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Magnetic resonance spectroscopy and imaging methods for measuring tumour and tissue oxygenation

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Summary It is well known that low levels of tissue oxygen (pO_2) protect tumour cells from ionising radiation and some chemotherapeutic agents. Thus, numerous studies have been aimed at developing methods to measure tissue oxygenation. An initial discussion of some of the traditional methods for measuring oxygenation is included, followed by a discussion of magnetic resonance spectroscopy (MRS) and magnetic resonance imaging (MRI) methods for measuring tumour and normal tissue oxygenation. The latter methods are of interest because of the non-invasive nature of magnetic resonance (MR). Some of the MR methods described herein include: ^{31}P MRS, ^{1}H MRS and MRI, and ^{19}F MRS and MRI. Each method is detailed, including a brief assessment of its ability to measure tumour oxygenation and its potential for clinical application.

Keywords: myoglobin; lactate; nitroimidazole probe; perfluorocarbon; electron spin resonance; magnetic resonance spectroscopy

It has been known since the 1950s that ionising radiation (Thomlinson and Gray, 1955) and some chemotherapeutic agents are less effective at low tissue oxygen (pO_2) levels. Since then, many methods have been developed for measuring tissue pO_2 which is also crucial for cellular viability and plays a key role in cellular regulation under physiological and pathophysiological conditions. Some methods for measuring tissue oxygenation or related parameters include: the standard arterial-venous (A - V) difference measurement of haemoglobin (Hb) saturation (see Vink, 1992 and references within, and Jue, 1994 and references within), O_2 electrode methods (see Vaupel *et al.*, 1981), phosphorescence (see Wilson and Cerniglia, 1992), near infrared (NIR) absorption spectrophotometry (Jobsis, 1977) or measurement of metabolites (such as lactate, pyruvate or phosphocreatine) by NADH fluorescence (see Chance *et al.*, 1978). Although these methods provide measures of tissue oxygenation, they have their limitations. They are either invasive in nature (A/V difference of Hb saturation and O_2 electrodes), may not accurately reflect cellular O_2 levels (NIR spectrophotometry), may be unreliable for measuring pO_2 in glycolytic tumours (NADH fluorescence), or may be unsuitable for use in humans (phosphorescence).

Another method for measuring tissue oxygenation that involves non-invasive detection and has proved to be clinically useful is nuclear magnetic resonance (NMR). NMR is divided into magnetic resonance spectroscopy (MRS) which provides a plethora of biochemical information and magnetic resonance imaging (MRI) which provides anatomical information about the spatial distribution of a specific nucleus. There are many reviews and books available on the basic principles of NMR (Andrew *et al.*, 1990) and related topics (e.g. MR measurements of blood flow; for review see Evelhoch, 1994) which are outside the scope of this article. At the present time, MRS and MRI do not provide a direct measure of oxygenation. However, several indirect methods based on examination of endogenous signals or the use of exogenous probes are currently in use and will be described herein.

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^{31}P MRS

Phosphorus (^{31}P) MRS is widely used for measuring tissue bioenergetics non-invasively. It can be used to measure several parameters such as high energy phosphates (ATP), inorganic phosphate (P_i), phosphocreatine (PCr) and intracellular pH, that all depend on tissue oxygenation. The relationship between ^{31}P MRS and tissue oxygenation can be described by the familiar equation for ATP production via oxidative phosphorylation:

$$3ADP + 3P_i + NADH + H^+ + \tfrac{1}{2}O_2 \rightarrow 3ATP + NAD^+ + 4H_2O \quad (1)$$

However, ATP levels are not only dependent on tissue pO_2 levels, but also to some extent on the concentrations of all the substrates in equation (1).

In the case of tumours, there are growth-related changes observed in the ^{31}P spectrum that are presumed to be related to tumour pO_2. These changes are closely coupled to the energy status of a tumour and are presumed to be a result of the tumour growing so rapidly that some areas become chronically deprived of O_2 and nutrients (i.e. chronic or diffusion-limited hypoxia). It has also been suggested that transient fluctuations in the delivery of O_2 and nutrients may contribute to some of the changes observed in the ^{31}P spectrum (i.e. acute or perfusion-limited hypoxia; Brown, 1979).

