Non-invasive $^{19}$F MR spectroscopy of 5-fluorocytosine to 5-fluorouracil conversion by recombinant Salmonella in tumours

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The aim of this study was to evaluate the applicability of fluorine-19 magnetic resonance spectroscopy ($^{19}$F MRS) for monitoring in vivo the conversion of 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU) after using an attenuated Salmonella Typhimurium strain recombinant to provide cytosine deaminase (TAPET-CD). The $^{19}$F MRS measurements were done on mice bearing the human colon tumour xenograft (HCT116). The intratumoural conversion is greater when TAPET-CD/5-FC is delivered intratumourally (i.tu.) than when TAPET-CD is delivered intravenously (i.v.) and 5-FC intraperitoneally (i.p.). Repeat measurements of the same tumour also yielded important information on the tumour colonization by TAPET-CD through the correlated 5-FC to 5-FU conversion efficacy. The in vivo MRS spectra were confirmed by in vitro $^{19}$F MRS of perchloric acid extracts of the tumour tissue. No 5-FU metabolites were detectable in vivo in the tumours. However, the in vitro measurements revealed, besides 5-FC and 5-FU, the presence of small amounts of catabolites. Finally, spectra obtained in vitro from liver extracts of tumour-bearing mice treated i.tu. with TAPET-CD/5-FC showed no 5-FU and only little amounts of catabolites. Our data illustrate most importantly the potential of $^{19}$F MRS to monitor biologically-based treatments involving cytosine deaminase.

Keywords: $^{19}$F MRS; 5-fluorouracil; cytosine deaminase; xenograft human tumour; mouse; recombinant bacteria

New developments for cancer treatment are allowing individualized and biologically-based therapies. As part of these developments one needs to accurately define both physical and biological characteristics of tumours, and to monitor the efficacy of treatment on an individual basis. Techniques such as computed tomography (CT) or magnetic resonance imaging (MRI) have been shown extremely useful in defining the physical limits of the tumour. Progress has also been made with regards to the evaluation of biological parameters of the tumour microenvironment (eg, hypoxia) and dynamic monitoring of therapy-induced changes (eg, 5-fluorouracil (5-FU) chemotherapy). Yet, further progress is of crucial importance for the evaluation of new biologically-based therapies that may be more tumour specific than traditional therapies, and which necessitate a stringent individualized follow-up. Technologies that are being explored in this context are positron emission tomography (PET) and magnetic resonance spectroscopy (MRS). The application of biological imaging methodologies is essentially non-invasive, and thus biopsies before and during the treatment do not need to be taken, leaving the tumour microenvironment undisturbed.

Furthermore, these methods allow potential screening of tumours at many sites of the body and enable the direct comparison with normal tissues.

The use of MRS to non-invasively assess biochemical properties of tumours (eg, pH and lactate), and to evaluate concentrations and pharmacology of fluorinated chemotherapeutics has been reported. Compounds such as 5-FU lend themselves to this methodology because fluorine-19 ($^{19}$F) has high sensitivity (83% relative to $^2$H), and because only negligible amounts of $^{19}$F are normally present in the body. 5-FU is a well-known chemotherapeutic agent, and has been evaluated along with the various metabolites. Although 5-FU measurements are often done in plasma samples, studies indicate that plasma levels do not reflect the intratumoural activity of 5-FU (Finan et al, 1987; Hull et al, 1988; Findlay et al, 1996). Using $^{19}$F MRS, 5-FU and metabolites could be detected directly in the tumour and in the liver, in both preclinical animal investigations as well as in patient studies of systematically delivered 5-FU (Stevens et al, 1984; Wolf et al, 1990; reviewed in Martino et al, 2000). Studies in animals and patients suggest that the trapping of 5-FU and the amount of anabolites in the tumour correlate with response and may yield prognostic information on treatment outcome (McSheehy et al, 1989, 1992; Wolf et al, 1990, 1998; Findlay et al, 1993; Presant et al, 1994; Schlemmer et al, 1999). Furthermore, in MRS the area of the spectral peak is proportional to the concentration of the corresponding metabolite (absolute concentrations require calibration). Although PET is more sensitive than MRS, the former
technique lacks the capacity to distinguish in vivo between the prodrug, the drug and the different metabolites.

