The effect of blood flow modification on intra- and extracellular pH measured by $^{31}$P magnetic resonance spectroscopy in murine tumours

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Summary
Intra- and extracellular pH ($p_{\text{H}}$ and $p_{\text{H}^+}$) were measured simultaneously by $^{31}$P magnetic resonance spectroscopy (MRS) in CaNT tumours before and after blood flow modification. Before modification, $p_{\text{H}}$ was 7.1 ± 0.09 ($n$ = 11) and $p_{\text{H}^+}$ [measured with an MRS-visible extracellular marker, 3-aminopropyl phosphonate (3-APP)] was 6.7 ± 0.05 ($n$ = 8). Chemical shift imaging and localised MRS experiments showed that the 3-APP signal was only from the tumour, not surrounding tissue. After modification by vascular occlusion, independent of whether tumours were maintained at room temperature (22–24°C) or kept warm (33–35°C), there was a decrease in $p_{\text{H}}$ and $p_{\text{H}^+}$ with $p_{\text{H}}$ decreasing to a greater extent. Qualitatively similar results were found using flavone acetic acid (FAA) as a blood flow modifier; only four out of nine tumours responded to FAA. Concomitant with the reduction of the pH gradient after modification was a decrease in the phosphorylation state of the adenine nucleotides measured either as ATP/P, by MRS or [ATP]/[ADP]/[P], in tumour extracts. These results indicate that the intracellular uptake of chemotherapeutic drugs which are dependent on the transmembrane pH gradient will not be enhanced in cells made ischaemic as a result of vascular shutdown.

Keywords: intracellular pH, extracellular pH; magnetic resonance spectroscopy; murine tumour; blood flow modifier

In the quest for new anti-cancer strategies for solid tumours, many approaches have been tried using pH (for review see Wike-Hooley et al., 1984). These include various strategies for altering pH: lowering the pH to make the tumour cell more sensitive to a particular treatment modality, e.g. hyperthermia (Hiraoka and Hahn, 1989), or accentuating pH gradients ($\Delta p_{\text{H}}$) between intra- and extracellular compartments (Gerweck et al., 1991). To achieve the latter, various methods have been tried: inducing hyperglycaemia to increase lactic acid production and thus lower pH (Evelhoch et al., 1984; Hwang et al., 1991; Jahde et al., 1992); inhibiting the Na$^+$/H$^+$ exchanger with amiloride and its analogues (Newell et al., 1992; Maidorn et al., 1993) and using compounds [e.g. hydralazine, flavone acetic acid (FAA)] that specifically reduce blood flow to tumours (Vorhees and Babbs, 1982; Evelhoch et al., 1991; Perkins et al., 1994a) to make the tumours more anoxic and therefore more acid. However, by using these various strategies, it is hoped that the $p_{\text{H}}$ differences can be exploited either by activating cytotoxic agents selectively within tumours (Tannock and Rotin, 1989; Newell et al., 1992) or by altering the distribution of drugs that are weak acids or bases (Gerweck et al., 1991) in such a way that they will be taken up more effectively by the tumour than by the normal surrounding tissue. Several drugs (e.g. mitomycin C, chlorambucil) have been shown to have increased cytotoxicity in vivo in isolated cell experiments at low extracellular pH ($p_{\text{H}^+}$) (Maidorn et al., 1993; Perkins et al., 1994b). These drugs have also caused growth delay in vivo in some tumour types (Newell et al., 1992; Perkins et al., 1994b).

Following the classical experiments of Warburg in the 1920s, many years tumours were thought to be acidic, but it is now generally accepted (Vaupe1 et al., 1989; Griffiths, 1991) that tumour intracellular pH ($p_{\text{H}^+}$) is close to neutrality. $^{31}$P magnetic resonance spectroscopy (MRS) has confirmed non-invasively that $p_{\text{H}^+}$ in both human and animal tumours is on the alkaline side of neutrality: pH 7.1–7.2 (Vaupe1 et al., 1989; Griffiths, 1991; Evelhoch, 1992; Nengdank, 1992) which is similar to that in most normal tissues. Confirmation that the measurement of tumour pH by MRS is largely representative of $p_{\text{H}}$, has been made in animal tumours (Stubbs et al., 1992). It is now possible, with the aid of an MRS-visible extracellular marker 3-aminopropyl phosphonate (3-APP) (Gillies et al., 1994) to measure $p_{\text{H}}$ in vivo. 3-APP is not toxic to C6 glioma or Ehrlich ascites tumour cells at concentrations up to 20 mM (Gillies et al., 1994). Thus it may be used for monitoring the course of the $\Delta p_{\text{H}}$ of solid tumours in vivo after therapy. Since in vitro and in vivo total experiments with CaNT tumour cells have shown dependence of drug cytotoxicity on $p_{\text{H}}$, enhancement of cell kill at low $p_{\text{H}}$ and dependence of ischaemia-induced cell death on temperature (Parkins et al., 1993, 1994a,b), the purpose of the work reported here was to monitor $p_{\text{H}}$ and $p_{\text{H}^+}$ simultaneously by $^{31}$P MRS in CaNT murine tumours before and after blood flow modification. The $\Delta p_{\text{H}}$ was monitored in three cohorts of mice, before and up to 2 h after total vascular occlusion. The core temperature was maintained at preoclusion values (33–35°C) or allowed to cool naturally to room temperature (22–24°C) after cessation of blood flow. The results showed that the $\Delta p_{\text{H}}$ decreased after vascular occlusion and that there was a decrease in both $p_{\text{H}}$ and $p_{\text{H}^+}$ after treatment with FAA in the four out of nine tumours that responded to FAA.