Correlations have been found between ^{31}P MRS spectra and results from O_2 electrode measurements (Vaupel *et al.*, 1989, 1994; Sostman *et al.*, 1991), ^{15}O perfusion measurements (Evelhoch *et al.*, 1986), intracapillary oxyhaemoglobin saturation (Rofstad 1988*a*), bioluminescence (Mueller-Klieser *et al.*, 1990; Vaupel *et al.*, 1994), optical spectrophotometry (Sostman *et al.*, 1991), and fraction of radiobiological hypoxic cells (Rofstad 1988*b*; Sostman *et al.*, 1991) in some tumour lines. However, none of the ^{31}P MRS parameters measured give information on the oxygenation status of tumours in general.

Phosphorus MRS has also been used to measure the bioenergetic status of normal tissues. The normal tissue most widely studied by this method is muscle. The pioneering work on the relationship between muscle bioenergetics and oxygenation (Chance *et al.*, 1985) showed that the P_i/PCr ratio can be used as a measure for muscle pO_2.

An explanation can be found by re-examining equation (1) since it establishes the close link between $[O_2]$, ADP and P_i.

Using Michaelis–Menten kinetics, the relationship in equation (1) can be rewritten as the relative velocity (V) of the reaction to its substrates under steady-state conditions:

$$V/V_{max} = 1/(1 + \{K_{ADP}/[ADP]\} + \{K_{O_2}/[O_2]\}) \quad (2)$$

where V_{max} is maximal velocity, and K_{ADP} and K_{O_2} are the respective Michaelis constants for ADP and O_2. This equation predicts that there is an inverse relationship between [ADP] and tissue pO_2. Thus, is would seem that changes in ADP levels would be useful for monitoring tissue oxygenation. Unfortunately, *in vivo* [ADP] is ~20 μM which is below the MR limit of detection. However, in normal tissues like muscle, ADP levels can be calculated from the MRS-detectable levels of PCr and ATP on the assumption that the creatine kinase reaction is near equilibrium:

$$PCr + ADP + H^+ \rightleftharpoons ATP + Cr \quad (3)$$

and

$$K_1 = \{[ATP][Cr]\}/\{[PCr][ADP]\} \quad (4)$$

where K_1 is the equilibrium constant for the creatine kinase reaction and equals $K_{Cr}[H^+]$. Provided that [ATP] and K_1 are constant, equation (2) can be rewritten as:

$$V/V_{max} = 1/1 + (\{K_{ADP} \cdot K_1 \cdot [PCr]/[ATP] \cdot [Cr]\} + \{K_{O_2}/[O_2]\}) \quad (5)$$

Thus PCr/Cr will be directly proportional to tissue oxygenation (i.e. a decrease in PCr/Cr corresponds to a decrease in pO_2). P_i can be substituted for Cr on the basis that coupled hydrolysis of PCr and ATP will give equimolar amounts of Cr and P_i. Thus, the P_i/PCr ratio from muscle can reflect changes in [ADP] and therefore changes in tissue oxygenation (for discussion, see Tozer and Griffiths 1992; Vink, 1992; McCully *et al.*, 1994).

Recently, it has also been shown that the P_i/PCr ratio in muscle is correlated with tissue oxygenation measured by NIR spectroscopy of oxyhaemoglobin (HbO_2) saturation, as long as muscle pH is maintained near neutrality (McCully *et al.*, 1994). Thus, although there is in some cases a direct relationship between ^{31}P MRS spectral parameters and tissue pO_2, because of confounding factors, it seems likely that ^{31}P MRS alone may be of limited use to measure oxygenation in either tumour or normal tissue (Nordsmark *et al.*, 1995; Gerweck *et al.*, 1995).