Others and we have been investigating a new biologically-based cancer therapy involving the cytosine deaminase (CDase) enzyme/prodrug system. The non-mammalian enzyme CDase converts the non-toxic 5-FC to the cytostatic drug (5-FU). Previous results showed that recombinant bacteria can be used as a delivery vector to transfer CDase selectively to the compromised intratumoural microenvironment. The tumour-selective applicability of this strategy has recently been demonstrated in vitro and in vivo with non-pathogenic clostridia (Minton et al, 1995; Fox et al, 1996; Theys et al, 2001) and attenuated Salmonella Typhimurium (msbB· purI· strain; Vion Pharmaceuticals Inc.) (Low et al, 1998; Lee et al, 2001; King et al, 2002; Mei et al, 2002). Since such bacteria-based vectors are tumour selective, the enzyme/prodrug system may provide maximal tumour dosing with prolonged drug presence and circumvent treatment-limiting side effects (reviewed by Aghi et al, 2000).

MRS has already been shown to be able to detect conversion of 5-FC to 5-FU in tumours in which cells have been engineered to express CDase or by using a monoclonal tumour targeting antibody-CDase conjugate (Aboagye et al, 1998; Stegman et al, 1999).

The objective of the present study is to demonstrate the applicability of 19F MRS to assess non-invasively biological therapies. Specifically, 19F MRS is applied in a situation in which the expression of the therapeutic gene, and thus the conversion efficacy, is expected to be dependent on the microenvironment and biological characteristics of the tumour. Attenuated S. Typhimurium engineered to express CDase (TAPET-CD) were investigated in a mouse-xenografted human tumour model.

Our results show that the selective intratumoural production of 5-FU by a biologically-based therapy such as the TAPET-CD/5-FU strategy, can be monitored using 19F MRS.

MATERIALS AND METHODS

In vivo tumour model

HCT116 human colon tumour was xenografted subcutaneous (s.c.) in nu/nu mice using 1 × 106 cells per 100 μl. The cells were inoculated into the lower flank. At the time of TAPET-CD injection and of 19F MRS, the tumour volumes were between 300 mm3 and 2000 mm3 (tumour volumes were measured with Vernier calipers in three orthogonal directions and calculated with the formula L × H × W × π/6).

All animal experiments were approved by the Animal Ethics Committee of the University ‘KU Leuven’, and procedures were according to the guidelines defined by the UKCCCR (Workman et al, 1998).

Bacterial strain and non-MRS evaluation

The bacteria used were attenuated Salmonella Typhimurium, recombinant for cytosine deaminase (coded VNP20047, provided by research by VION Pharm. Inc. New Haven CT 06511, and hereafter referred to as TAPET-CD). The bacteria were grown in liquid modified LB medium at 37°C for 24 h, at which time the colony-forming units (cfu) per gram of tissue were determined.

The preparation of the bacterial lysates for in vitro analysis of CDase activity has been described previously (Theys et al, 2001). Briefly, aliquots of an exponentially growing TAPET-CD culture were taken at various optical densities (OD600nm) and centrifuged. The pellets were washed, sonicated, and the lysates were incubated at 37°C for 24 h with a 10 mg/ml solution of 5-FC to which Tris HCl (1 M) was added.

Preparation of tissue perchloric acid extracts (PCA extracts)

Animals were sacrificed by cervical dislocation and immediately afterwards the tumour and liver were removed and snap frozen in liquid nitrogen. The whole tumour or liver was homogenized at 0°C in 5 ml of cold HClO4 (1 M) per gram tissue. After homogenization, the sample was incubated on ice for 1 h followed by centrifugation for 15 min at 1000 x g (4°C). The supernatant was decanted and neutralized with KOH (10 M) and KHCO3 (1 M). The resulting precipitate was discarded following a second centrifugation. The supernatant was subsequently freeze-dried and the lyophilized was dissolved in 0.5 ml of a potassium phosphate buffer (pH 7.0; 1 M).

