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# Effect of carbogen breathing on the pharmacodynamics of 5-fluorouracil in a murine colon carcinoma

Y.J.L. Kamm [a,*], A. Heerschap [b], D.J.Th. Wagener [a]

[a] *Department of Medical Oncology, University Hospital, PO Box 9101, 6500 HB Nijmegen, The Netherlands*
[b] *Department of Radiodiagnostics, University Hospital, PO Box 9101, 6500 HB Nijmegen, The Netherlands*

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**Abstract**

To determine whether carbogen breathing has an effect on 5-fluorouracil (5-FU) uptake, retention and metabolism in C38 murine colon tumours grown in C57Bl/6 mice, we used *in vivo* $^{19}F$ nuclear magnetic resonance (NMR) spectroscopy. Eleven tumour-bearing mice were treated with 150 mg/kg of 5-FU given intraperitoneally (i.p.). Five mice received carbogen gas (95% $O_2$ and 5% $CO_2$) for 9.5 min, starting 1 min before 5-FU administration. We found increased levels of 5-FU and its anabolites and catabolites by sequential [$^{19}F$] NMR spectroscopy in the group treated with 5-FU in combination with carbogen compared with the group treated with 5-FU alone. The maximum of normalised values of 5-FU and its metabolites, reached after carbogen breathing, was almost 2-fold higher than after treatment with 5-FU alone. Despite these increased concentrations no significant effect of carbogen on growth inhibition of the tumour by 5-FU was observed, which may be related to the size as well as the well vascularised and perfused conditions of the tumours studied. 

*Keywords:* [$^{19}F$] NMR spectroscopy; 5-Fluorouracil; Carbogen

## 1. Introduction

Colorectal cancer is one of the most common cancers in the western world. Moreover, a significant number of patients develop metastases and will die of the disease. Treatment of advanced colorectal cancers with chemotherapy is difficult. Only 20–30% of patients will respond to treatment with 5-fluorouracil (5-FU), the main cytostatic agent for this disease. It is also difficult to predict whether a patient will respond to treatment or not. Strategies to improve treatment outcome which are already used in clinical practice are biomodulation of 5-FU, administration by continuous infusions or hepatic arterial infusion instead of bolus injections [1–3]. In an earlier study, we observed that certain biomodulators of 5-FU metabolism increased the tumour levels of anabolites or the ratio of anabolites to catabolites [4].

To exert their effect, cytostatics should reach and enter the tumour cells. In tumours with poorly perfused areas this may become a limiting factor and treatment of these tumours may benefit from perfusion-enhancing approaches. Modulation of the oxygenation of tumours has been attempted by breathing carbogen (95% $O_2$, 5% $CO_2$). It has been reported that carbogen raises distending pressure in capillaries by increasing systemic arterial blood pressure and decreasing the tone of resistance of arterioles [5]. Carbogen breathing produces in some cases an increase in blood flow/perfusion [6]. The heterogeneity in blood flow increase highlights the disordered structure of tumour vascular architecture. In Morris hepatoma, carbogen breathing increased both tumour $pO_2$ and magnetic resonance (MR) image intensity which is sensitive to changes in deoxyhaemoglobin [7]. No changes were seen in blood flow measured by laser Doppler flowmetry. This in contrast with other tumour types, where carbogen has been shown to change tumour blood flow, suggesting that this may be a tumour-specific phenomenon [8]. Carbogen is applied in experimental settings in the field of radiotherapy in order to increase the $pO_2$ and radiosensitivity of tumours [9]. Carbogen breathing is often combined with the use of perfluorochemical emulsions (PFCE) as an adjuvant to radiation or chemotherapy [10]. The rationale is that solid tumour masses contain

* Corresponding author. Tel.: +31-24-361-5215; fax: +31-24-354-0788.
*E-mail address:* y.kamm@onco.azn.nl (Y.J.L. Kamm).