^1H MRS

The proton (^1H) nucleus is the most sensitive stable NMR nuclide; furthermore, it is 99.99% naturally abundant, and most living tissues contain high concentrations of hydrogen atoms. One consequence of this is that a typical *in vivo* ^1H spectrum contains a peak from tissue water (^1H atoms in tissue water are ~80 M) and lipids (~1 M) which overlap all of the other ^1H resonances. Fortunately, technical developments have allowed adequate suppression of the water signal so that *in vivo* ^1H MRS can be performed routinely (for reviews see van Zijl and Moonen, 1992; Howe *et al.*, 1993*a*).

The simplest way to measure tissue pO_2 is by direct ^1H MRS observation of oxygen-binding proteins but some assumptions about the spectrum have to be made (see Jue, 1994). Firstly, there must be no contamination from other metabolites or proteins (such as Hb). Secondly, there should be a difference between ligated and unligated states of O_2. Finally, the signal which is observed should be present at a concentration that is high enough to be detected by *in vivo* ^1H MRS. To date, the use of ^1H MRS to measure normal tissue oxygenation is mainly restricted to measurements of myoglobin. ^1H MRS can also be used to measure lactate (although its use as an indicator of tissue oxygenation is questionable). This method is mainly applicable to human brain tumours, although there is a preliminary report of ^1H spectroscopy of a subcutaneous animal tumour (Shungu *et al.*, 1992).

Myoglobin

Myoglobin meets the above-mentioned criteria and tissue pO_2 can be determined directly because of the equilibrium relationship between oxymyoglobin (MbO_2) and deoxymyoglobin (Mb) (see Jue and Anderson, 1990). It is the ^1H NMR signals from the proximal histidyl NH and the valine E11 γ-CH_3 resonances that allow measurement of Mb and MbO_2 respectively (Kreutzer *et al.*, 1992). In perfused organs, the ^1H signals from both MbO_2 and Mb are detectable (Kreutzer *et al.*, 1992) while *in vivo* only the ^1H signal from Mb is MR visible. The Mb signal can be obtained within seconds from human forearm (Wang *et al.*, 1992) and it appears that there is minimal contamination from Hb (Wang *et al.*, 1993). But, in spite of its success in muscle, this method is of limited use for measuring oxygenation in tumours since they have low concentrations of myoglobin.

Lactate

The concentration of lactate ranges from 0.5 mM in normal brain to 25 mM in exercised muscle. These concentrations may become elevated under pathological conditions such as cerebral ischaemia or in tumours. In order to detect the lactate signal *in vivo*, editing techniques must be employed to minimise contamination from fat (for discussion see Howe *et al.*, 1993*a*). This is especially important for measurements in diseased muscle in which the relative amount of fat may be increased. Although there is a report in the literature of a systematic evaluation of tumour lactate and pO_2 (Vaupel *et al.*, 1994), the correlation between tissue lactate and pO_2 is questionable in highly glycolytic tissues such as tumours because many other factors (e.g. substrate supply, the presence of appropriate metabolic pathways, etc.) will be involved. Also, lactate is found to accumulate in the extracellular fluid as well as in cells. Thus, although tissue lactate is a product of anaerobic metabolism, it is unlikely to be a direct indicator of oxygen content in either tumour or normal tissue.

^1H MRI

Blood can act as an endogenous contrast agent in MRI. Like Mb, Hb is paramagnetic, hence the magnetic field around venous blood vessels is perturbed and because of this some MRI methods [particularly those using gradient recalled echo (GRE) sequences; Haase *et al.*, 1986] are very sensitive to this blood oxygen level dependent (BOLD) effect. Thus, changes in blood flow and oxygenation can be imaged in some detail.

Recently developed functional magnetic resonance imaging (fMRI) techniques can measure changes in cerebral oxygenation and/or blood flow in response to an external stimulus in both animals and humans (Ogawa *et al.*, 1990; Kwong *et al.*, 1992). These MRI methods are rapid (in the order of seconds per acquisition) and in the images the flowing arterial/venous blood appears bright relative to stationary spins which are saturated owing to the fast repetition rate. Regions containing high concentrations of paramagnetics appear dark due to dephasing (ie. T_2^* effects). GRE ^1H MRI sequences are thus sensitive to local susceptibility variations near the blood vessels and hence offer a non-invasive approach to studying changes in the concentration of Hb due to physiological challenge such as blood flow modification or the degree of desaturation of O_2 in the blood.