In vivo and in vitro MRS measurements

In vivo 19F MRS experiments on mice bearing the HCT116 tumour were performed at 188 MHz in a Bruker Biospec (Karlsruhe, Germany), equipped with a horizontal 4.7 Tesla superconducting magnet with 30 cm bore, using a 10 mm transmit-receive surface coil. Prior to the measurement, the mice were anaesthetized with intraperitoneally (i.p.) injected sodium pentobarbital (1 mg/g body weight Nembutal®, Sanofi, Belgium). After administration of the 5-FC (i.tu. 100 μl of a 7.5 μg/μl solution in saline by one injection central to the tumour since multiple injections may lead to multiple peaks (Guerquin-Kern et al, 2000), or i.p. 300 mg/kg (ie, on average 9 mg per mouse); SIGMA, Belgium), the mice were placed on a Perspex plate such that the tumour was positioned directly above the circular surface coil. Following shamming on the water proton signal, serial 19F MRS spectra were acquired every 13 min during 1 to 2.5 h (90 degree pulse of 11 μs, total repetition time TR=0.75 s, number of averages NA=1024, spectral width = 27 kHz; acquisition size = 2048 points; no proton decoupling). The signals were processed by zerofilling to 4096 points and exponential multiplication of 6 Hz.

Most 19F MRS measurements were performed at day 0 and day 1. Some animals were also evaluated 1 or 2 weeks after the injection of TAPET-CD. Before a new injection of 5-FC was given, a spectrum was first recorded to determine whether there were still signals present from the previous injection (24 h earlier). The chemical shift of the 5-FU resonance was set to 0 ppm. The 5-FC signal was observed around 1.2 ppm. The signal positions were verified with the i.tu. injection of 5-FC (as before) and 5-FU (40 μl of 50 mg/ml Fluroblastine®, Pharmacia, Belgium) in tumour-bearing mice treated with TAPET-CD and were also correlated to the 1H chemical shift of the tumour H2O resonance. A spherical external reference sample (diameter = 7.5 mm, volume ~100 μl) containing 4-fluorobenzoic acid (FBA; ACROS, Belgium) in deuterated water saturated with chromium acetylacetonate (Cr(acac)3, ACROS, Belgium) was placed ~9 mm under the coil to monitor stability of the MRS technique and coil load changes for
the different mice/tumours under evaluation. Absolute concentrations were determined in a separate experiment using a cylindrical water phantom sample (diameter = 25 mm, height = 22 mm) containing 1.25 mM 5-FC, 2.5 mM 5-FU and 10 mM NaF.

During the in vivo $^{19}$F MRS measurements, the body temperature of the mice was kept at 36°C with the use of warm air ventilation in the magnet bore.

In vitro analysis of tissue extracts of TAPET-CD/5-FC-treated animals and in vitro analysis of lysates of TAPET-CD cultures were performed in a high resolution AMX 360 (8.4 Tesla) spectrometer (Bruker, Karlsruhe, Germany). Specific settings for the tissue extracts (278 K) were, TR = 2.5 s, NA = 3072 or 12288, $^{1}$H WALTZ-16-decoupling during the 1 s acquisition; and for the lysates (295 K), TR = 2.5 s, NA = 256, no proton decoupling. The tissue extracts were measured at 278 K to exploit the substantially shorter relaxation time at low temperature (typically a factor three) (Kamm et al, 1994). Absolute concentrations were determined using a phantom sample of 5-FU in water (2.8 mm, 500 μl) with a coaxial FBA reference sample insert.

Statistics

All results are expressed as averages ± SEM (with indication of the number of experiments). Linear regression analysis was performed where appropriate.