Materials and methods

Tumours
Moderately differentiated murine adenocarcinoma NT (CaNT) tumours were grown subcutaneously on the lower dorsum of syngeneic CBA/Gy ItO mice and examined when they were about 10 mm in diameter. The mice were divided into two groups for MRS measurements and freeze clamping. The mice were anaesthetised intraperitoneally (i.p.) with ketamine (30 mg kg$^{-1}$) (Parke-Davis, UK) and diazepam (25 mg kg$^{-1}$) (Phoenix Pharmaceuticals, UK) to avoid motion artefacts during MRS measurements.

Injection of 3-APP and FAA
The mice were injected with 0.3 ml of 128 mg ml$^{-1}$ of 3-APP (Sigma, UK) i.p. (12–15 mmol kg$^{-1}$ body weight), 30 min before the spectra were collected. In the studies with FAA...
Total vascular occlusion
Because remotely controlled non-magnetic occlusion devices are difficult to make, vascular occlusion was achieved by an intravenous (i.v.) injection of a lethal dose of euthalial via an in-dwelling tail vein cannula with a line for remote injection inserted before the mouse was placed in the magnet.

MRS measurements
MRS measurements were made on a Isisco 200–330 at 4.7 T using images-guided localised spectroscopy by ISIS (image-selected in vivo spectroscopy) (Ordidge et al., 1986), with adiabatic pulses, a recycle time of 3 s and a gradient strength of 7.5 × 10−4 T cm−1. On average a volume of 0.8 cm3 was selected using a two-turn 1 or 2 cm solenoid coil (depending on tumour size and shape). Pre- and post-occlusion spectra were obtained with an interleaved ISIS localisation acquired in 320 scans (total) with the transmitter frequency set on α-NTP in one spectrum (Figure 3a) and on 3-APP in the other (see Figure 3b). Because the chemical shift difference between 3-APP and α-NTP was >30 p.p.m. there was a significant chemical shift artefact and the double interleaved ISIS acquisition was used to minimise this problem (see Maxwell et al., 1994, and Discussion). To obtain adequate signal/noise for the 3-APP signal it was necessary to double the dose (0.3 ml of 128 mg ml−1) 12–15 μmol g−1 body weight given by Gilles et al. (1994) which produced a peak between 32 and 34 p.p.m. (relative to α-NTP). The mice tolerated this increased dose very well and there were no deaths that could be attributed to it.

For the chemical shift imaging (1D-CSI) experiment, 64 transients were acquired for each of 32 phase-encoding steps over a 4 cm field of view. The acquisition time was 128 ms with a repetition time of 2 s and 8 kHz spectral width.

Data processing
The MR spectra were analysed using VARPRO, a time domain fitting routine (van der Veen, et al., 1988). Because the chemical shift artefact causes more distortion to the 3-APP peak than the γ-NTP, the latter has been used to calculate the NTP/P ratios. The contribution from free NDP is considered negligible and likely to be in the micromolar range (Stubbs et al., 1989).

pH measurements
pH was measured from the difference in chemical shift between the P resonance and that of α-NTP at −7.57 p.p.m. according to Pritchard et al. (1983). The value for pH was measured from the chemical shift difference between 3-APP and α-NTP. In the occlusion experiments, the reference signal (α-NTP), disappeared after 30–40 min and in these cases the chemical shift value obtained for the α-NTP peak before the α-NTP signal disappeared was used for calculation of pH and pH. For this analysis a standard curve was constructed in vitro by setting up solutions at 19 pH intervals between pH 4.93 and 9.01 containing 0.154 M sodium chloride, 5 mM potassium chloride, 30 mM Pi, 30 mM ATP, 30 mM PCr, 30 mM magnesium chloride and 30 mM 3-APP. 31P MR spectra were collected under our standard conditions from the solutions in a glass sphere, similar in shape to the tumours we examined. The chemical shift was referenced to α-ATP.

