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Current issues in the utility of ^{19}F nuclear magnetic resonance methodologies for the assessment of tumour hypoxia

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It is now well established that uncontrolled proliferation of tumour cells together with the chaotic and poorly regulated blood supply of solid tumours result in tissue hypoxia, and that hypoxic regions of tumours are resistant to radiotherapy and chemotherapy. The development and application of non-invasive methods to rapidly determine the degree and extent of tumour hypoxia in an individual tumour would clearly enhance cancer treatment strategies. This review describes the current status of two ^{19}F nuclear magnetic resonance (NMR) methodologies that have been exploited to investigate tumour hypoxia, namely: (i) ^{19}F NMR oximetry following administration of perfluorocarbons, from which tumour p_{O_2} measurements can be made; and (ii) ^{19}F NMR measurements of the tumour retention of fluorinated 2-nitroimidazoles.

Keywords: hypoxia; ^{19}F NMR; perfluorocarbon; 2-nitroimidazole

1. INTRODUCTION

It is now well established that the characteristically chaotic and poorly regulated blood supply of tumours causes tissue hypoxia, and that hypoxic regions of tumours are resistant to radiotherapy and many kinds of chemotherapy (Horsman 1998). With the recent advances in the understanding of its molecular basis, hypoxia has been revealed as a cause of genetic instability, tumour progression and angiogenesis (Harris 2002).

The development and application of methods to rapidly determine the degree and extent of tumour hypoxia in an individual tumour would clearly enhance cancer treatment strategies. The most commonly used technique for determining the tumour oxygenation status has been the invasive Eppendorf polarographic electrode. Measurements of oxygen tensions in a range of human tumours have clearly demonstrated the presence of hypoxia and that the level of this hypoxia is prognostic for increased tumour aggressiveness, metastasis and poor response to treatment (Hockel *et al*. 1991, 1996; Brizel *et al.* 1994, 1996, 1997; Nordsmark *et al.* 1996; Fyles *et al.* 1998).

A non-invasive method for measuring the heterogeneous distribution of hypoxia in a tumour would be of considerable use in the clinic. We review two ^{19}F nuclear magnetic resonance (NMR) methodologies that have been used to investigate tumour hypoxia. The first is ^{19}F NMR measurements of tumour oxygenation following the administration of perfluorocarbon (PFC) emulsions, and the second involves ^{19}F NMR measurements of the retention of fluorinated 2-nitroimidazoles by hypoxic tumour tissue. The aim of this review is to describe the principles behind both approaches, highlight some pertinent experimental studies and discuss some current issues concerned with the utility of both techniques.

2. TUMOUR OXYGENATION MEASUREMENTS BY ^{19}F NMR OF PERFLUOROCARBONS

(a) *Principles of ^{19}F NMR oximetry*

^{19}F NMR spectroscopy and imaging of PFC emulsions, hydrocarbons whose protons have been replaced with fluorine nuclei, has been extensively exploited to measure the oxygen tension of biological systems in preclinical studies. There are several modes of administration: (i) intravenous administration of emulsions; (ii) direct intratumoral injection of emulsions; and (iii) administration of PFC in oxygen-permeable alginate capsules, either by direct intratumoral injection or by growing the tumour around the capsules (see below). Whatever the mode of administration, the ^{19}F MR signal of the PFC is sensitive to the p_{O_2} of the surrounding tumour tissue, and acts as an oximeter. It is also possible to perform low-resolution ^{19}F magnetic resonance imaging (MRI) and to overlay these images onto anatomical ^{1}H MRI images, demonstrating the location from which the signal is obtained.

The principle behind ^{19}F MR oximetry relies on the linear increase of the NMR spin-lattice relaxation rate R_1 ($= 1/T_1$) of PFC emulsions with increasing oxygen tension (Parhami & Fung 1983). Molecular oxygen has a very high solubility in PFC emulsions, thus permitting oxygen tension measurements in locations where the PFC is sequestered from the PFC spin-lattice relaxation rates *in vivo*. ^{19}F MR oximetry provides a sensitive measure of apparent tissue oxygen tension and promises to be a powerful approach for monitoring tumour hypoxia. PFCs are primarily selected on their ability to form stable emulsions with a good biodistribution and a reliable R_1 response. One PFC that fits these criteria is perfluoro-15-

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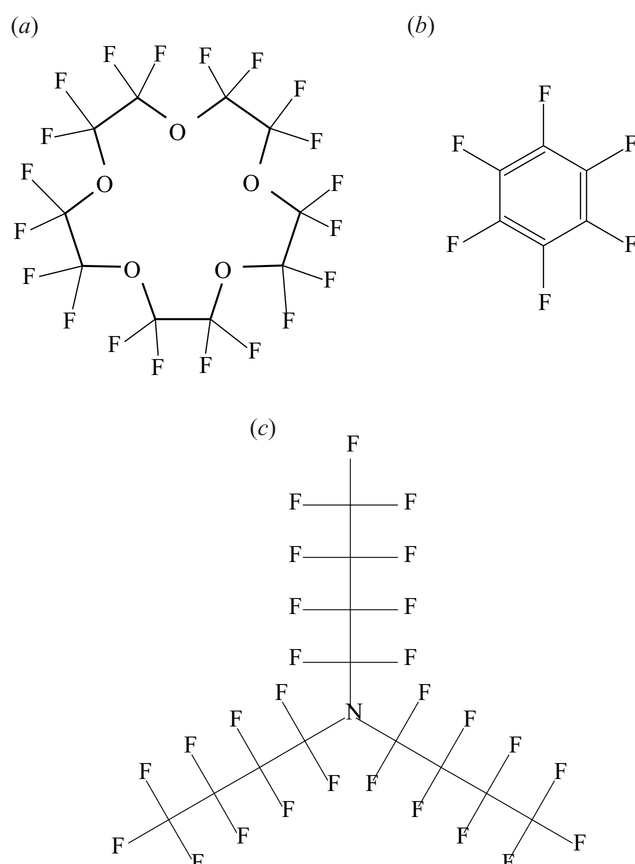


