 2 h—for an explanation, see Methods. ■ 5-FU. ▨ 5-FU, carbogen. □ Carbogen alone.

Table 1

Levels of 5-FU, FdUMP and 5-FU-RNA in liver and small intestine of rat after either air or carbogen breathing [a]

Tissue	Air/Carbogen	Time	5-FU (pmol/mg wet wt)	5-FU-RNA (pmol/μg RNA)	FdUMP (fmol/mg wet wt)
Liver	−	2 h	2.96 ± 1.50 (5)	0.322 ± 0.150 (4)	9.86 ± 2.14 (5)
Liver	+	2 h	4.54 ± 3.47 (5)	0.409 ± 0.177 (4)	16.2 ± 5.5 (5)
Liver	−	24h	0.80 ± 0.61 (4)	0.536 ± 0.123 (4)	1.23 ± 0.76 (3)
Liver	+	24 h	0.16 ± 0.05 (4)	0.249 ± 0.213 (5)	5.5 ± 3.5 (5)
Liver	−	10 days	n.d. (5)	0.094 ± 0.054 (4)	n.d. (5)
Liver	+	10 days	n.d. (5)	0.111 ± 0.044 (5)	n.d. (5)
Small intestine	−	2 h	75.9 ± 7.2 (4)	0.481 ± 0.120 (4)	25.0 ± 5.8 (5)
Small intestine	+	2 h	64.3 ± 26.7 (4)	0.434 ± 0.209 (5)	21.4 ± 9.39 (5)
Small intestine	−	24h	16.5 ± 4.8 (5)	0.607 ± 0.249 (5)	n.a.
Small intestine	+	24 h	12.4 ± 3.3 (5)	0.656 ± 0.295 (5)	n.a.
Small intestine	−	10 days	n.d. (5)	0.012 ± 0.007 (5)	n.d. (5)
Small intestine	+	10 days	n.d. (5)	0.009 ± 0.005 (5)	n.a.

[a] Values are means + SD for 3–5 animals. Samples from some rats were not available (n.a.) or the metabolite was not detectable (n.d.). In the liver, FdUMP levels at 2 h were significantly different when the air-breathing animals were compared with the carbogen-breathing animals ($p < 0.05$) but not at 24 h ($p > 0.05$). FdUMP levels were significantly different between liver and small intestine ($p = 0.003$) at 2 h but by 10 days undetectable in both liver and small intestine (when enough samples were available for testing).

Cancer cells are known to metabolize 5-FU to a variety of toxic FNuct, including 5-fluorouridine triphosphate (FUTP), which can be incorporated into RNA, 5-fluorodeoxyuridine triphosphate, which can be incorporated into DNA, 5-fluorouridine diphosphate sugars, which may interfere with glycosylation of proteins and lipids, and 5-fluorouridine monophosphate (FdUMP), which inhibits thymidylate synthase, a key enzyme in DNA synthesis (17). In normal tissue toxicity studies in rats, we focused on FdUMP as the most toxic of the anabolites. HPLC studies from extracts of animal tumours have suggested that 5-FUTP is the predominant fluoronucleotide seen in the MR spectrum. FdUMP is formed at concentrations that are too low to be visible by ^{19}F MRS. Nevertheless, FNuct signals in animal and human tumour xenografts have been shown to predict response to 5-FU treatment (10, 18, 19). Apparently, the extent of FNuct synthesis is related to that of FdUMP, resulting in a correlation between FNuct synthesis and response.

Detection of FNuct in normal tissues could have some practical significance. Hepatic toxicity is noted when 5-FU is administered directly into the liver, by arterial infusion, to treat hepatic tumours (note that in the present study it was given intravenously). Furthermore, the activity of 5-FU as a single agent is quite low (usually less than 20%) and it is often given with adjuvants. It might be possible to predict the effects of such adjuvants on normal tissue from changes in the FNuct signal intensity. In view of these opportunities and concerns regarding toxicity to normal tissues, we were interested to see whether carbogen breathing enhanced the toxicity of 5-FU in normal rats. Our previous study (9) had demonstrated that drug uptake (ifosfamide) by the liver and other major organs (11) was not enhanced by carbogen breathing, but in view of our on-going clinical trial with 5-FU, it was still important to demonstrate that other normal tissues, and particularly those most sensitive to anticancer agents—bone marrow, intestinal mucosa and white blood cells—were also unaffected by using carbogen as an adjuvant for 5-FU.

Taken together, the lack of significant differences in WBC counts, histology and 5-FU analyses implies that there is no additional normal tissue toxicity caused by the carbogen breathing. This is important in relation to our ongoing clinical studies, especially as we have shown that toxic FdUMP is not significantly enhanced by carbogen breathing in the liver (or in the small intestine). Furthermore, the incorporation of 5-FU into RNA did not increase after carbogen breathing either in the liver or small intestine; 5-FU-RNA is thought to be responsible for gut toxicity (2, 20).

In summary, we have demonstrated that ^{19}F MRS can be used to monitor toxic FNuct in the normal human liver of patients undergoing chemotherapy with 5-FU. We have also shown in rats that the use of carbogen to enhance the uptake of 5-FU into tumours does not enhance toxicity to liver, bone marrow, white blood cells or the intestinal mucosa. The preliminary report of this ongoing clinical study suggests that carbogen breathing may increase FNuct in colorectal metastases of the liver.

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