GRE imaging has been used to observe the response of tumours to other blood flow modifiers. The response to the

hypotensive agent calcitonin gene-related peptide (CGRP) in GH3 prolactinomas was observed by interleaved ^{1}H GRE imaging and ^{31}P MR spectroscopy (Howe *et al.*, 1993*b*). A decrease and subsequent recovery of normalised image intensity, $\beta NTP/P_i$ and intracellular pH was observed, consistent with transient hypoxia induced by the short-lived effect of CGRP. Vasodilation of the host vessels by CGRP presumably caused the redirection of blood flow away from the tumour and hence an increase in Hb within the tumour, resulting in the decrease in image intensity. A similar response was seen in GRE images of RIF-1 tumours in response to acute hypoxia induced by 5 mg kg^{-1} hydralazine (Bhujwalla *et al.*, 1993), which further suggests that GRE image contrast is sensitive to the oxygenation state of the tumour.

One of the first reports using this method was on the effects of hyperoxia on T_2^* weighted images of rat R3230AC mammary adenocarcinomas (Karczmar *et al.*, 1994). More recently the same group showed that T_2^* weighted images could differentiate tumours from normal tissue (Kuperman *et al.*, 1995). Significant image intensity increases were again observed within the tumour centre and rim, while little change was observed in muscle tissue during hyperoxia. Another recent study reports the effects of breathing the chemical modifier carbogen (95% oxygen/5% carbon dioxide) on tumour GRE image intensity (Robinson *et al.*, 1995*a*). Carbogen breathing caused increases of up to 100% in normalised image intensity in GH3 prolactinomas grown in Wistar Furth rats; reversion to air breathing caused a subsequent fall in image intensity. These changes in signal intensity are consistent with an increase in oxygen content of the blood, tumour cell oxygenation or tumour blood flow (for a representative example, see Figure 1). Three-dimensional GRE imaging, which suppresses the signal from flowing blood, still shows increases in image intensity in response to carbogen, which implies that improvements in both tumour oxygenation and blood flow contribute to the signal intensity increase seen in the two-dimensional images (Howe *et al.*, 1995). The response of mammary tumours, chemically induced by MNU, to carbogen has also been measured and increases in signal intensity typically one-third of that seen in the transplanted GH3 prolactinomas were observed (Robinson *et al.*, 1995*b*). This reduced response was probably due to a different vascular architecture that develops in chemically induced tumours. These observations all support the use of fMRI methods in the clinic, both to improve radiotherapy treatment protocols and as a diagnostic technique.

^{19}F MRS and MRI

Fluorine (^{19}F) MR is almost as sensitive as ^{1}H MR, so it is possible to detect exogenously administered probe molecules. The stable isotope of fluorine (^{19}F) is suitable for MRS studies because it has a spin of ½, low background and 100% natural abundance, coupled with a relatively high sensitivity for detection (0.83 relative to protons).

Nitroimidazole probes

It is a well-known phenomenon that some nitroimidazoles undergo nitroreduction and selective binding within hypoxic tissues (Chapman *et al.*, 1981). If a fluorinated 2-nitroimidazole is administered, it is possible to detect residual ^{19}F signal non-invasively from the tumour following the 'wash-out' of unmetabolised, unbound nitroimidazole.

The low concentration of bound adducts, however, means that generally only probes with multiple fluorine substitution or which can be given in relatively high doses (in the order of 0.1 mmol kg^{-1} magnetically equivalent fluorine atom) are suitable for MRS/MRI studies (Workman *et al.*, 1992). Relevant to the design of suitable fluorinated 2-nitroimidazoles are pharmacokinetic/toxicity considerations and in particular tumour and plasma half-lives of the parent 2-nitroimidazole, as well as tumour–plasma and brain–plasma partition coefficients (Workman and Brown, 1981). In addition to these, it is also necessary to consider the signal attenuation due to macromolecular binding and its influence on the stoichiometry of the retention of these compounds (Workman *et al.*, 1992).