RESULTS

$^{19}$F MRS to evaluate in vitro the conversion capacity of the TAPET-CD

In vitro $^{19}$F MRS measurements of the conversion of 5-FC to 5-FU were initially performed in lysates from a series of TAPET-CD cultures to be used in the experiments. The conversion capacity as a function of TAPET-CD growth status (OD$_{600}$) and was optimal when lysates were evaluated at the maximum of the growth phase (Table 1). This clearly demonstrated the relationship between the number of viable TAPET-CD and the 5-FU production ($R^2 = 0.945$) and confirmed the quality of the TAPET-CD stock used for the in vivo experiments.

In vivo $^{19}$F MRS of i.tu. injected TAPET-CD and 5-FC

We subsequently assessed the applicability of $^{19}$F MRS to evaluate in vivo non-invasively the conversion capacity of the TAPET-CD in the tumour microenvironment. The proof-of-principle was first demonstrated using HCT116 tumour-bearing mice and local i.tu. injection of both TAPET-CD and 5-FC. Figure 1 displays an example of these in vivo $^{19}$F MRS measurements performed 1 day after the TAPET-CD injection, starting immediately after the 5-FC injection. The 5-FC signal was maximal in the first acquisition that started within 10 min after the 5-FC administration (time needed for positioning of the animal, for shimming and for acquisition of a water signal used for calibration of the chemical shift), and declined slowly during the experimental time of up to 2 h. The 5-FU resonance peak became visible during the first 30 min of the measurement, increasing continuously thereafter as shown with the serial spectra. Both 5-FC and 5-FU resonances were measured with adequate spectral resolution. No clear catabolites or anabolics of 5-FU could be observed during the total experimental time.

Spectra recorded 24 h after the initial 5-FC to 5-FU conversion measurement, showed complete absence of fluorine signals. Conversion of 5-FC to 5-FU was however again detectable in these tumours following a repeat 5-FC i.tu. injection.

Intratumoral conversion of 5-FC to 5-FU was however not always present at day 1 following the i.tu. TAPET-CD injection. We hypothesized that this was the result of insufficient bacterial colonization in these tumours. We therefore repeated the $^{19}$F MRS analysis at a later time point without any additional administration of TAPET-CD. A representative example of such a measurement is illustrated in Figure 2A. While very little 5-FC to 5-FU conversion was detectable at day 1 after the TAPET-CD was locally inoculated, a substantial conversion was measured during the measurement on day 7. For both time-points (day 1 and day 7), the $^{19}$F MRS analysis was started within 10 min after the i.tu. 5-FC injection. Figure 2B shows the time course for 5-FC and 5-FU derived from the top set of spectra in Figure 2A. The time course was similar for...
all experiments (slopes were $-6.1 \pm 0.7$ A.U./h for 5-FC and $2.0 \pm 0.4$ A.U./h for 5-FU, based on linear regression; $n = 6$). These data highlight the importance of repeat measurements for detecting the presence of TAPET-CD conversion efficacy during the tumour colonization period.

**In vivo $^{19}$F MRS of i.v. injected TAPET-CD and i.p. 5-FC**

The potential clinical applicability of TAPET-CD/5-FC involves not only i.tu. application but also the systemic delivery of the enzyme/prodrug system. Intratumoural production of 5-FU following i.v. injection of TAPET-CD and i.p. injection of 5-FC (300 mg/kg) was measured in vivo in the HCT116 colon tumour (Figure 3A). The 5-FU concentration in the tumours following i.p. injection was comparable to the i.tu. administration (3-4 mM). A clear conversion of 5-FC to 5-FU was seen with this systemic application of TAPET-CD/5-FC, although the 5-FU signals were less than with the i.tu. delivery route (compare Figure 1 and Figure 3A).

Because of the reduced 5-FU signal, the conversion quality was further analysed and confirmed with *in vitro* high resolution $^{19}$F MRS (8.4 Tesla) of extract preparations from these tumours. Intratumoural concentrations of 5-FU were about 0.5 ± 0.2 mM ($n = 6$) for the i.tu. and 0.15 ± 0.07 mM ($n = 7$) for the systemically TAPET-CD plus 5-FC treated tumours respectively. A representative example of this analysis (Figure 3B) illustrates the efficacy of the TAPET-CD/5-FC conversion to 5-FU in this i.v./i.p. administration modality.