Figure 1. Chemical structures of some commonly used perfluorocarbons: (*a*) perfluoro-15-crown-5-ether (15C5), (*b*) hexafluorobenzene, (*c*) perfluorotributylamine (Oxypherol). Both 15C5 and Oxypherol have been typically administered as emulsions, whereas hexafluorobenzene has been administered neat.

crown-5-ether (15C5) (figure 1). It exhibits a strong dependence of R_1 on p_{O_2} and has a single resonance resulting from 20 chemically equivalent fluorine atoms, giving a simple spectrum (suitable for MRI) and excellent sensitivity.

(i) *Intravenous administration of emulsion*

Because of their low toxicity, large doses of PFC emulsions can be injected intravenously. The circulating PFC emulsion particles are then cleared from the vasculature by the reticulo-endothelial system, mainly into the liver and spleen, but some are sequestered by macrophages in abcesses and solid tumours. In tumours, which have characteristically leaky vasculature, the PFC particles can also pass through the vessel walls and accumulate in the interstitial space. In preclinical studies, PFC emulsions have generally been administered in this way to rodents bearing subcutaneous transplanted tumours in their flanks. However, the majority of the PFC is sequestered in the liver and spleen and the amount taken up by the tumour is often insufficient for determination of the tumour p_{O_2}. Typically the PFC emulsion is cleared within a few days, thus confining the p_{O_2} measurements to a limited time-window, although intravenously administered Oxypherol has been shown to be retained in tumour tissue for longer (Mason *et al.* 1994).

(ii) *Direct intratumoral injection of emulsions*

This method affords greater sensitivity and precision of p_{O_2} measurements (Mason *et al.* 1996; McIntyre *et al.* 1999). However, the PFC can only record p_{O_2} in the regions of the tumour into which it has been injected. Furthermore, the characteristically raised interstitial fluid pressure could force the PFC out of the tumour. Another problem is that the bleb of emulsion can migrate within the tumour. As in the case of PFC emulsion administered intravenously, the small boluses of PFC emulsion are cleared within a few days.

(iii) *PFC in oxygen-permeable alginate capsules*

PFC-loaded alginate capsules overcome many of the problems of direct intratumoral injection. The capsules do not elicit a host immune response, they do not migrate once implanted and they are retained indefinitely in the tumour. These PFC-loaded capsules can be used for a unique experiment in which the p_{O_2} of an experimental tumour is monitored from the time that the tumour cells are implanted. This is done by mixing PFC-loaded alginate capsules with the tumour cells prior to injecting into the flanks of rodents (Nöth *et al.* 2001). The alginate capsules become incorporated into the growing solid tumour (figure 2). In this way, repeated measurements of tumour p_{O_2} can be made by ^{19}F NMR over the whole period of tumour growth.

(b) *Applications of ^{19}F NMR oximetry*

Numerous studies by ^{19}F NMR oximetry have reported tumour p_{O_2} measurements in a range of different rodent tumour models (Hees & Sotak 1993; Dardzinski & Sotak 1994; Mason *et al.* 1994, 1996, 1998; Baldwin & Ng 1996; Le *et al.* 1997; Hunjan *et al.* 1998, 2001; McIntyre *et al.* 1999; van der Sanden *et al.* 1999; Worden *et al.* 1999; Nöth *et al.* 2001; Song *et al.* 2002; Zhao *et al.* 2002) and these have been summarized in table 1. For comparison, literature p_{O_2} values measured for the same tumour type by the invasive Eppendorf electrode are also shown (Yeh *et al.* 1995; Adam *et al.* 1999; Robinson *et al.* 1999). ^{19}F NMR oximetry has been predominantly used to monitor changes in tumour p_{O_2} in response to adjuvants that modify tumour oxygenation, principally for enhancing radiosensitivity. One such approach for increasing tumour p_{O_2} is through breathing high-oxygen content gases such as carbogen (95% O_2/5% CO_2), to increase the amount of dissolved oxygen in the plasma, provide more oxygen at the capillary level and hence promote diffusion of oxygen into hypoxic regions in order to radiosensitize them (Rojas 1991). Several studies have shown that carbogen breathing enhances rodent tumour radiosensitivity and, in combination with nicotinamide (which reduces the occurrence of intermittent tumour blood flow and thereby also increases tumour oxygenation and hence radiosensitivity; Chaplin *et al.* (1991)), is currently being re-evaluated in the clinic as a radiosensitizer (Kaanders *et al.* 2002*a*). Several ^{19}F NMR oximetry studies have demonstrated significant increases in tumour oxygenation when the breathing gas was switched from air to either oxygen (Hees & Sotak 1993; Mason *et al.* 1996, 1998; Le *et al.* 1997) or carbogen (Dardzinski & Sotak 1994; Hunjan *et al.* 1998, 2001; McIntyre *et al.* 1999; van der Sanden *et al.* 1999; Worden *et al.* 1999; Nöth *et al.* 2001; Zhao *et al.* 2002).