The selective retention of both Ro-07-0741 [1-(3-fluoro-2-hydroxy propyl)-2-nitroimidazole] and CCI-103F [1-(3-(1, 1, 1, 3, 3, 3-hexafluoropropan-2-oxy)-2-hydroxypropyl)-2-nitroimidazole] in mouse tumours with high radiobiological hypoxic fraction (eg. EMT6 and KHT) has been reported (Maxwell *et al.*, 1988). In the same study, less intense residual ^{19}F signals were observed from RIF-1 tumours (which have a low hypoxic fraction). Similar studies have also been reported

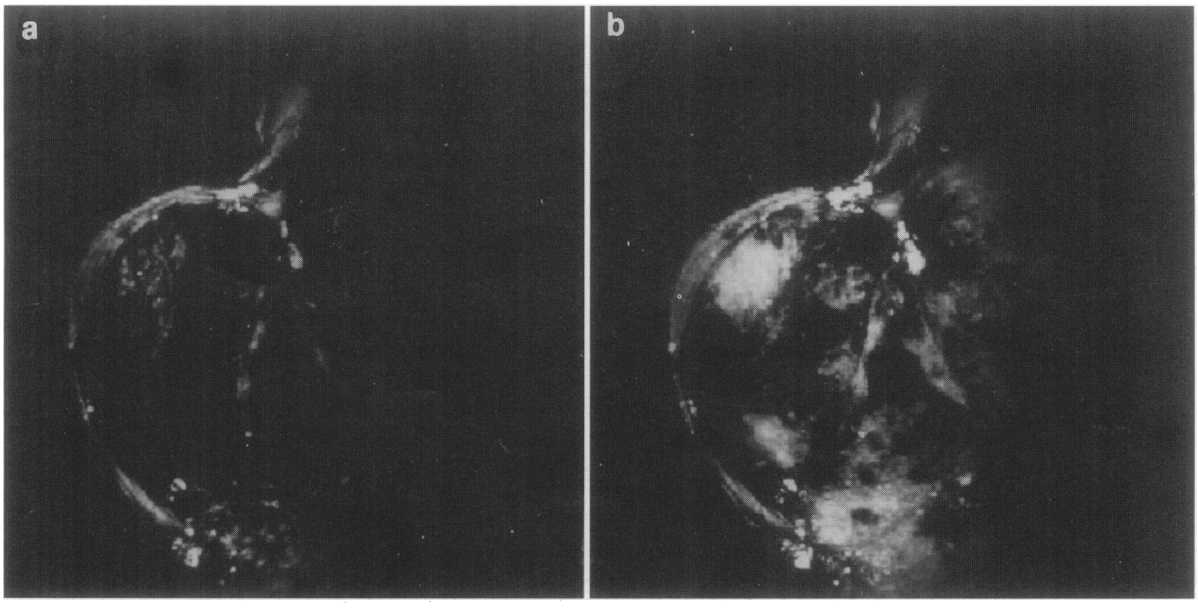

Figure 1 GRE ^{1}H MR images acquired from a transplantable rat GH3 prolactinoma in response to (**a**) air and (**b**) carbogen, administered at 2 l min^{-1}. TE, 20 ms; TR, 80 ms; $\alpha = 45°C$. The images clearly show a heterogeneous response, with regions of intense signal becoming more so during carbogen breathing, corresponding to well-perfused areas of the tumour. Other regions giving rise to little or no signal were unaffected by carbogen and probably correspond to areas of low blood flow or necrosis.

in mouse SCCVII tumours (Jin *et al.*, 1990), and in rat Dunning R3327 prostate adenocarcinomas (Kwock *et al.*, 1992) and Walker-256 carcinosarcomas (Raleigh *et al.*, 1991). In addition, the selective retention of the fluorinated 2-nitroimidazole SR-4554 [*N*-(2-hydroxy-3,3,3-trifluoropropyl)-2-(2-nitro-1-imidazolyl) acetamide] was found to correlate to RIF-1 tumour weight; the bigger tumours have a higher hypoxic fraction (Aboagye *et al.*, 1994). The relationship between residual ^{19}F retention of SR-4554 and the pO_2 measured using microelectrodes (Eppendorf) in mouse tumours has been reported elsewhere (Aboagye *et al.*, unpublished results).

