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Journal

International Journal of Radiation Biology, 84(3)

ISSN

0955-3002

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Publication Date

2008

DOI

10.1080/09553000801902174

International Journal of Radiation Biology

ISSN: 0955-3002 (Print) 1362-3095 (Online) Journal homepage: www.tandfonline.com/journals/irab20

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To cite this article: Michael N. Hoff, Donald T. Yapp, Andrew C. Yung, Thomas S. Oliver & Piotr Kozlowski (2008) *In vivo* measurement of the hypoxia marker EF5 in Shionogi tumours using ^{19}F magnetic resonance spectroscopy, International Journal of Radiation Biology, 84:3, 237-242, DOI: 10.1080/09553000801902174

To link to this article: https://doi.org/10.1080/09553000801902174

Published online: 03 Jul 2009.

Int. J. Radiat. Biol., Vol. 84, No. 3, March 2008, pp. 237–242

informa
healthcare

In vivo measurement of the hypoxia marker EF5 in Shionogi tumours using ^{19}F magnetic resonance spectroscopy

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(Received 1 June 2007; accepted 8 October 2007)

Abstract

Purpose: ^{19}F magnetic resonance spectroscopy (MRS) was used to non-invasively detect EF5 [2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl) acetamide] adducts in the Shionogi tumour model of prostate cancer to evaluate hypoxia.

Material and methods: ^{19}F MRS signal of EF5 in Shionogi mouse tumours was acquired using a 2 cm diameter solenoid volume coil with a 7.05 T Bruker scanner. MRS signal was observed in mouse tumours longitudinally following intraperitoneal (IP) injection of EF5. Another mouse group was injected intravenously (IV) with EF5, and *in vivo* MRS signal was obtained two hours after injection. This data was compared with the *ex vivo* percentage of hypoxic cells present in the corresponding excised tumours, determined by flow cytometry of bound EF5.

Results: Longitudinal ^{19}F MRS signal attributable to EF5 began to decline within five hours of EF5 administration. Flow cytometry comparisons yielded an inverse correlation (p-value < 0.006) between the MRS signal and tumour hypoxic cell percentage. The tumours exhibited an average cell viability of $34 \pm 26\%$.

Conclusions: The results confirmed that MRS of EF5 in mice is an unsuitable technique for the determination of EF5 binding as a measure of tumour hypoxia.

Keywords: *^{19}F magnetic resonance spectroscopy, 2-nitroimidazole binding, Hypoxia probes, Shionogi tumour model*

Introduction

In 1936, Mottram suggested that hypoxic, non-oxygenated regions in cancerous tumours are insensitive to radiation therapy (Mottram 1936). Gray et al. (1953) would later show that cellular response to radiation depended on oxygen levels. These discoveries have provoked extensive research into tumour oxygenation. Many clinically applicable techniques have been developed, most notably the use of invasive oxygen electrodes (Clark et al. 1953) to measure oxygen concentration in tumours (Kolstad 1968). The development of an *in vivo*, non-invasive hypoxia measurement technique could provide clinicians with knowledge of tumour oxygenation prior to treatment. Tumour re-oxygenation through hyperbaric oxygen or carbogen breathing, or hypoxic cell sensitization using radiosensitizers could then be implemented to improve the efficacy of hypoxic tumour therapy.

The mechanism of 2-nitroimidazole bioreduction has been used to elucidate hypoxia on a cellular level (Hodgkiss 1998). Nitroimidazoles were originally developed as radiation sensitizers (Varghese & Whitmore 1980), but are now predominately used as exogenous markers of hypoxia (Hodgkiss 1998). Upon introduction of 2-nitroimidazoles into living organisms, nitroreductases initially reduce the drug to form a highly reactive radical. In the presence of oxygen, this reduction is reversed to reform

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ISSN 0955-3002 print/ISSN 1362-3095 online
DOI: 10.1080/09553000801902174

the parent compound. Under hypoxic conditions (when $pO_2 < 10$ mmHg, Lord et al. 1993), further reductions occur, giving rise to highly unstable intermediates that subsequently bind to cellular macromolecules. The degree of binding is correlated with the level of hypoxia, and so the detection of adducts may provide a measure of tumour hypoxia (Chapman et al. 1981).