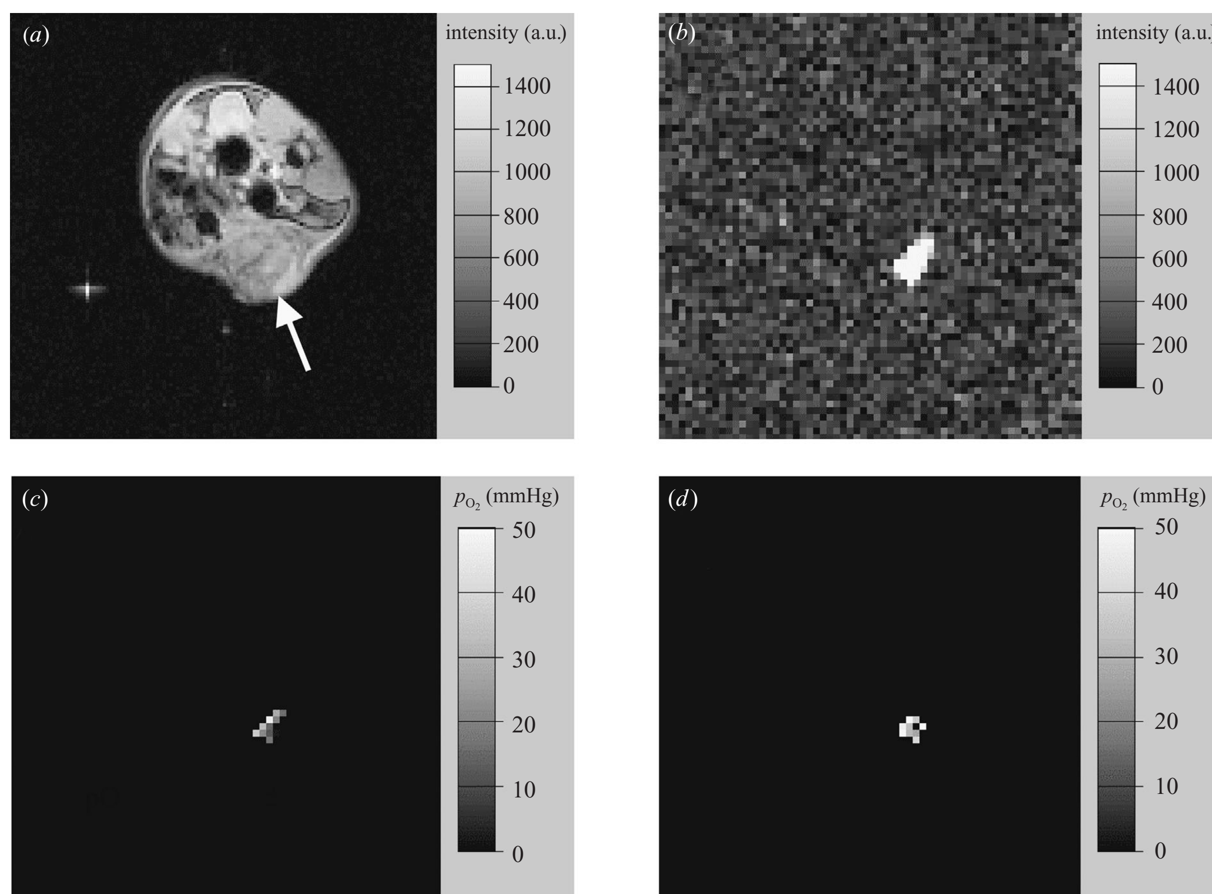


Figure 2. (*a*) Transverse ^{1}H gradient echo scout image and (*b*) ^{19}F gradient echo image through the abdomen of a rat bearing a GH3 prolactinoma containing 15C5-loaded alginate capsules. The arrow indicates the tumour. The signal intensity is given in arbitrary units. In (*b*) only the ^{19}F NMR signal of the 15C5-loaded alginate capsules within the tumour can be seen. (*c*) Calculated ^{19}F NMR p_{O_2} maps of the same slice, acquired while the rat was breathing air and (*d*) carbogen (95% O_2/5% CO_2). The p_{O_2} values are given in mmHg. The average p_{O_2} for this tumour was 22.8 mmHg during air breathing and increased to 42.7 mmHg during carbogen breathing. This set of images was acquired 10 days after the co-inoculation of GH3 tumour homogenate and PFC-loaded alginate capsules. (Data courtesy of Dr Ulrike Nöth.)

^{19}F NMR oximetry has also been used to examine changes in the oxygenation status of RIF-1 tumours in response to treatment with nicotinamide (Hees & Sotak 1993). Consistent with the hypothesis that nicotinamide improves tumour perfusion and hence oxygenation, there was a significant increase in p_{O_2} for treated tumours versus control tumours.

(c) *Current issues*

As previously described, the major application of ^{19}F NMR oximetry to oncology has been in the evaluation of tumour oxygenation and the effects of approaches to overcome tumour hypoxia and enhance the response to irradiation. Whereas normal tissues typically have oxygen tensions of *ca.* 40 mmHg, the large majority of tumours have overall oxygen tensions less than 10 mmHg, with localized tissue areas of less than 2.5 mmHg. These areas of less than 2.5 mmHg are termed radiobiologically hypoxic, since three times more radiation is required to kill hypoxic than normally oxygenated cells (Hall 1994). From a radiation oncologist's point of view, this means that tumours including such regions have impaired radiotherapeutic response.

^{19}F NMR oximetry is currently the only NMR methodology from which absolute tumour p_{O_2} measurements can be derived. Despite this, the limited distribution of PFC emulsions and hence the large sampling volume for ^{19}F NMR oximetry is clearly a limiting factor. Leaving aside these drawbacks, the absolute p_{O_2} values determined from a range of different tumour models listed in table 1 highlight two issues. First, in the majority of the studies, the basal tumour p_{O_2} measured by ^{19}F NMR oximetry is generally much higher than those reported by the Eppendorf electrode. Second, these high PFC p_{O_2} results would predict very little radiobiological hypoxia, a prediction that is clearly at variance with many results showing radioresistance in these and other rodent tumour models.