In vivo experiments
Temperature was monitored throughout the experiments via a rectal probe and maintained by a bath of circulating warm water. During vascular occlusion mouse core temperatures were maintained at preocclusion values (33–35°C) or allowed to cool naturally to room temperature (22–24°C). During the FFA experiments the core temperature was maintained at about 31–33°C.

Statistical analyses
The results from the in vivo experiments were analysed using the Student’s t-test and are reported as mean ± s.e.m.

Freeze clamping
Two hours after vascular occlusion some of the tumours were freeze clamped. Extracts of the tumours were assayed for adenine nucleotides by high-performance liquid chromatography (HPLC), lactate according to Bergmeyer (1974) and P, according to Lowry and Lopez (1946) as modified by Chandra Rajan and Klein (1976).

Results
Occlusion Studies
The results in Figure 1 show the chemical shift dependence on pH for 3-APP. The data were fitted to the Henderson–Hasselbalch equation to obtain an estimated pK values of 6.91 for 3-APP with a limiting acid chemical shift of 34.30 p.p.m. and base chemical shift of 31.11 p.p.m. Using similar experimental conditions, Gillies et al. (1994) reported a slightly higher value (7.1) for the pK of 3-APP. They also noted that standard curves constructed at different temperatures or ionic strengths did not significantly affect the values reported for pK 3-APP.

Preliminary experiments were performed to ascertain that 3-APP was present in the tumours. The CSI experiment (Figure 2), which gives an overall view of where the MR signal is coming from, demonstrates that the majority of the 3-APP signal is from the tumour along with signal from P, and the α, β and γ-phosphates of NTP. The PCR signal comes only from the muscle of the body wall and there appears to be no 3-APP signal coming from this region. The sensitivity of this experiment is insufficient to make any statements about the homogeneity of the 3-APP in the tumour.

The values for pH in CaNT tumours in the absence and presence of 3-APP were 7.1 ± 0.09 (n = 11) and 7.08 ± 0.06 (n = 8) respectively, indicating that the presence of 3-APP did

Figure 1 pH dependence of the chemical shift of 3-APP. pK 3-APP was calculated as 6.91 (see Materials and methods for details). It should be noted that phosphonate resonances shift to lower frequencies at higher pH, the opposite to phosphates, which shift to higher frequencies at higher pH values (Pritchard et al., 1983).
not affect pH\textsubscript{i}. When pH\textsubscript{i} and pH\textsubscript{e} were measured simultaneously in the CaNT tumours, the value for pH\textsubscript{i} was 6.7 ± 0.05 (n = 8) which is about 0.4 pH units more acid than pH\textsubscript{e} and is significantly different from pH\textsubscript{i} (P<0.005). Spectra obtained with an interleaved ISIS acquisition (see Materials and methods for details) from one of the tumours examined are shown in Figure 3a and b. The findings are consistent with the results of Gillies et al. (1994) for RIF-1 tumours.

The plots in Figure 4, before and up to 128 min after occlusion, demonstrated that both pH\textsubscript{i} and pH\textsubscript{e} decreased after occlusion. The values for pH\textsubscript{i} after occlusion decreased on average by 0.51 and 0.68 pH units for the room temperature and 33–35°C experiments respectively. On the other hand, pH\textsubscript{e} decreased by less, 0.35 for the room temperature and 0.41 for the 33–35°C experiments. Values for pH\textsubscript{pot} measurements reported previously (Parkins et al., 1994a) also showed decreases in pH\textsubscript{pot} after occlusion, but in those experiments a relatively larger decrease (0.5 pH units) was observed at 33–35°C when compared with room temperature (0.2 pH units) after 120 min occlusion.

Although the ΔpH (i.e. the difference between pH\textsubscript{i} and pH\textsubscript{e}) appeared smaller in both the tumours maintained at room temperature and tumours maintained at 33–35°C after occlusion, decreasing from 0.31 ± 0.13 to 0.17 ± 0.015 and from 0.53 ± 0.04 to 0.28 ± 0.13 respectively, these differences were not statistically significant.