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areas of hypoxia which are therapeutically resistant. Several anticancer agents are enhanced in antitumour activity by the co-administration of PFCE and carbogen. As carbogen breathing may affect tumour blood perfusion, it could also serve as a tool to enhance the uptake of antitumour compounds like chemotherapeutics, such as cyclophosphamide, ifosfamide and 5-FU. With carbogen breathing tumour growth delay by cyclophosphamide is enhanced [11]. In addition, it has been shown that carbogen breathing increases the uptake and cytotoxicity of ifosfamide in rat prolactinomas [12]. Recently it has been demonstrated by [$^{19}$F] nuclear magnetic resonance (NMR) spectroscopy that carbogen breathing increases 5-FU uptake and cytotoxicity in hypoxic murine RIF-1 tumours [13]. Carbogen may also exert its effect by changing the extracellular pH. It is known that cellular uptake of 5-FU is pH-dependent [14]. After carbogen breathing a fall in the extracellular pH is observed, which can cause an increased 5-FU uptake.

The purpose of this study was to determine the effect of carbogen breathing on the pharmacodynamics of 5-FU uptake in the C38 murine colon tumour monitored by [$^{19}$F] NMR spectroscopy. Furthermore, the effect of carbogen breathing on tumour growth inhibition by 5-FU was studied.

## 2. Materials and methods

### 2.1. Chemicals

5-FU was obtained from Teva (Mijdrecht, The Netherlands) as a saline solution at 50 mg/ml. Carbogen gas (95% $O_2$–5% $CO_2$) was obtained from Hoekloos (Schiedam, The Netherlands).

### 2.2. Tumour model

Female C57BL/6 mice of 8–12 weeks of age were obtained from the Central Animal Laboratory of our university. The C38 murine colon tumour, which is known to be sensitive to 5-FU, was acquired from Dr P. Lelieveld of the REPGO-TNO Institute, Rijswijk, The Netherlands, and is described elsewhere [15]. Tumour tissue fragments with a diameter of 3 mm were implanted subcutaneously (s.c.) in the right flank of the mouse. Treatment was performed after 2 weeks, when the tumours reached a weight of 0.4–0.8 g. The maximum tumour size was $\leqslant$7% body weight. The tumour was measured with a calliper. The tumour was considered to be an ellipsoid and its volume was estimated from three orthogonal diameter measurements using the following formula: tumour volume ($mm^3$) = $X \times Y \times Z \times 0.5$ [15]. The experimental procedures were approved by the local ethics committee for the use of animals.

### 2.3. Treatment

At the time of the experiment the tumour-bearing mice were cannulated intraperitoneally (i.p.) with polythene tubing (inner diameter 0.4 mm, outer diameter 1.8 mm) under inhalation anaesthesia using a mixture of enflurane (1.5%), oxygen (29.5%) and nitrous oxide (69%) applied through a nose-cone and positioned in the magnet. The temperature of the mice was maintained at 37°C using a warm water blanket with a feedback system and controlled with a rectal temperature probe. To determine whether carbogen breathing causes an effect on the pharmacodynamics of 5-FU, the following experiment was performed. After shimming, carbogen breathing (2 l/min) was switched on for 9.5 min, whilst turning off the nitrous oxide and oxygen. After the first minute of carbogen breathing 5-FU was administered as a bolus i.p. at a dose of 150 mg/kg in normal saline (0.1 ml/g) within 20 s. At the moment of 5-FU administration [$^{19}$F] NMR was also started. At the end of the first block of 8.5 min, so after 9.5 min in total, carbogen was switched off and replaced by oxygen and nitrous oxide again. The dose of 5-FU used, i.e. 150 mg/kg, is well tolerated and corresponds to 457 mg/$m^2$ in man, which is in the therapeutic range (370–500 mg/$m^2$ for 5 days) [16]. A total of five mice were treated in this way. The same procedure was applied to a control group of tumour-bearing mice ($n = 6$) but omitting the carbogen.