One reason for this discrepancy could be the presence of a systematic error in the ^{19}F NMR measurement. Tumour p_{O_2} maps are derived from quantitative ^{19}F T_1 relaxation maps of the PFC emulsion by interpolation onto a calibration curve. This curve is usually derived *in vitro* from ^{19}F NMR T_1 measurements of pure PFC emulsion equilibrated to different oxygen tensions using nitrogen, air or oxygen. Using the resulting curve, the oxygen tension can then be derived using the equation

$$p_{O_2} = (1/T_1 - A)/S,$$

where S is the slope of the linear fit of $1/T_1$ versus p_{O_2} and A the anoxic intercept. The calibration constants A and

Table 1. Summary of ^{19}F NMR oximetry-derived p_{O_2} measurements reported from a range of different rodent tumour models.
(For comparison, literature p_{O_2} values measured in the same tumour type by the invasive Eppendorf electrode, where available, are also shown.)

tumour	route of PFC administration	^{19}F NMR-derived p_{O_2} (mmHg)	reference	Eppendorf-derived p_{O_2} (mmHg)	reference
RIF-1 fibrosarcoma	intravenous	min. 1.1, max. 6.2	Hees & Sotak (1993)	median 1.2	Adam *et al.* (1999)
R3327-AT1 Dunning prostate adenocarcinoma	intravenous	range 0–86	Mason *et al.* (1994)	mean 3.5	Yeh *et al.* (1995)
RIF-1 fibrosarcoma	intravenous	range 9–37, mean 26.4	Dardzinski & Sotak (1994)		
KHT sarcoma	intravenous	range 0–80	Baldwin & Ng (1996)	median 0.8	Adam *et al.* (1999)
R3327-AT1 Dunning prostate adenocarcinoma	intratumoral	mean 1.6	Mason *et al.* (1996)		
R3327-AT1 Dunning prostate adenocarcinoma	intratumoral	mean 3.3	Le *et al.* (1997)		
R3327-AT1 Dunning prostate adenocarcinoma	intratumoral	tumour core range 1.4–6.4, periphery range 7.9–78.9	Hunjan *et al.* (1998)		
R3327-AT1 Dunning prostate adenocarcinoma	intratumoral	mean 19	Mason *et al.* (1998)		
R3327-AT1 Dunning prostate adenocarcinoma	intratumoral	small tumours (< 2 cm^3) mean 39, large tumours (> 3.5 cm^3) mean 3	Mason *et al.* (1999)	small tumours (< 2 cm^3) mean 11, large tumours (> 3.5 cm^3) mean 7	Mason *et al.* (1999)
R3327-AT1 Dunning prostate adenocarcinoma	intratumoral	mean 40	Worden *et al.* (1999)		
E49 human glioma xenograft	intravenous	range 0.03–49.7, mean 8.8 ($n = 13$)	van der Sanden *et al.* (1999)	range 0.4–3.3	van der Sanden *et al.* (1999)
GH3 prolactinoma	intravenous	mean 34.7	McIntyre *et al.* (1999)	median 2.8	Robinson *et al.* (1999)
GH3 prolactinoma	intratumoral	mean 12.5	McIntyre *et al.* (1999)		
R3327-AT1 Dunning prostate adenocarcinoma	intratumoral	mean 32.3	Hunjan *et al.* (2001)		
GH3 prolactinoma	encapsulaed	mean 18	Nöth *et al.* (2001)		
13762NF breast adenocarcinoma	intratumoral	range 17–74, mean 24	Song *et al.* (2002)		
R3327 Dunning prostate HI subline	intratumoral	small tumours (< 2 cm^3) mean 39, large tumours (> 3.5 cm^3) mean 13	Zhao *et al.* (2002)		
R3327 Dunning prostate MAT-Lu subline	intratumoral	small tumours (< 2 cm^3) mean 24, large tumours (> 3.5 cm^3) mean 8	Zhao *et al.* (2002)		

S for a PFC emulsion depend on temperature, magnetic field strength and the environment of the PFC molecules. Small changes in the anoxic intercept *A* can result in large differences in measured p_{O_2} (Lutz *et al.* 1997). Temperature can also give rise to a significant source of error in absolute p_{O_2} determination. For example, in the case of Oxypherol, a 2 °C error in tumour temperature can lead to an error of 12 mmHg in tissue p_{O_2} (Mason *et al.* 1994). Tumour temperature is not usually determined or controlled in routine experiments, even when the animal's body temperature is carefully maintained. Subcutaneous tumours or those on the extremities of the limbs might be significantly below core body temperature, and deepening anaesthesia could change the gradient between core and peripheral temperature. Worse still, some of the interventions intended to modify tumour oxygenation (e.g. hypotensive drugs such as hydralazine, which 'steal' blood from the tumour into the host tissues) act by altering blood flow, and may thus change tumour temperature.

Another problem is that the environment of the PFC emulsion in a test tube will be very different from that experienced within a solid tumour, which could result in artificial p_{O_2} measurements. For instance, if the tumour contains metal ions (such as Mn^{2+}) or free radicals that can diffuse into the PFC, they may alter T_1 and thus lead to a false estimate of p_{O_2}. ^{19}F NMR oximetry studies in which a p_{O_2} calibration curve is accurately derived from a phantom that more closely resembles the *in vivo* situation at a carefully controlled temperature would be highly revealing in this regard.