Both invasive and non-invasive methods have been utilized in the detection of bound 2-nitroimidazoles. Invasive methods include autoradiography using radioactive ^{14}C (Chapman et al. 1983), and antibody-based methods such as immunohistochemistry and flow cytometry (Lord et al. 1993). Non-invasive techniques include positron emission tomography (PET) of the radioactive fluorine isotope ^{18}F (Rasey et al. 1987), and magnetic resonance spectroscopy (MRS) of ^{19}F (Raleigh et al. 1986). PET's limited spatial resolution and the inherent difficulties which accompany the use of radionuclides have spurred interest in the development of 2-nitroimidazole detection using MRS (Hodgkiss 1998).

Raleigh et al. (1986) made the first attempt at detecting a 2-nitroimidazole using ^{19}F MRS for the purpose of measuring hypoxia. Although the drug used (Hexafluoromisonidazole, CCI-103F) yielded limited results, the technique showed promise. Aboagye et al. (1995) developed and tested the trifluorinated 2-nitroimidazole SR-4554, and then showed a correlation between its ^{19}F retention index and the reported radiobiological hypoxic fraction values for four different murine tumour models (Aboagye et al. 1997). The retention index was computed by normalizing ^{19}F signal by signal values acquired at early time-points, when signal was maximal and related mostly to uptake of the parent drug. Unfortunately, the correlation of the retention index with radiobiological hypoxic fraction could only be shown following tumour modulation through hydralazine administration or carbogen breathing (Aboagye et al. 1997, Seddon et al. 2002). Recently, the visibility of another trifluorinated 2-nitroimidazole (TF-MISO) was established in *ex vivo* hypoxic conditions with ^{19}F MRS, and localization studies were performed using chemical shift imaging (Procissi et al. 2007).

One study has attempted to measure the 2-nitroimidazole EF5 [2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl) acetamide] *in vivo* using MRS. Salmon et al. claimed that all observed EF5 MRS signal in tumours was generated by the preferential retention of parent EF5, while broad line widths rendered *in vivo* EF5 adducts invisible. The subsequent conclusion that the technique is inadequate for hypoxia measurement is potentially valid, although the conclusion was not fully supported by the results (Salmon & Siemann 2004). No quantitative comparisons were made between the level of hypoxia and EF5 retention measured using MRS.

The current study is the first to compare *in vivo* MRS signal of a 2-nitroimidazole with *in vitro* measurements of hypoxia using flow cytometry in an attempt to quantify tumour hypoxia using MRS. Following administration of EF5 in DDS mice with Shionogi tumours, ^{19}F MRS signal acquired from tumours was compared with the percentage of hypoxic cells found using flow cytometry of the same excised tumours. EF5 ^{19}F MRS signal was also observed over the first 7 h following injection in order to gain insight into the retention of the drug over time.

Materials and Methods

Drugs and reagents

EF5 (10 mM in saline) and the Cy5-tagged monoclonal antibody ELK5-31 were generously provided by Dr Cameron Koch from the University of Pennsylvania, USA. The ^{19}F MRS external standard, a 0.05 ml glass sphere containing 0.12 M TFA (trifluoroacetic acid), and the anaesthetic Avertin (2,2,2 tribromoethanol) were acquired from Sigma-Aldrich, St. Louis, Missouri, USA. All chemicals used for flow cytometry and tumour disaggregation were of analytical grade.