Within the total *in vivo* experimental time, no metabolic activity was observed in the tumours, i.e., the concentrations of the catabolites and anabolites of 5-FU were below the detection threshold of the *in vivo* $^{19}$F MRS. Using the much more sensitive *in vitro* MRS on tumour extracts, we observed a small catabolite signal around −17 p.p.m. (i.tu. 0.1 ± 0.1 mM ($n = 4$), i.p. 0.025 ± 0.025 mM ($n = 5$)) and in some cases also a very small anabolite signal between 3.5 and 5.5 p.p.m. (∼0.015 mM) (an example is shown in Figure 4).

Those tumours, which were not further investigated with *in vitro* MRS, were analysed for bacterial colonization. Levels of $5 \times 10^8$ ± $2 \times 10^8$ cfu per gram tissue were found.

**In vitro $^{19}$F MRS of liver PCA extracts of mice treated with TAPET-CD/5-FC**

It is obviously important to evaluate normal tissue in parallel with tumour tissue, as both ultimately determine the therapeutic window of treatment. Besides the perchloric acid extracts of tumours, also extracts of snap-frozen livers were thus analysed with *in vitro* $^{19}$F MRS. In all cases where conversion to 5-FU was detected in the tumour (both after 5-FC i.p. or i.tu.), small catabolite signals of $\alpha$-fluoroureido-$\beta$-propionic acid (FUPA) at −17.3 p.p.m. and $\alpha$-fluoro-$\beta$-alanine (FBAL) at −18.6 p.p.m. were observed in the liver extracts (Figure 5). Although the conversion to 5-FU that was observed in the tumour after the i.tu. TAPET-CD/5-FC application was stronger than after the systemic route, the catabolite levels in the liver were similar for the i.tu. method (0.05 ± 0.04 mM ($n = 3$)) and the systemic administration (0.09 ± 0.02 mM ($n = 6$)).

**DISCUSSION**

In the present $^{19}$F MRS study we demonstrate the non-invasive detection of the dynamic production of 5-FU from 5-FC in the human HCT116 colon tumour xenograft following the use of cytotoxic deaminase-(CDase-) recombinant *S. Typhimurium* (TAPET-CD). The results show intratumoural conversion of 5-FC to 5-FU within 30 min which proceeded throughout the MRS measurement time extending up to 1.5–2 h after the 5-FC injection. This suggests that continued intratumoural production of 5-FU is greater than the diffusion of 5-FU from the tumour into
the systemic circulation of the animal. The high resolution in vitro 19F MRS analysis of the snap-frozen whole tumours confirmed the in vivo results. At the end of the MRS measurement, the i.tu. 5-FU signal corresponded to about 0.5 ± 0.2 \text{nmol}.

With investigations involving the injection of i.v. TAPET-CD and i.p. 5-FC, we measured an intratumoural 5-FU concentration of about 0.15 ± 0.07 \text{nmol} two hours post 5-FC injection. Although this is more than three times less than what is found after an i.tu. injection (0.5 ± 0.2 \text{nmol}), this still represents about 20 \text{ng/ml} 5-FU (based on 300 mg/kg 5-FC given i.p. in the present experiments). Lambin and colleagues discussed the potential of the bacteria-based CDase/5-FC strategy to improve the therapeutic outcome from the combination with radiotherapy (Lambin et al., 2000). Bacteriolytic published data, they estimated that an intratumoural production of 0.6–0.9 \text{ng/ml} of 5-FU would lead to a radio-sensitization factor of 1.1–1.2 when combined with standard 2 Gy radiotherapy schedule. Therefore the 5-FU levels obtained with the present bacterial CDase/5-FC system should lead to a highly significant radio-sensitization.