The route of administration of PFC emulsion could also result in relatively high p_{O_2} values (McIntyre *et al.* 1999). By definition, chronically hypoxic areas of tumours arise where the rapidly proliferating tissue outstrips its blood vessels, resulting in deprivation of oxygen and nutrients from the tumour cells. Intravenous administration tends to deliver PFC emulsion to well-perfused tumour regions where it is sequestered in macrophages around the blood vessels, and thus reports relatively high p_{O_2} values. Intravenously administered PFC emulsion has impaired access to the poorly vascularized areas that are likely to be radiobiologically hypoxic. By contrast, direct intratumoral administration of PFC emulsion has been shown to give lower p_{O_2} values, with a skewed oxygen-tension-frequency histogram that more closely resembled that obtained by the Eppendorf electrode (McIntyre *et al.* 1999). In this situation the PFC emulsion does appear to be interrogating poorly vascularized tumour regions that are hypoxic, and thus affords a more realistic tumour hypoxic profile that would be more informative to the radiation oncologist (figure 3). The approach does, however, suffer from poor distribution of the PFC emulsion, as described earlier.

Tumour p_{O_2} measurements derived from implanted PFC-loaded alginate capsules also appear to be higher than those obtained by the Eppendorf electrode (Nöth *et al.* 2001). In the light of the above discussion, these data are surprising, as there is no reason to think that the capsules would be preferentially distributed near blood vessels as the tumour grows, which would be one reason for their reporting misleadingly high p_{O_2} values. In this situation, it is more likely that a systematic error in the ^{19}F NMR oximetry method has arisen from the translation of the *in vitro* p_{O_2} calibration curve derived from neat PFC emulsion to the data acquired *in vivo*. Calibration curves are often derived using PFC emulsion equilibrated with very high-oxygen-content gases and then extrapolated back to the much lower radiobiologically significant oxygen tensions in solid tumours. It would be more pertinent to derive these calibration curves using gas mixtures with oxygen tensions of less than 10 mmHg, as used by Mason *et al.* (1996). Experiments should also be performed to investigate the possibility that PFC T_1 values are systematically altered *in vivo*.

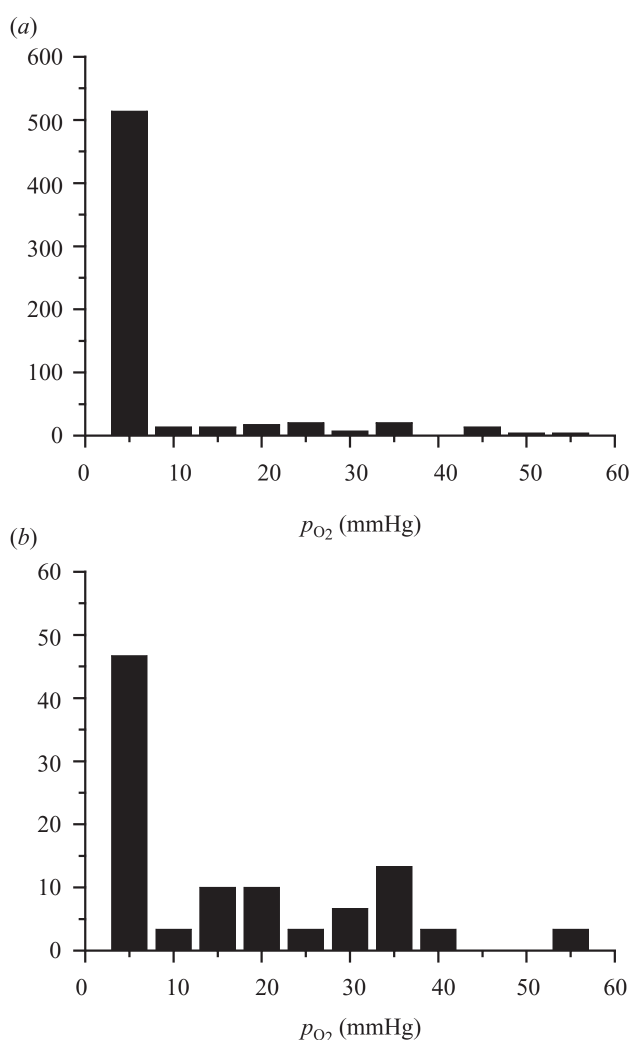


Figure 3. Oxygen-tension-frequency histograms obtained from GH3 prolactinomas measured by (*a*) Eppendorf histography and (*b*) ^{19}F MR oximetry following intratumoral administration of hexafluorobenzene. Note that the skewed histogram profile obtained closely resembles that obtained by the Eppendorf electrode, suggesting that the PFC is interrogating poorly vascularized tumour regions that are hypoxic. (Data courtesy of Dr Dominick J. O. McIntyre.)

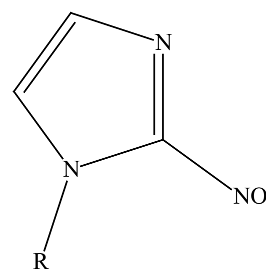
Despite being the only NMR methodology currently affording absolute p_{O_2} tumour measurements, ^{19}F NMR oximetry has not evolved into a routine pre-clinical NMR experiment for the assessment of tumour oxygenation, largely because of the numerous issues described herein. The ability of ^{19}F NMR oximetry to detect *changes* in tissue oxygenation in response to modifiers of the tumour microenvironment is less questionable, but its application and validity in determining absolute p_{O_2} values from unperturbed tumours needs further validation.