Animal and tumour models

Adult DDS mice weighing 25–35 g were bred in the Prostate Centre at Vancouver General Hospital, Vancouver, Canada. They were injected subcutaneously into the upper back with 5×10^6 cells of the Toronto subline of the transplantable Shionogi SC-115 androgen-dependent mouse mammary carcinoma (Minesita & Yamaguchi 1965). *In vivo* tumour volumes were periodically calculated from tumour diameters measured with digital callipers. All mice were castrated when tumours reached 1.5 cc in size, approximately 2–3 weeks following inoculation. When mice with Shionogi tumours are castrated, the tumours regress, and then re-grow in a manner independent of the androgen levels. When androgen-independent tumours reach mature size, they have been shown with flow cytometry to exhibit a significant amount of uniformly distributed EF5 binding relative to the more unpredictable binding of pre-castrated androgen-dependent tumours (Skov et al. 2004). The androgen-independent mice were prepared for MRS analysis once the tumours had re-grown to 1.5 cc in size (5–10 weeks). All care and use of mice in this study were in accordance with the guidelines of the Canadian Council for Animal Care and were approved by the University of British Columbia Animal Care Committee.

Magnetic resonance spectroscopy

MRS experiments were carried out on a 7.05 T/30 cm animal MRI scanner (Bruker BioSpin MRI GmbH, Ettlingen, Germany). A four turn radio-frequency (RF) solenoid coil (20 mm inner diameter, 14 mm length) tuneable to both ^{1}H (300.21 MHz) and ^{19}F (282.55 MHz) frequencies was custom built for spin excitation and signal reception in the tumour alone. The TFA reference standard was positioned in the lower part of the RF coil for all *in vivo* MRS experiments. Tumours were centred in the coil, and shimming was performed on ^{1}H signal. The coil was then tuned to ^{19}F frequency, and spectra were acquired using a single pulse acquisition. Scan parameters were: pulse length = 50 μs, flip angle = 90°, and spectral width = 25 kHz. T_R (repetition time) = 1.5 s and N_A (number of averages) = 1200 were utilized for the longitudinal experiments, while $T_R = 1.0$ s and $N_A = 600$ were used for the MRS – flow cytometry comparison. The T_R values used in this study are too short to acquire fully relaxed fluorine signal from a given metabolite, but this is permissible since only relative signal between mice was compared. The use of shorter T_R allows for a greater total amount of signal for a given total scan time.

In vivo *MRS protocol – longitudinal EF5 retention*

Mice were anaesthetized with Avertin: 600 μl injected intraperitoneally (IP) to start, 800 μl IP 15 min later, and then 600 μl IP every 35 min following. Mice were restrained in the scanning bed in the supine position, with the tumour on the upper back resting in the upper part of the RF solenoid coil. Body temperature was regulated with a heating pad and monitored with a thermometer. The bed was then placed inside the magnet. One 600 μl IP injection of 10 mM EF5 was made at various times, depending on the mouse: after anaesthesia, before anaesthesia, or remotely via a catheter inside the magnet. Seven androgen-independent mice with re-growing tumours were given continuous scans up to 7 h after injection of EF5.

In vivo *MRS protocol – EF5 signal vs. flow cytometry*

Seven mice with androgen-independent tumours were anaesthetized with isofluorane in medical air (4% induction, 1% maintenance) and positioned in an animal bed with the tumour resting in the upper section of the RF coil. EF5 (600 μl of 10 mM) was injected intravenously (IV) and a ^{19}F spectrum was acquired 2 h following injection, when it was perceived that all of the parent EF5 had cleared from the tumour (in accordance with previous half-life studies, Laughlin et al. 1996). When scans were completed, the mice were euthanized with CO_2, and their tumours were excised for flow cytometry.