Concomitant with the decrease in pH\textsubscript{i} and pH\textsubscript{e} after occlusion, there was a decrease in NTP/P\textsubscript{i} ratio with time (Figure 5). Spectra from one of the tumours before and after occlusion, with the temperature maintained at 33–35°C, are shown in Figure 6. There are significant decreases in γ-NTP/P\textsubscript{i} with time up to 48 min post-occlusion (Figure 5) at both temperatures, after which no further changes were observed. The rate at which the NTP/P\textsubscript{i} ratio decreased at the higher temperature was not significantly different from the NTP/P\textsubscript{i} ratio of the tumours maintained at room temperature.

Metabolites measured in extracts of CaNT tumours at the end of the experiment (Table I) showed that there had been a significant breakdown of adenine nucleotides after occlusion (P<0.01) although no significant differences were observed between tumours maintained at room temperature and tumours maintained at 35°C. This is in accordance with the observations made by MRS (Figure 5) that no further decreases in NTP/P\textsubscript{i} were observed after 48 min of occlusion when a plateau appears to have been reached.

**FAA studies**

pH\textsubscript{i} and pH\textsubscript{e} were also followed for 2 h after treatment of the animals with FAA, an agent that reduces blood flow by coagulopathy (Murray et al., 1989). The NTP/P\textsubscript{i} ratios (Figure 7) and pH\textsubscript{i} and pH\textsubscript{e} measurements obtained indicated that there were two different responses to FAA. Nine mice
measurements represent (for Directing before Hill times unchanged the 6.59 non-responders, after 7.08 ± 0.02 were four the upper spectrum. pH₃ was measured from the chemical shift difference between α-NTP and P₁ in the upper spectrum. pH₄ was measured from the chemical shift difference between 3-APP in the lower spectrum and α-NTP in the upper spectrum to correct for the chemical shift artefact (for further details see text).

were treated with FAA, five showed no significant decrease in the NTP/P ratio 2 h after treatment whereas in the other four tumours the NTP/P ratio started to decrease after 68 min and was significantly different from the control values after 100 min of treatment. In the group of four tumours, the pH₃ correspondingly decreased (but not significantly) from 7.08 ± 0.02 to 6.85 ± 0.05 and the pH₄ from 6.66 ± 0.08 to 6.23 ± 0.16 by the end of the experiment. In contrast, in the non-responders, pH₃ (7.24 ± 0.1 at the beginning of the experiment and 7.16 ± 0.05 at the end) was virtually unchanged as was pH₄ (6.75 ± 0.05 at the beginning and 6.59 ± 0.05 at the end).

The decreases in the responders were not significant but in the same direction and of a similar magnitude to those found for total vascular occlusion (see Figure 4). This is probably because FAA takes a long time to exert its effect (relative tumour perfusion ~21% of control after 200 mg kg⁻¹; see Hill et al., 1989). However it is not possible to look at later times in these experiments because the 3-APP has to be given before the FAA, when the vasculature is still patent. The 3-APP gradually disappears from the tumour with time leaving no marker of extracellular space at the later times and the signal/noise deteriorates.

Discussion

Direct non-invasive measurements of pH in tumours have changed the view that existed for many years after Warburg’s experiments of the 1920s, namely that tumours were ‘acidic’ (for review see Griffiths, 1991). Now it is possible to make direct, non-invasive (except for injecting the marker, 3-APP) measurements of pH. However these measurements do represent volume-average pH values and this may not be ideal in tumours that are known to be histologically heterogeneous. However, some strength lies in using volume averages, especially since they are directly comparable with subsequent metabolic assays done on freeze-clamped tumours which also represent volume averages. One drawback of the volume-average method is that pH values obtained by 31P MRS are likely to be different from pH values obtained by
Table 1  Effect of vascular occlusion for 2 h on adenine nucleotides [ATP]/[ADP][Pi] and lactate in extracts of CaNT tumours

| Treatment          | Total adenine nucleotides (μmol g⁻¹) | [ATP]/[ADP][Pi] M⁻¹ | Lactate (μmol g⁻¹) |
|--------------------|-------------------------------------|---------------------|-------------------|
| Control (n = 3)    | 2.16 ± 0.14                         | 1107 ± 268          | 7.8 ± 0.6         |
| Occlusion (n = 5)  | 1.2 ± 0.08*                         | 51 ± 8.2*           | 21 ± 2.3*         |
| at 22-24°C         |                                     |                     |                   |
| Occlusion (n = 7)  | 0.99 ± 0.17*                        | 80 ± 31*            | 19 ± 2.2*         |
| at 33–35°C         |                                     |                     |                   |

*Significantly different from control value (P<0.05).

Figure 6 Representative 31P spectra showing preoclusion (a) and the decrease in γ-NTP/P, with time 16–32 min (b) and 32–48 min (c) post occlusion. Mouse core temperature was maintained at (33–35°C).