Leaving aside these issues, could ^{19}F NMR oximetry have a clinical role? Of the two main methods for administering PFCs, intratumoral implantation is clearly invasive, and it would have to compete with established invasive methods, notably the Eppendorf electrode. Most interest has therefore centred on the possibility of administering PFC emulsions to patients intravenously, but a combination of factors makes this approach appear impractical, at least for the present generation of clinical MRI/magnetic resonance spectroscopy (MRS) instruments. The first problem is sensitivity: in the currently available 1.5 T routine clinical MRI instruments the signal-to-noise is about one-third of that in a typical 4.7 T laboratory instrument. This, combined with the small fraction of the emulsion taken up by the tumour means that very high doses (of the order of hundreds of millilitres) of emulsion would have to be infused into a patient, even if the optimum PFC, 15C5, were used. The toxicity of PFCs, though very low, is not zero, and at these doses, it can begin to be a problem. The majority of the agent is sequestered in the liver and spleen where very large quantities are stored in the reticuloendothelial cells. High-molecular-weight PFCs such as 15C5 have extremely low biological elimination rates and this state of hepatic and splenic overload could last indefinitely. Lower-molecular-weight PFCs are eliminated more quickly, probably through the lungs, but they have lower NMR sensitivity so even higher doses would be required. Also, there is a risk of a vapour embolus forming in a blood vessel when a volatile agent is administered intravenously. All of these issues currently prevent clinical application of PFC oximetry. One consequence of this is that ^{1}H MRI methodologies, with their high temporal and spatial resolution, are being investigated to provide surrogate markers of tumour hypoxia (Robinson *et al.* 1998; Fan *et al.* 2002; Wang *et al.* 2002).

3. FLUORINATED 2-NITROIMIDAZOLES AS ^{19}F MRS PROBES FOR TUMOUR HYPOXIA

(a) *Principles of ^{19}F MRS of 2-nitroimidazoles*

A number of 2-nitroimidazole probes, such as pimonidazole (Raleigh *et al.* 1998), have been developed specifically for immunohistochemical assessment of tumour hypoxia. Nitroimidazoles undergo a hypoxia-dependent bioreductive metabolism of the nitro group catalysed by cellular nitroreductase enzymes via a number of reactive intermediates. One of these intermediates, the hydroxylamine four-electron nitroimidazole, is unstable and highly reactive, and undergoes binding to cellular macromolecules such as proteins, nucleic acids and non-protein sulphydryl compounds. It is this bound hydroxylamine that reflects cellular hypoxia and can be detected by immunohistochemistry with monoclonal antibodies, giving detailed morphological information about the distribution of hypoxia at the cellular level (Evans *et al.* 1997). In an extensive pre-clinical study, pimonidazole binding was shown to correlate with both tumour p_{O_2} and the radiobiological hypoxic fraction under different levels of tumour oxygenation, with strong adduct formation occurring when the p_{O_2} fell below 10 mmHg (Raleigh *et al.* 1999). Similar investigations are now being implemented in the clinic (Nordsmark *et al.* 2001; Kaanders *et al.* 2002*b*).



R	name
$-CH_2CONHCH_2CH_2CF_2CF_3$	EF5
$-CH_2CH(OH)CH_2OCH(CF_3)_2$	CCI-103F
$-CH_2CH(OH)CH_2F$	Ro 07-0741
$-CH_2CONHCH_2CH(OH)CF_3$	SR-4554
$-CH_2CH(OH)CH_2N$	Pimonidazole

Figure 4. Chemical structures of 2-nitroimidazoles used as hypoxia markers.

Fluorinated 2-nitroimidazoles have also been developed to detect the reduced adducts and hence tumour hypoxia non-invasively using ^{19}F MRS or MRI. These include hexafluoromisonidazole (CCI-103F) (Raleigh *et al.* 1986, 1991; Maxwell *et al.* 1989), the monofluorinated 2-nitroimidazole Ro 07-0741 (Maxwell *et al.* 1989; Workman *et al.* 1992), EF5 (Lord *et al.* 1993) and SR-4554 (Aboagye *et al.* 1997; figure 4). To quantify tumour hypoxia, it is necessary to measure the generation and accumulation of the reduced adducts. In pre-clinical studies, this has been done by comparing the ^{19}F MR spectrum of the retained, reduced adducts acquired at a late time-point after administration of 2-nitroimidazole to the ^{19}F MR spectrum acquired at an early time-point, when parent 2-nitroimidazole predominates. The degree of tumour hypoxia is then described by a retention index, i.e. how much of the 2-nitroimidazole is retained within the tumour. In contrast to immunohistochemical detection of 2-nitroimidazoles, the retention index gives a global readout on hypoxia over the whole tumour and no spatial resolution. Figure 5 shows a representative ^{19}F MR spectrum acquired from a tumour 45 min after administration of SR-4554.

(b) *Applications*

As the formation of reduced 2-nitroimidazole adducts occurs under radiobiological hypoxic conditions, the majority of ^{19}F MRS studies of 2-nitroimidazoles have focused on their utility for the assessment of tumour hypoxia. The selective retention of CCI-103F and Ro 07-0741 in murine tumours with a high radiobiological hypoxic fraction (EMT6 and KHT) has been measured by ^{19}F MRS (Maxwell *et al.* 1989). Similar observations have been made in SCCVII and Dunning R3327 tumours (Jin *et al.* 1990; Li *et al.* 1991; Kwock *et al.* 1992). In addition, Kwock *et al.* (1992) used immunohistochemical staining of the 2-nitroimidazole to correlate and validate the ^{19}F retention index. The retention index of SR-4554 has been shown to correlate with the reported radiobiological