Carbogen was given during 9.5 min. This duration was based on the following findings. After 1 min of carbogen breathing the oxyhaemoglobin concentration and deoxyhaemoglobin concentration in human glioma xenografts are close to a steady state [8]. Therefore carbogen breathing was started 1 min before 5-FU administration. Upon carbogen breathing an increasing tumour $pO_2$ has been reported in both animals and patients, which is maximal in patients after 1–6 min [17]. Return to precarbogen levels of oxygenation occurred in 1 min after the end of gas exposure [18]. In clinical radiotherapeutical studies, carbogen breathing is applied 4 min before and also during radiotherapy, in total for approximately 15 min [9]. Within approximately 10 min carbogen breathing reaches its maximal effect. Because we wanted to obtain tumour trapping of 5-FU after bolus injection by inducing a short vasodilatation by means of carbogen, carbogen breathing was continued for one block of 8.5 min, in total for 9.5 min.

### 2.4. $^{19}$F NMR spectroscopy

$^{19}$F NMR spectra were measured at 169.457 MHz on a 4.3 Tesla (T) Oxford vertical bore magnet equipped with a SMIS console. A home-built [$^1$H]/[$^{19}$F] double tunable radio frequency (RF) coil with an internal diameter of 13 mm and two windings was placed around the tumour. The RF coil was fitted with a Faraday

shield to eliminate spurious signals from normal tissue adjacent to the tumour. Shimming was performed until a $H_2O$ signal line width at half-height of less then 70 Hz was obtained. [$^{19}$F] NMR data acquisition parameters were: spectral width 10 kHz, repetition time 0.5 s, flip angle 90° with a rectangular RF pulse of 20 μs. No [$^{1}$H] decoupling was used. Spectra were recorded from the moment of 5-FU injection. Measurements took place during 2 h in blocks of 8.5 min in which 1024 acquisitions or measurements were recorded.

Before and after this session a [$^{1}$H] NMR spectrum at 180.130 MHz was recorded by the [$^{19}$F] coil for quantification using the signal of $H_2O$ as an internal reference. Sixty-four scans were recorded with a repetition time of 1 s. Because the [$^{1}$H] NMR spectra were recorded before and 2 h after the carbogen breathing, the influence of carbogen breathing on the relaxation of the [$^{1}$H] spins of water was excluded.

### 2.5. Analysis of [$^{19}$F] NMR spectra

The [$^{19}$F] NMR resonance frequency of 5-FU was set to a chemical shift value of 0 parts per million (ppm). The integral for the signals of 5-FU (F), the FU anabolites (A), and the FU catabolites (C) (α-fluoro-β-ureidopropionic acid [FUPA] and α-fluoro-β-alanine [FBAL]) was determined. Signals in [$^{19}$F] and [$^{1}$H] NMR spectra were quantitated by iterative fitting of the time domain signal with the variable projection method (VARPRO) using MRUI software [19,20]. All the peak areas were obtained by fitting the spectral lines to Lorentzian line shapes. The catabolite content was determined as the sum of the FUPA and FBAL signal intensities. All [$^{19}$F] curves are normalised by dividing the integrals by the mean integral of the $H_2O$ signal in the two [$^{1}$H] spectra. This assumes that the amount of hydrogen measured is correlated with the amount of measured tumour tissue, taking into account that a constant part of the tumour (85–90%) consists of water. By this semi-quantitative method it is possible to compare metabolite levels between the different treatment modalities. The area under the concentration–time curve (AUC) for A, C and F was calculated.

### 2.6. Tumour histology

Tumour sections were also offered for histological examination. Special attention was given to the presence of necrosis.

### 2.7. Tumour vascularity and perfusion

Six separate mice (without any treatment) were studied for tumour vascularisation and perfusion [21]. This method has been described by Rijken and colleagues [21].