The in vivo 19F MRS indicated the lack of 5-FU metabolites in tumours. Yet, the in vitro measurements of PCA extracts of the same tumours enabled the detection of small amounts of catabolates and sometimes anabolics. The formation of fluoronucleosides and fluoronucleotides from 5-FU is known to be a slow process and may also depend on the tumour type under investigation. The concentration of the free anabolics could on the other hand remain below the sensitivity of the 19F MRS, although cytosine activity is present, a possibility discussed by McSheehy and Griffiths (1989).

We investigated in vitro the extracts of several snap-frozen livers from TAPET-CD/5-FC treated tumour-bearing mice that presented with a strong intratumoural 5-FU signal. This 19F MRS analysis revealed the absence of 5-FU and only small amounts of catabolates in the liver, at least up to 2 h after the in vivo measurements. Our results agree with the observations made by King et al., (2002) using high-performance liquid chromatography. The difference between the small catabolite levels in our study and the large levels usually found with conventional 5-FU chemotherapy, is obvious for two reasons: (1) it is expected that the ‘leakage’ of 5-FU from the tumour microenvironment into the systemic circulation is poor (eg, indirectly shown by Wallace et al., 1994), and (2) this type of conversion occurs mainly in the liver and little FBAL enters the systemic circulation (discussed by eg, Prior et al., 1990).

The difference in spectral quality for mice treated with i.p. versus i.tu. 5-FC injection is, besides the conversion quality, also partially related to the broader linewidths that are observed after an i.p. injection. This increases the overlap of the 5-FC peak with the 5-FU peak and decreases the signal-to-noise ratio. For this reason the 5-FU signal was most clearly observed when the 5-FC signal had significantly decreased (about 2.5 h after injection).

The 19F MRS measurements allowed us to gain a better understanding of some of the characteristics of the TAPET-CD/5-FC treatment strategy. First, a period ranging from 1 to 7 days seems necessary for the bacteria to establish and proliferate selectively in the tumours and express sufficient levels of CDase. This agrees with our previous work using the TAPET-CD/5-FC, in which the rat rhabdomyosarcoma tumour model was used (Mei et al., 2002). Using in vitro analysis of bacterial lysates, we also clearly demonstrated the positive correlation between the number of TAPET-CD and the amount of 5-FC that is converted to 5-FU for a given incubation period. Second, the TAPET-CD persist in the tumours without the need for a repeat injection, similar to the findings of King et al., (2002) and Mei et al., (2002). Indeed, in the present study, the production of 5-FU after the i.p. 5-FC administration was measurable even at 14 days after the injection of the bacteria. Third, intratumoural variation in the production of 5-FU in vivo was present. This can likely not be explained on the basis of differences in TAPET-CD colonization of the tumours. Indeed, the analysis for intratumoural presence revealed colonization levels of 5 × 10^8 ± 2 × 10^9 \text{cfu per gram of tumour tissue}. However, tumours are inherently morphologically heterogeneous, a fact which may partly explain differences of drug levels (as discussed by Kamm et al., 1996).

CONCLUSION

The present results are to our knowledge the first demonstration of the applicability of 19F MRS to monitor in vivo the intratumoural activity of CDase-recombinant bacteria. This suggests that MRS may be useful for monitoring therapy with TAPET-CD/5-FC since it allows repetitive examinations without the need to interfere with the microenvironment. The use of non-pathogenic strains of bacteria to transfer effector genes and therapeutic proteins selectively to the tumour microenvironment is being intensively investigated (Minton et al., 1995; Zheng et al., 2000; Theys et al., 2001; King et al., 2002; Liu et al., 2002). Based on the promising preclinical in vitro and in vivo data, including the positive safety profile, the application of the attenuated Salmonella strain (both i.tu. and i.v. administration) is being tested in clinical phase I studies (Cunningham and Nemunaitis, 2001; Tosó et al., 2002). From the perspective of the broader application of the novel anti-cancer treatment (eg, optimisation of protocol, combining with radiotherapy or chemotherapy), the non-invasive MR methodology thus offers a significant advance for individual guidance and longitudinal monitoring of treatment.

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