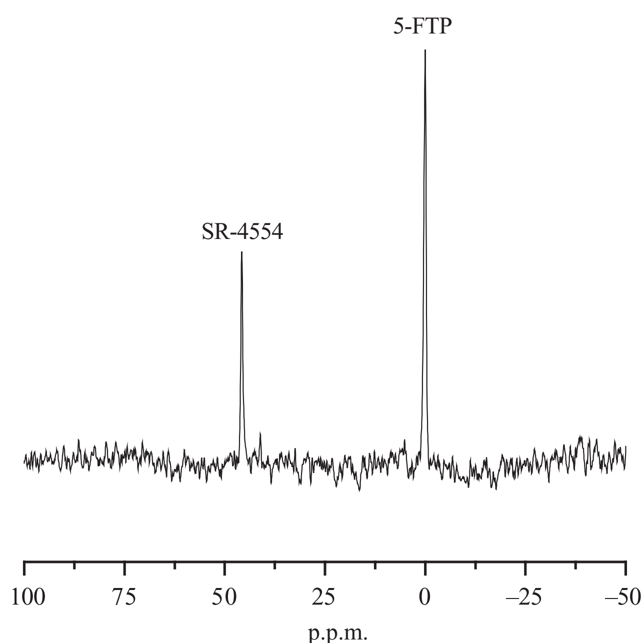


Figure 5. ^{19}F NMR spectrum obtained from a RIF-1 fibrosarcoma 45 min after administration of 180 mg kg^{-1} SR-4554 intraperitoneally. SR-4554 resonates at *ca.* 45 p.p.m. relative to a 5-fluorotryptophan (5-FTP) external standard.

hypoxic fraction of four different murine tumour models (Aboagye *et al.* 1997), but no correlation has been observed between the retention index of SR-4554 and tumour p_{O_2} measured by the Eppendorf electrode (Aboagye *et al.* 1998; Seddon *et al.* 2002). SR-4554 has entered a Phase 1 clinical trial in which retention of ^{19}F MRS signals has been detected in a range of human tumours (Seddon *et al.* 2003).

Several studies have also investigated the effects of modifiers of the tumour environment on tumour hypoxia by measuring the retention of reduced adducts by ^{19}F MRS under modulated conditions. For example, decreases in the tumour ^{19}F retention index of SR-4554 have been shown when the host was administered nicotinamide and breathed carbogen, consistent with an increase in tumour oxygenation. By contrast, administration of the vasodilator hydralazine, which causes vascular steal by the systemic circulation away from the tumour, or the anti-vascular drug combretastatin A-4 phosphate, which results in a decrease in tumour perfusion, causes increases in the ^{19}F retention index of SR-4554, consistent with an increase in tumour hypoxia (Aboagye *et al.* 1997; Seddon *et al.* 2002).

(c) *Current issues*

One aspect that has not yet been resolved is the nature of the adducts formed by the fluorinated bioreductive probes detected by ^{19}F MRS. It has been generally assumed that bioreductive binding of the hydroxylamine takes place on tissue macromolecules. Histochemical studies using radioactive bioreductives or antibody detection clearly detect macromolecular adducts, as any low-molecular-weight adducts would have been washed away during histological processing. The situation is not so clear in the case of ^{19}F MRS *in vivo*. One problem is that narrow spectroscopic peaks are seen only if the molecule bearing the label is free to tumble in the magnetic field, hence the length of the fluorine-bearing side chain (figure 4). Significant loss of ^{19}F signal from the 2-nitroimidazole can occur due to a reduction in the NMR transverse (spin–spin) relaxation time T_2. This is associated with broadening of the MRS signals, which accompanies the binding of small molecules to macromolecules, such as occurs with the reduced 2-nitroimidazole adducts. It is not impossible that a bound nitroimidazole moiety retains sufficient freedom on the surface of a protein for the ^{19}F atom(s) to behave as though they were free in solution, but this would need to be experimentally verified. The alternative possibility is that some of the adduct molecules are small enough to tumble rapidly in the magnetic field. One small molecule that might form adducts with 2-nitroimidazoles is the non-protein sulphydryl compound and free-radical scavenger glutathione, which is present in most tissues and tumours at millimolar concentrations and protects cells against foreign oxidizing agents. According to this hypothesis, some of the fluorinated 2-nitroimidazole binds to macromolecules and can be detected by immunohistochemical methods, whereas the bulk of the ^{19}F MR signal observed *in vivo* arises from glutathione binding. Nitroimidazole bioreduction would thus be associated with glutathione depletion, and the intensity of the retained ^{19}F signal would depend, in part, on the initial concentration of glutathione in the tumour. Another question that arises in this context is whether oxidation of a large proportion of cellular protein sulphydryl groups could be tolerated without evidence of toxicity.

Magnetic resonance spectroscopy is inherently an insensitive method, and concentrations of adduct in the hundreds of micromoles per litre of cell water usually have to be present in a tissue for an adequate signal to be observed. At such concentrations, many nitroimidazoles are significantly neurotoxic. It should be noted that in addition to binding to thiol-rich macromolecules, the hydroxylamine four-electron product has a number of other possible fates, including conjugation and ring fragmentation, such that only a proportion of the administered parent 2-nitroimidazole will be detectable.

A third observation suggesting that most fluorinated bioreductive adducts detected by ^{19}F MRI are small molecules is their pharmacokinetics: the retained signal is usually undetectable 24 h after administration. This could be explained by a rapid reversal of the bioreduction reaction, or some other process eliminating the fluorine, but a simple explanation would be that the bioreductive adducts are small enough molecules that they can be lost from the tissue quite rapidly, albeit more slowly than the parent compounds.