### 2.8. Tumour growth

All mice were followed for tumour volume and body weight after the spectroscopy experiment plus nine additional ones receiving 5-FU alone plus an additional one receiving 5-FU plus carbogen. Those two parameters were determined twice a week for a period of 4 weeks. In order to compare tumour growth, the relative tumour volumes were taken. Therefore, reference was made to the value obtained on day 0, which was the day of treatment. At the end of the experiment the mice were sacrificed.

### 2.9. Statistics

The spectroscopy experiment was performed on six mice who received 5-FU alone and five mice who received 5-FU together with carbogen breathing. For each group of mice medians are presented with the minimum and maximum for the spectroscopy parameters. Differences between the two groups were analysed by the unpaired Student *t*-test. For the parameters under $C_{max}$ concerning maximum concentration, tests were performed after log transformation, because of oblique distribution. For the parameters under AUC concerning surface, tests were performed after root transformation. A two-sided significance level of 0.05 was used. For the half life ($t_{1/2}$) of 5-FU the Wilcoxon 2-sample test was used.

The experiment concerning tumour growth inhibition was performed on 15 mice who received 5-FU alone and six mice who received 5-FU together with carbogen breathing. To express the growth rate after the minimal tumour volume was reached, the tumour volume doubling time was calculated on the assumption of exponential growth. Parameters concerning tumour volume (growth) were analysed after log transformation.

## 3. Results

### 3.1. Comparison of [$^{19}$F] NMR in vivo serial spectra of C38 colon tumour with and without carbogen breathing

In sequential [$^{19}$F] NMR spectra of C38 tumours after administration of 5-FU, signals from 5-FU and its anabolites and catabolites could be observed (Fig. 1). The 5-FU peaks, and its anabolite and catabolite peaks were often higher in the presence of carbogen. The 5-FU signal almost completely disappeared one hour after its application, both in the absence and in the presence of carbogen. The anabolites were presented as one single peak (peak A between 4.5 and 5 ppm). The catabolites were reflected by two different peaks, the first representing FUPA and the second representing its conversion product FBAL (peaks C between −16.5 and −17 ppm and between −18 and −18.5 ppm).