Figure 7 Time course of γ-NTP/P ratios measured after treatment with 200 mg kg⁻¹ of FAA. (O) are the responders (n = 4) and (□) are non-responders (n = 5). The 0 min value was obtained from control animals with no treatment (n = 3). Symbols represent mean ± s.e.m. *Indicates significant difference (P<0.05) from values observed at 36 min, the first post-FAA treatment value obtained.

Microelectrodes since microelectrode measurements are invasive and can only sample a few microenvironments within a tumour. The values found in untreated tumours for pH by 31P MRS were somewhat lower than those found by microelectrode studies in the same tumour type (although performed on different batches at different times (see Parkins et al., 1994a), and this may reflect differences between the techniques. The wide range of pH (5.8–7.52) found in tumours by microelectrode measurements (see Wike-Hooley et al., 1984) suggests that they are sampling a mixture of compartments (see also Vaapel et al., 1989) whereas the range of pHiso is very narrow (6.5–6.8) in untreated tumours (see Gilles et al., 1994 and this paper).

In spite of these discrepancies both microelectrodes and 3-APP methods confirm that the extracellular compartment is more acid than the intracellular compartment (see also Vaapel et al., 1989; Griffiths, 1991; Stubbs et al., 1994). Further studies, in which pH will be measured on the same tumour by both 31P MRS-3-APP and microelectrodes are planned to begin shortly and we hope these studies will resolve the differences between the two methods.

In this study, ISIS localisation of the tumours was chosen to ensure that the intense PCr signal from the body wall did not contribute to the tumour spectra (Howe et al., 1992). However, because the chemical shift difference between 3-APP and α-NTP is > 30 p.p.m. there would be a significant chemical shift artefact (Howe et al., 1992). The consequence of this would be that the volumes from which the 3-APP and the α-NTP signals were obtained would be displaced by 2 mm (in three dimensions). In a 0.8 cm³ volume of interest (chosen for most of our experiments), the volumes from which the signals arose would have overlapped by only 43%. To ensure that the chemical shift artefact was minimised and that α-NTP and 3-APP signals came from an identical volume, the double interleaved ISIS acquisition (Maxwell et al., 1994) was used for all ISIS experiments. It should be noted that the chemical shift artefact is negligible when calculating pH, because the chemical shift difference between P and α-NTP is < 10 p.p.m.

In theory, absolute concentrations can be obtained from 31P MRS since the area under the signals in the MR spectrum are directly proportional to the concentration of the
metabolites. However, since ISIS localisation was used, the results presented here have been expressed as peak ratios (i.e. NTP/P) since absolute sensitivity depends on coil loading and the relative position of the selected voxel to the coil. It should be noted that this ratio (NTP/P) only approximates the energy status of the tumour in real time. In order to obtain information about the metabolic status of the tumour that might indicate thermodynamic information [ADP] would have to be ascertained. While [ADP] cannot be determined directly from in vivo 31P MR spectra, total [ADP] (free + bound) can be ascertained by freeze clamping and making extracts of the tumour with subsequent assays of ATP, ADP and P. Although this does not give true thermodynamic information, it does give the direction, and some indication of the magnitude of the changes observed.

At equilibrium it is clear that the MR spectrum includes all the nucleotide phosphates under the peaks which accounts for the conventional label of NTP, although the major proportion of this peak is ATP (Stubbs et al., 1989). However, the extracts provide data on ATP since HPLC separates ATP from other trinucleotides. The maintenance of adenine nucleotides in tumours compared with control tissue (e.g. liver or kidney) over relatively long periods of ischaemia is surprising and may indicate a new role for adenosine triphosphate (ATP) which in tumours is catabolised to adenosine monophosphate (ADP) and inorganic phosphate (Pi).

The proton MR spectrum of a cell is determined by its pH value, and for a pH of 7.0 the peaks due to ATP at 3.57, 3.90 and 4.10 ppm, to ADP at 3.70 and 3.77 ppm, and to Pi at 5.30 ppm are all relatively strong. Reliable quantitation of the pH value is possible by comparing the peak area of the Pi peak with the area of one of the other phosphorus metabolite peaks. Once the pH of the cell is determined, it is possible to calculate the pH of the interstitial fluid with reasonable accuracy. In most tumours this is about 7.3. The pH range found in tumours is wider than that encountered in normal tissues. This property could be exploited to detect and quantify tumours since the MR spectra of tumours and the pH of the interstitial fluid in normal tissues are unlikely to overlap.

It is also possible to estimate the pH of the intracellular space, which is around 6.8. It may be possible to gain an indication of the pH of the extracellular space, which is about 7.5, by comparing the areas of the Pi peaks from the intracellular and extracellular spaces.

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