Flow cytometry analysis

After scanning, mouse tumours were examined for hypoxic cells with flow cytometry as described previously (Skov et al. 2004). Briefly, tumours were excised, weighed, minced with scalpels, and placed in disaggregation mix. Following incubation, Medicones and a Medimachine™ (BD Biosciences, San Jose, CA, USA) were used to disaggregate any remaining tissue to obtain cell suspensions. Representative portions were partitioned for cell counting with a microscope. Live cells and dead cells were counted to attain an estimate of the viability (live cells/total cells). Total cell concentrations for each cell solution were determined, and 1×10^7 cells were separated for flow cytometry. Tumour cells were fixed with paraformaldehyde (4%) and blocked with mouse serum. Samples were then incubated with the EF5-specific monoclonal antibody ELK3-51, conjugated to the fluorescent dye Cy-5. Flow cytometry analysis was carried out on the FACSCalibur™ system (BD Biosciences, San Jose, CA, USA). Hypoxic cells generated *in vitro* in the presence of EF5 were stained alongside each set of tumor cells and used to determine the thresholds for positive staining with ELK3-51.

MRS data analysis and quantification

All MRS data was processed off line using the Java-based Magnetic Resonance User Interface (JMRUI, see Acknowledgements). The CF_3 peak was used for EF5 quantification, since there is no known metabolic bias between CF_3 and CF_2 groups. The peak integrals were estimated using curve fitting with the time-domain based algorithm Advanced Method for Accurate, Robust and Efficient Spectral fitting (AMARES – Vanhamme et al. 1997). Standard linear regression analyses were used in the analysis of the MRS and flow cytometry data, with all *p*-values being two-sided.

Results

In vivo *MRS – longitudinal EF5 retention*

Figure 1 shows a typical sample MRS spectrum acquired 5 h after IP injection of EF5 in a mouse bearing an androgen-independent tumour. EF5 MRS signal for each mouse in this study was represented by an EF5/TFA peak ratio: CF_3 peak area for EF5 in the tumour was normalized to the corresponding reference TFA peak area at each time

point. This ratio permits the observation of the relative retention of EF5 in the tumour by correcting for systematic errors in total ^{19}F MRS signal. MRS CF_3/TFA peak ratios were calculated for each spectrum, and are plotted as a function of time for seven mice with androgen-independent tumours in Figure 2. Within several hours after EF5 administration, all mice exhibited a decline in signal.

In vivo *MRS – EF5 signal vs. flow cytometry*

MRS spectra were acquired 2 h following IV injection of EF5, and flow cytometry data were acquired from each tumour following excision.

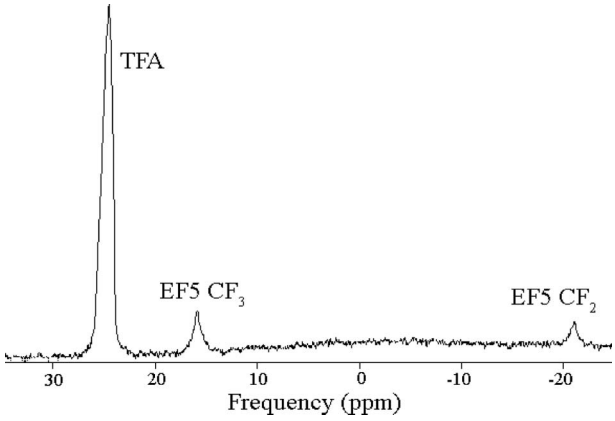

Figure 1. *In vivo* ^{19}F magnetic resonance spectrum from a Shionogi tumour detected by a tumour volume coil. The spectrum was acquired 5 h after intraperitoneal injection of EF5 in a DDS mouse (mouse "e" in Figure 2).

Figure 3 plots the CF_3/TFA MRS peak ratios as a function of the percent of hypoxic cells measured with flow cytometry for the six mice with androgen-independent tumours. A statistical regression analysis of the data revealed an inverse correlation between MRS signal and flow hypoxia that is statistically significant (p-value < 0.006). Cell counting performed on each tumour yielded an average viability level of $34 \pm 26\%$, where the error is given by the standard deviation of the viability.