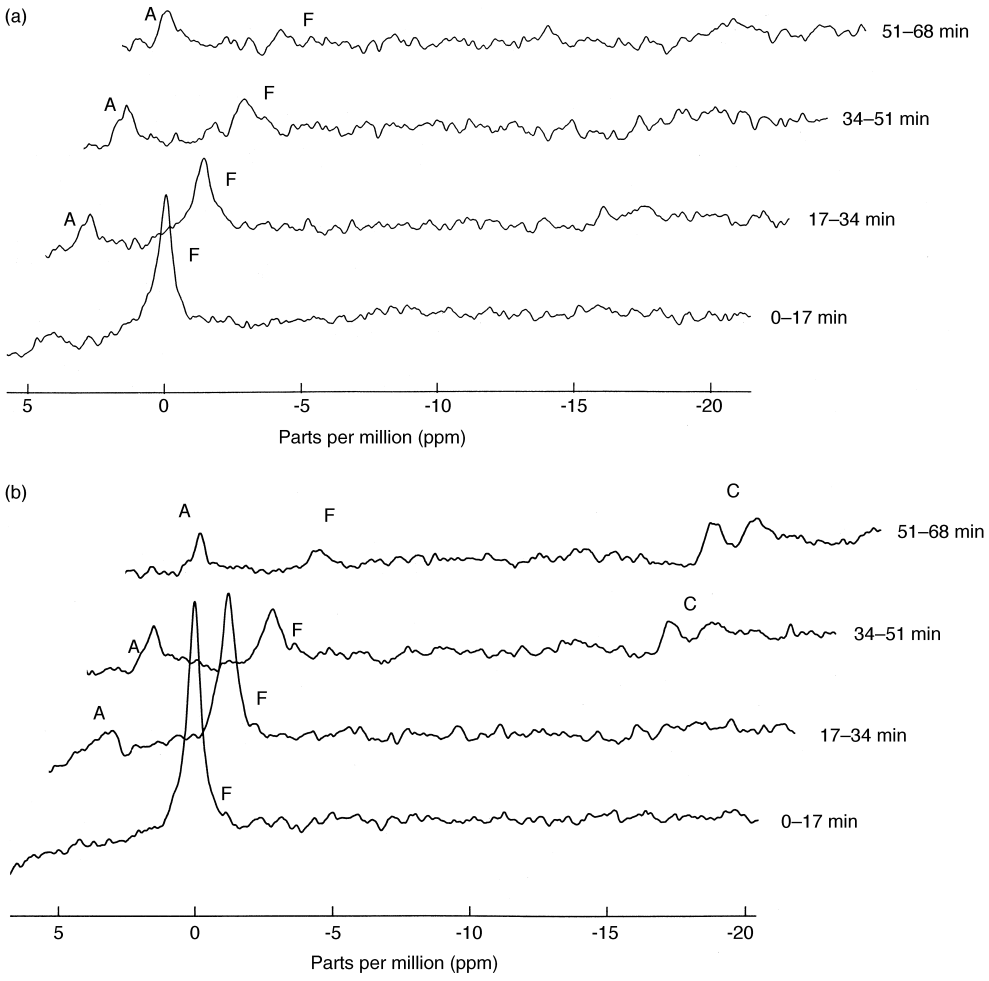


Fig. 1. Effect of carbogen breathing on the $^{19}F$ nuclear magnetic resonance (NMR) spectrum of a C38 murine colon tumour. Shown are sequential spectra at 17 min intervals (two spectra of 8.5 min summed) after intraperitoneal (i.p.) injection of 150 mg/kg 5-FU. Plotted beside each spectrum is the time in min of the acquisition interval after injection of 5-FU. (a) Treated with 5-FU alone; and (b) treated with 5-FU plus carbogen breathing during the first 8.5 min. A, anabolites; C, catabolites; F, 5-FU.

### *3.2. Time dependence of the tumour 5-FU metabolite pattern with and without carbogen breathing*

Carbogen breathing resulted in a more rapid increase to a higher level of 5-FU in the tumour. Also the levels of its anabolites and catabolites were enhanced (Table 1). As a consequence the total amount of fluorine-containing compounds was higher as well. 5-FU increased during the first 17 min and decreased thereafter within two hours (Fig. 2). The anabolites became detectable mostly within 17 min after 5-FU administration and were increasing until they reached a plateau level during the first hour. The catabolites followed slightly later, after 25–34 min post 5-FU, and reached a plateau at approximately 60–68 min. The aforementioned time points were not shifted by carbogen with the exception of catabolites which reached a plateau at 76–85 min. Two groups of mice treated with 5-FU alone ($n=6$) or plus carbogen breathing ($n=5$) were compared. The maximum peak area ($C_{max}$) of anabolites, 5-FU and total fluorine containing compounds was significantly higher after treatment with carbogen (Table 1). The AUC of A, C, F and T was also higher after treatment with carbogen, and showed a tendency for significance. The median $t_{1/2}$ of 5-FU retention in the tumour after treatment with 5-FU alone and after addition of carbogen breathing was not different in both cases.

### *3.3. Histological characterisation and perfusion of tumours*

Histological examination of six separate tumours of comparable weight was performed. The tumours consisted of a poorly differentiated adenocarcinoma which contained 15–30% necrosis.