As with other fields of nitroimidazole research, the exact nature of the MR-visible adducts is not known. The data from studies reported so far, however, suggest that soluble molecules (possibly glutathione) are labelled in greater proportion, since macromolecules rarely give such narrow MRS peaks. This is supported by preliminary data (EO Aboagye, unpublished results) which show the presence of ultrafiltrable hypoxia-dependent SR-4554 metabolites in microsomes and *in vivo* in EMT-6 murine tumours.

Perfluorocarbon probes

Perfluorocarbons (PFCs) are organic molecules in which all the hydrogen atoms have been replaced with fluorine, giving a large ^{19}F MR signal. Perfluorocarbons are biologically inert and non-toxic and liquid PFCs are commercially available as lipid emulsions (suspensions of micelles). These emulsions may be injected into the bloodstream since the particles are smaller than blood corpuscles. Oxygen is highly soluble in many PFCs, which allows them to be used in two ways: either as blood substitutes (see Rockwell, 1985 and references within) or to enhance tumour oxygenation to assist radiotherapy (for review see Teicher, 1992). Immediately after injection, ^{19}F MRI may be used to image arteries and veins, including those in tumours. The PFCs are then absorbed by macrophages over several days after injection. Most will be taken up by the liver and the spleen, while tumours containing large numbers of macrophages will also take up enough to be imaged. When these substances bind oxygen, their ^{19}F MR signal characteristics are modified and their MR relaxation times T_1 and T_2 are inversely proportional to the concentration of dissolved O_2 (Parhami and Fung, 1983). Thus, T_1 images of projections through tissues may be used to measure the pO_2 of tumours or other macrophage-containing organs (i.e. liver and spleen) (see Dardzinski and Sotak, 1994; Sotak *et al.*, 1993).

Recently, PFCs have been designed specifically to simplify MRI studies, with all the resonances close or overlapping. Blood flow modification agents such as carbogen have been used to increase the uptake of PFC into tumours, improving the uniformity of uptake and allowing the imaging of slices through tumours (see Figure 2) (McIntyre *et al.*, 1995; McCoy *et al.*, unpublished results).

Electron spin resonance (ESR)

NMR methods for measuring tissue oxygenation are inherently insensitive (with the exception of the BOLD method, see above), so it would be useful to have a spectroscopic method with higher sensitivity. One such method that has recently been used to measure tissue pO_2 *in vivo* is electron spin resonance (ESR) spectroscopy (Swartz *et al.*, 1992). Recently, this method has been used to measure tissue oxygenation in perfused rat heart (Kuppusamy *et al.*, 1995). It is also possible to monitor the oxygenation of tumours by injecting them with an inert compound such as india ink (Goda *et al.*, 1995*a*). Although it has been used to monitor tumour pO_2 after x-ray irradiation (Goda *et al.*, 1995*b*), and has been combined with MRI to measure pO_2