Another anomaly is that some studies have failed to find evidence of ^{19}F retention in certain animal tumour models. Raleigh *et al.* (1991) reported that no ^{19}F signal could be measured 40 min after administration of CCI-103F in 50% of the *excised* R3327 Dunning prostate HI subline tumours that they studied. Under such conditions, these tumours should be extremely hypoxic and hence all the CCI-103F reduced, and thus a strong ^{19}F signal anticipated. These data do, however, correlate with the studies of Zhao *et al.* (2002), which demonstrate that these same tumours have a low hypoxic fraction. A more recent study attempted to measure the degree of retention of SR-4554

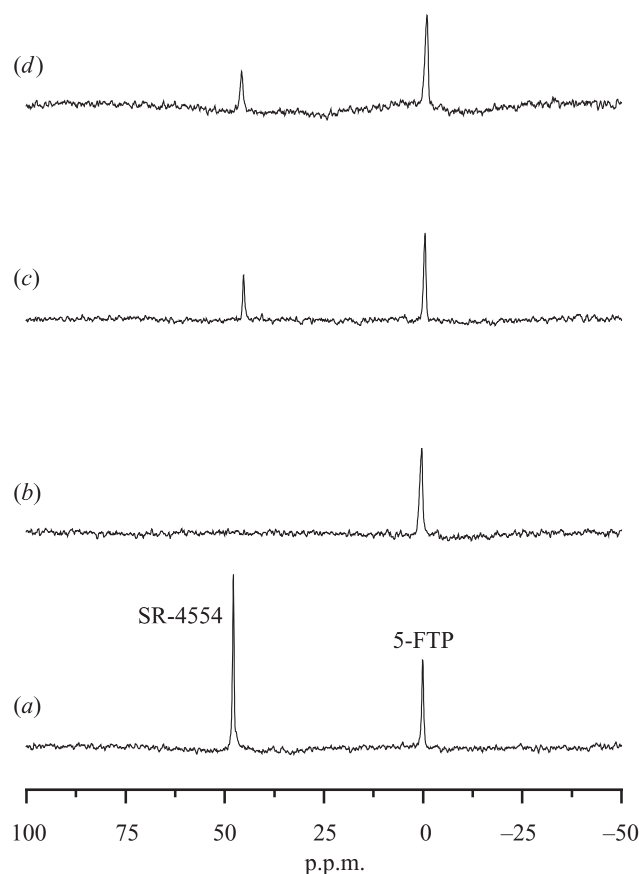


Figure 6. ^{19}F NMR spectra obtained from (*a*) a vial containing 6 mg ml^{-1} SR-4554 resonating at *ca.* 45 p.p.m. relative to a 5-fluorotryptophan (5-FTP) external standard, and *in vivo* ^{19}F spectra acquired from (*b*) a wild-type C6 glioma; (*c*) a RIF-1 fibrosarcoma; and (*d*) an HT29 colon adenocarcinoma all acquired 45 min after administration of 180 mg kg^{-1} SR-4554 intraperitoneally. The degree of retention of the reduced adducts of SR-4554, measured by ^{19}F MRS, affords a non-invasive assessment of tumour hypoxia. No ^{19}F resonance was detected in either C6 wild-type or D27 gliomas 45 min after administration of SR-4554. To confirm that this observation was not due to a technical failure, two other murine tumour models, the RIF-1 fibrosarcoma grown in C3H mice and HT29 colon adenocarcinoma grown in nude mice, were administered SR-4554 and ^{19}F MR spectra acquired under identical conditions. A clear ^{19}F resonance from SR-4554 was observed from these tumours 45 min after administration, thereby validating the acquisition protocol and demonstrating that the observation of a ^{19}F resonance from SR4554 is not dependent on the tumour host.

in vivo using ^{19}F MRS to assess the degree of hypoxia non-invasively in wild-type and mutated C6 gliomas grown in the flanks of nude mice (Robinson *et al.* 2002). Surprisingly, no ^{19}F resonance was detected in any of the tumours studied 45 min after administration of SR-4554 (figure 6). The lack of signal in the C6 gliomas was unexpected, especially as retention of pimonidazole had been detectable by immunohistochemistry in the same tumour types. Whether C6 gliomas express the appropriate nitroreductases required to metabolize SR-4554 specifically is unknown (Workman 1992; Joseph *et al.* 1994), but the data appear to suggest caution in their use with this particular tumour model.

The lack of visibility of the reduced adducts by ^{19}F MRS in these two studies may be a consequence of T_2 shortening. It has recently been suggested that all of the bound adducts of EF5 may have significantly short T_2 values, resulting in extremely broad line widths and thus calling into question the ability of ^{19}F MRS to detect the bound, hypoxia-related adducts *in vivo* (H. W. Salmon, M. Arreola and D. W. Siemann, personal communication). If true, this would imply that the signals detected by ^{19}F MRS *in vivo* are from the parent 2-nitroimidazole, and that the retention index is a measure of tumour blood flow *per se*, rather than hypoxia. In this context, it is also noteworthy that the majority of agents used to perturb the tumour environment, such as nicotinamide, hydralazine or combretastatin A-4 phosphate, are primarily modifiers of tumour blood flow that indirectly cause changes in tumour hypoxia. The reported changes in ^{19}F MRS retention index with these modifiers (Aboagye *et al.* 1997; Seddon *et al.* 2002) are also consistent with the expected changes in tumour blood flow, and hence could be reflecting trapping or a reduced clearance of parent 2-nitroimidazole within the tumour vasculature, rather than the reduced adducts. Alternatively, the tumours used in these studies may have abnormally low levels of glutathione, which, as discussed earlier, would result in less nitroimidazole bioreduction.

It is apparent that further experiments are required to establish categorically if ^{19}F MRS is reporting on parent or reduced 2-nitroimidazole. It would also be informative to measure the tumour ^{19}F MRS retention index of a 2-nitroimidazole in response to a modifier that directly perturbs tumour hypoxia, perhaps through modification of oxygen consumption by the tumour cells, which has been shown theoretically to be more efficient at affecting oxygen transport than direct modification of delivery (Secomb *et al.* 1995; Snyder *et al.* 2001).

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