One of the most important parameters for tumour vascularisation and perfusion is the perfusion fraction (PF), indicating the fraction of vessel structures that are perfused. The median PF was relatively high: 0.83 (range 0.72–0.97). A high PF means that the tumour is

Table 1
Effect of carbogen breathing on 5-FU pharmacodynamics in the C38 murine colon tumour obtained from *in vivo* [$^{19}$F] NMR spectra

| Treatment | $C_{max}$ of metabolites in arbitrary units[a] | | | |
|---|---|---|---|---|
| 5-FU | A | C | F | T = A + F + C |
| − carbogen median (range) | 0.23 (0.13–0.53) | 0.41 (0–0.66) | 0.66 (0.23–1.58) | 0.77 (0.50–1.74) |
| + carbogen median (range) | 0.47 (0.31–0.82) | 0.92 (0.33–2.41) | 1.41 (1.23–2.41) | 1.46 (1.23–3.56) |
| *P* value | $P = 0.03$ | $P = 0.08$ | $P = 0.01$ | $P = 0.01$ |
| Treatment | AUC of metabolites in arbitrary units[a] | | | |
| 5-FU | A | C | F | T = A + F + C |
| − carbogen | 16 (3–33) | 16 (0–28) | 31 (11–66) | 53 (32–115) |
| + carbogen | 26 (17–33) | 32 (15–142) | 43 (39–100) | 101 (71–269) |
| *P* value | $P = 0.11$ | $P = 0.05$ | $P = 0.06$ | $P = 0.04$ |
| Treatment | $t_{1/2}$ of 5-FU (min) | | | |
| 5-FU | F | | | |
| − carbogen | 34.0 (17.0–34.0) | | | |
| + carbogen | 34.0 (25.5–42.5) | | | |
| *P* value | $P = 0.40$ | | | |

[a] $C_{max}$ is the maximum peak area (by block of 8.5 min) given in normalised values, which are achieved by dividing integrals by the mean integral of the $^{1}$H spectra and multiplying by 1000; AUC is area under the concentration–time curve (from 0 to 119 min) also in normalised values of anabolites (A), catabolites (C), 5-FU (F) and total fluorine-containing compounds (T); and $t_{1/2}$ is half-life of 5-FU (after $C_{max}$) in the tumour after treatment with 5-FU 150 mg/kg alone ($n = 6$) or together with carbogen breathing ($n = 5$).

5-FU, 5-fluorouracil; NMR, nuclear magnetic resonance.

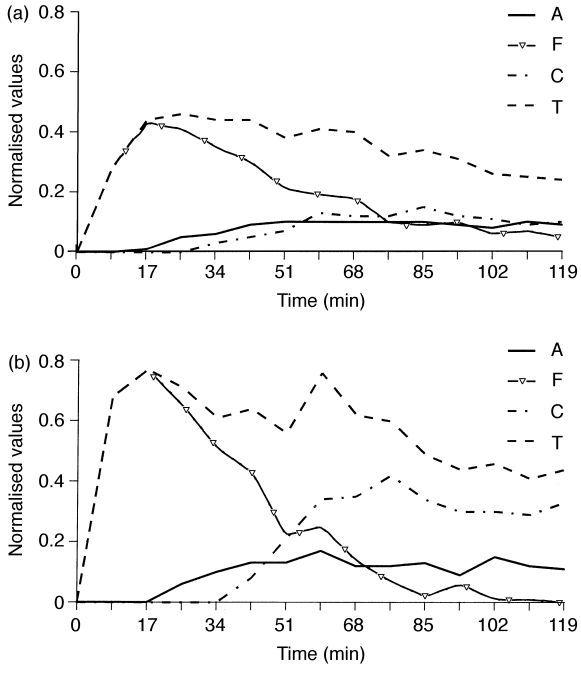


Fig. 2. Effect of carbogen breathing on time dependence of 5-FU metabolite pattern in the C38 murine colon tumour. Data are normalised values of the integrals of the signals of anabolites ( — ), catabolites (· — ·), 5-FU (–▽–) and total fluorine-containing compounds (– – –) 0–119 min after 5-FU injection. Points, mean values of 5–6 mice. (a) Treated with 5-FU alone; and (b) treated with 5-FU plus carbogen breathing during the first 8.5 min. Data time points represent the end of data accumulation periods, e.g. 8.5 min stands for a spectrum collected from 0 to 8.5 min.

well perfused. Images of immunohistochemical stained sections showed that the tumour was also well and regularly vascularised.