Discussion

The first attempt to detect EF5 adducts in mice using MRS was made by Salmon & Siemann (2004). Their published results raised doubts as to the utility of using ^{19}F MRS measurement of EF5 signals as an indicator of cellular oxygenation status. The results presented in this study present further evidence that ^{19}F MRS of EF5 is not a suitable methodology for hypoxia determination. No positive correlation was found between EF5 MRS signal and the measurement of EF5 binding determined by flow cytometry, a recognized method of detecting the macromolecular adducts of EF5 (Lord et al. 1993). On the contrary, the existence of a negative correlation between flow cytometry and MRS data could be construed as evidence of the measurement of non-macromolecular binding of EF5.

In any analysis of MRS data, the many variables which could influence the signal observed should first be considered. Extrapolating signal trends in

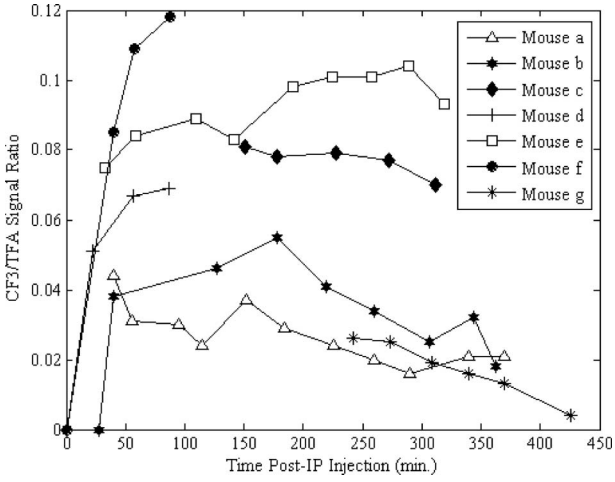

Figure 2. ^{19}F CF_3/TFA MRS signal ratio in seven DDS mice with androgen-independent, re-growing Shionogi tumours vs. post-EF5-injection time. MRS spectra were acquired longitudinally using a tumour volume coil following IP injection of EF5 for each mouse. ^{19}F CF_3/TFA signal ratios were calculated by normalizing CF_3 peak areas to TFA peak areas from each spectrum acquired at different times following IP EF5 injection. Ratio values were then plotted as a function of the post-injection time for each mouse.

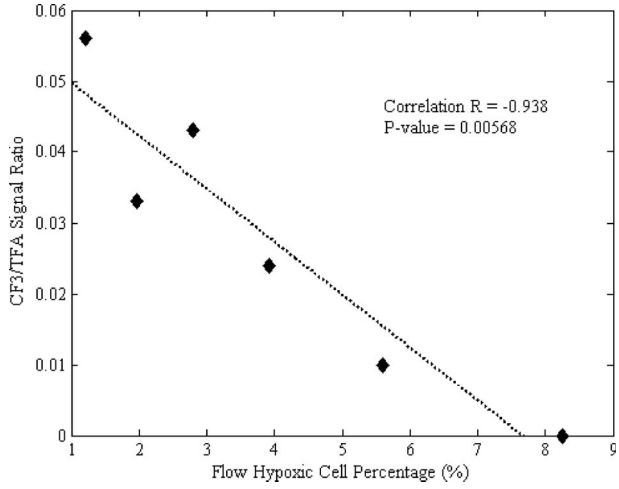

Figure 3. ^{19}F CF_3/TFA MRS signal ratio vs. flow cytometry hypoxic cell percentage for six DDS mice with androgen-independent tumours. MRS spectra were acquired 2 h following IV EF5 injection using a tumour volume coil. ^{19}F CF_3/TFA signal ratios were calculated by normalizing CF_3 peak areas to TFA peak areas from each spectrum for each mouse. The tumours were then excised, and hypoxic cell percentages were calculated using flow cytometry.