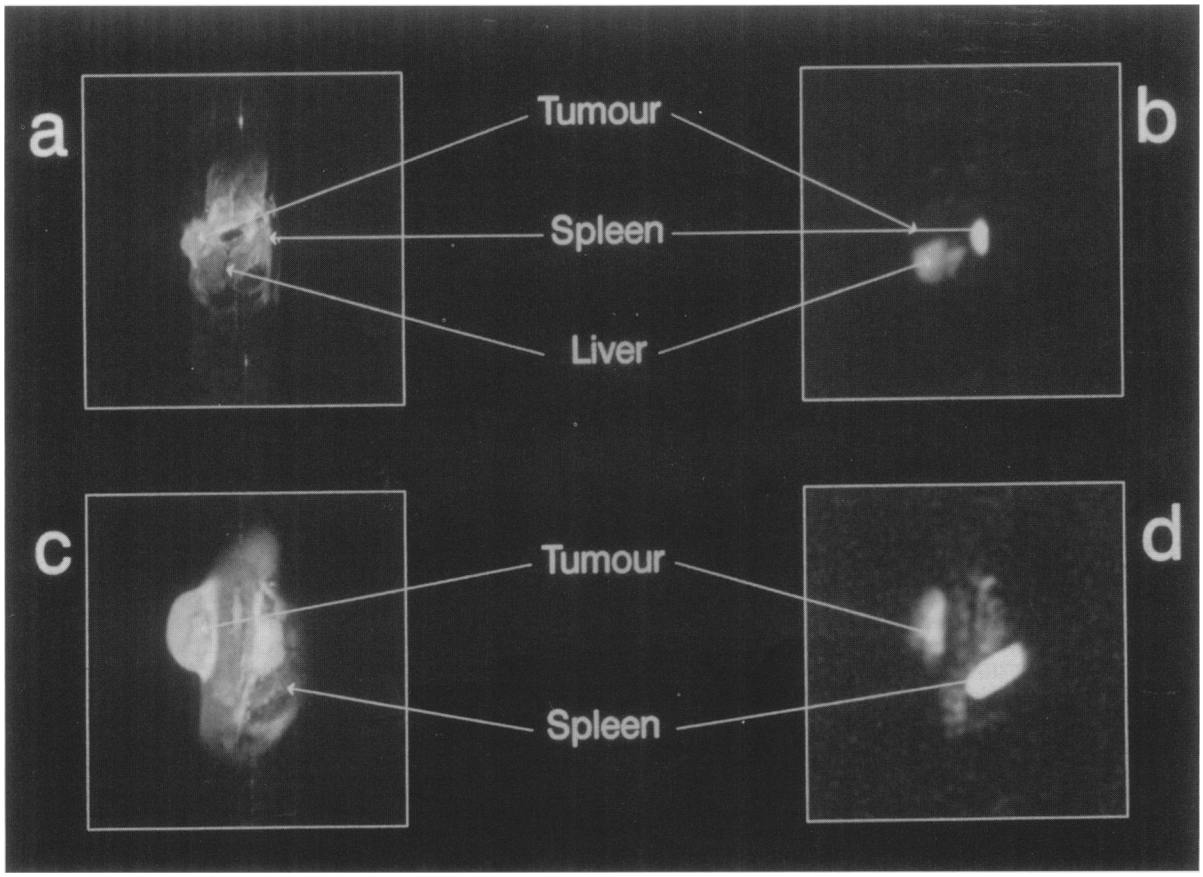

Figure 2 ^{1}H scout image (**a**) and ^{19}F image (**b**) from a C3H mouse bearing a RIF-1 tumour showing low uptake of fluorovist (9 mmol kg^{-1}). ^{1}H scout image (**c**) and ^{19}F image (**d**) from a C3H mouse bearing a RIF-1 tumour showing enhanced uptake of fluorovist (9 mmol kg^{-1}) after allowing animal to breathe carbogen for 15 min immediately after injection. Arrows indicate position of liver, spleen and tumour.

(Bacic *et al.*, 1993) this interesting method is still at a fairly early stage of development, and for tumours it is limited to those that are accessible to injection.

Future directions

Most magnetic resonance methods for measuring tumour oxygenation are generally less invasive than those currently available, and several give spatial maps. Most of the methods discussed in this paper can readily be used to study the actions of chemical modifiers on tissue or tumour oxygenation. For example, it is possible to detect the difference in oxygenation (e.g. BOLD MRI) induced by the modifier; in others the modifier itself is the probe that is detected (e.g. fluorinated nitroimidazoles and ESR). Although the MR methods discussed in this paper pertain to indirect methods of measuring tissue pO_2, the non-invasive nature of MR means that it offers some advantages over classical methods for measuring tissue oxygenation.

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