### 3.4. Effect of carbogen breathing on tumour growth and body weight

The tumour weight was 0.4–0.8 g. No difference in tumour growth behaviour was seen for the two treatment groups in this experiment. The minimal tumour volume after treatment (relative mean 0.40 and 0.33) and the tumour volume 14 days after treatment (1.88 and 2.93) were not significantly different for the groups treatment resp. without and with carbogen as well as the doubling time after the minimal tumour volume (4.34 and 3.14 days).

The mean maximal loss of body weight in 14 days after treatment was 7±4% standard deviation (S.D.) for the group treated with 5-FU alone and 11±9% for the group treated with 5-FU plus carbogen. This parameter was taken as an indicator for the maximal tolerated dose. In this period there was one toxic death in the group treated with 5-FU alone.

## 4. Discussion

According to Presant and colleagues an increased 5-FU uptake in the tumour, an improved metabolism to anabolites and a higher $t_{1/2}$ of 5-FU retention are predictive for a better response to therapy [22]. Carbogen

breathing can cause vasodilatation and increased perfusion and is a potential method to improve the effect of chemotherapy [12]. The purpose of this study was to answer the question of whether carbogen breathing increases the uptake, retention and metabolism of 5-FU in a murine colon tumour and thus results in an enhanced tumour growth delay.

We have found that carbogen breathing indeed increased tumour levels of 5-FU and its metabolites. In addition to vasodilatation and increased perfusion the drop in extracellular pH compared with the intracellular pH after carbogen breathing may have contributed to the increased cellular uptake of 5-FU [13,14]. Carbogen and nicotinamide can act on both diffusion-limited hypoxia and intermittent perfusion-limited hypoxia [23]. Alone and in combination they can decrease the number of mismatched and closed vessels, but this is dependent on tumour type.

However, the present study did not reveal an effect of carbogen breathing on tumour growth delay by 5-FU, despite the higher 5-FU and 5-FU metabolite levels attained with carbogen breathing. In addition, no enhancing effect of carbogen on 5-FU retention in the tumour was found. Several reasons may explain the lack of effect of carbogen on tumour growth inhibition notwithstanding increased 5-FU and 5-FU metabolite levels. One reason could be that in the C38 tumour the uptake of 5-FU and its immediate conversion are not rate limiting in the effect of 5-FU on tumour growth inhibition and that the tumour was already responding effectively to this treatment. Supporting this hypothesis is the high $t_{1/2}$ of 5-FU retention without (median 34 min, range 17–34) and with carbogen (median 34 min, range 25.5–42.5 min), whereas a $t_{1/2}$ of less than 20 min is found in other tumours [13,24]. It is possible that because carbogen did not increase this $t_{1/2}$ further, no additional effect on tumour growth inhibition was seen.

The particular reason for the lack of effect of carbogen on tumour growth inhibition is unclear, but could be related to some tumour characteristics such as tumour type and size [12]. In addition, the combination with other treatment strategies, such as multiple dosing, may be more effective. Differences in vascularisation may play an important role in differential effects. Recently, the effect of carbogen breathing on 5-FU uptake and cytotoxicity was studied by others in hypoxic murine RIF-1 tumours, also using [$^{19}$F] NMR spectroscopy [13].

In conclusion, this study showed increased levels of 5-FU and its anabolites and catabolites after carbogen breathing. This indicates that carbogen could be of importance to improve the uptake and metabolism of 5-FU in colorectal cancer. However, no effect of carbogen breathing on tumour growth inhibition was observed, possibly due to the small size and well perfused condition of the tumour.

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