Figure 2 leads to EF5 signal beyond 6–7 h in the tumours studied. Since the half-life of EF5 has been reported at values as low as 40 min (Laughlin et al. 1996), it is apparent that EF5 signal at such late times is the result of two possible scenarios: delayed clearance of parent drug, or binding of EF5 in the tumour. Unfortunately, the determination of the mechanism responsible for this MRS signal is clouded by the many potentially contributing factors. Peculiarities to specific mice/tumours, delayed drug uptake as a result of IP EF5 administration, or altered vascular diffusion and/or perfusion (Kwock et al. 1992) could all result in prolonged retention of parent drug. Physiological modifiers such as mouse temperature, exogenous drugs, and oxygen intake could affect the blood flow, tumour oxygenation, and consequently both the retention of parent EF5 and the metabolism of the drug. Perhaps the most potent modifier of the tumour environment is anaesthesia, as it has been shown to directly affect levels of tumour oxygenation (Milross et al. 1996).

The results obtained in the comparisons of MRS signal with flow cytometry strongly suggest that MRS signal does not provide a measure of EF5 binding to cellular macromolecules. Since flow cytometry is a proven method of quantification of these adducts (Lord et al. 1993), the lack of positive correlation depicted in Figure 3 between MRS signal and flow cytometry implies that the two techniques measure different phenomena. Salmon & Siemann (2004) concluded that EF5 adducts are undetectable to MRS due to the lack of mobility of EF5 bound to large molecules. This lack of mobility leads to shorter T_2 values and subsequently broad spectral lines which may be undetectable in frequency space (Jeffrey et al. 1989).

There are speculations as to the sources of the inverse correlations present in Figure 3. Standard high resolution MRS signal is typically only obtainable from relatively small and mobile compounds in aqueous solution. This has led to the prevalent theory that MRS of *in vivo* EF5 measures only sufficiently mobile adducts of low molecular weight (LMW) thiols such as glutathione (GSH) (Robinson & Griffiths 2004), which have shown to exhibit a marked component of *in vivo* 2-nitroimidazole binding (Varghese & Whitmore 1980, Varghese 1983, Raleigh & Koch 1990). The presence of the radical-scavenging agent GSH has also shown to inhibit macromolecular binding to 2-nitroimidazoles (Chapman et al. 1983, Raleigh & Koch 1990, Silver et al. 1986). Flow cytometry measures only cellular macromolecular adducts of EF5 (since its histochemical processing washes away any LMW adducts), and MRS potentially measures only LMW adducts. Thus an inhibitory process of GSH on macromolecular binding could conceivably result in a competition model between the two forms of metabolism. A result of this model would be that a negative correlation would be observed between data from MRS and flow cytometry.

While the competition model of binding used to explain the observed correlations is intriguing, further work will be necessary to substantiate the theorem. Assays of LMW thiol levels in tumours concurrent with MRS, flow cytometry and immunohistochemistry studies could help to determine the source of signal. Other tests should be made to provide validation of any conclusions. Measurements of blood flow, tumour perfusion, and the physiological effects of anaesthesia would be valuable if made in parallel with longitudinal MRS measurements over several days. By coupling the selection of a consistently viable tumour line with the normalization of the drug retention by the MRS signal at the point of maximal drug uptake (Aboagye et al. 1997), the influence of variable drug absorption, tumour vascularization, perfusion, and tumour size on MRS signal could be minimized.

In conclusion, ^{19}F MRS of EF5 in mice with Shionogi tumours is ineffectual as a means of measuring EF5 binding to macromolecules. However, the existence of a negative correlation between ^{19}F MRS signal of EF5 and values of hypoxic cell percentage obtained from flow cytometry in associated tumours indicates the possibility that the MRS technique measures adducts of EF5 with non-macromolecular LMW thiols. The refinement of such a measurement technique could yield a valuable tool for the study of tumour metabolism.

Acknowledgements

We would like to thank the Canadian Prostate Cancer Initiative for providing financial support for this study.

The jMRUI software package was kindly provided by the participants of the EU Network programmes: Human Capital and Mobility, CHRX-CT94-0432 and Training and Mobility of Researchers, ERB-FMRX-CT970160.

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