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An NMR compatible model for solid tumour*

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Abstract: An NMR model for the study of drug interactions with solid tumour is described. It is based on the use of packed cells in a simple medium. The system is capable of dealing with cellular biochemistry at a molecular level through experiments conducted in a homogeneous field by ^{1}H spin-echo NMR spectroscopy. However, through a simple imaging technique, it is also possible to introduce a spatial element into the chemistry of the packed cells. The use of these complementary NMR methods are illustrated by the action of the cytotoxic agent doxorubicin on cellular glycolysis. A pattern of signals observed in a homogeneous field experiment following oxidative stress are duplicated in the imaging experiment to illustrate the toxins' ability to penetrate the model tumour.

Keywords: *NMR spectroscopy; glycolysis; doxorubicin; drug diffusion; cultured cancer cells; tumour model.*

Introduction

^{31}P NMR spectroscopy has been used as a non-invasive probe of energy metabolism in cultured cancer cells [1, 2]. Studies using ^{1}H NMR, although less common, have been used to investigate phosphorylcholine increases during differentiation [3], membrane lipids [4–6], glycolysis [7] and drug stress [8, 9]. The experimental conditions in the NMR instrument required to sustain these cell cultures during prolonged metabolic studies create a situation, which comprises of low pO_2, high cell densities and restricted nutrition. These conditions are similar to those found in regions of bulk tumour tissue served by a limited vascular system. This has led us to postulate that experiments using packed cells may give rise to a plausible solid tumour model [8]. However, these experiments are still unable to address the spatial nature of solid tumour and the effects this has on its resulting biochemistry. We have attempted here, using a simple imaging technique on a sample of packed cells, to create a simple, cellular based one dimensional tumour model.

Experimental

IXRPMI 1640 medium (Dutch modification with 20 mM Hepes buffer, 1 g l^{-1} sodium bicarbonate and 6.4 g l^{-1} sodium chloride) and trypsin (2.5% Hanks balanced salt solution) were obtained from Flow Laboratories (UK) Ltd. Doxorubicin was a generous gift from Farmitalia plc.

A sensitive strain of small cell carcinoma of the lung was grown in enriched RPMI 1640-Hepes (500 ml), (1%, w/v) glutamine (5 ml), 7.5% sodium bicarbonate (2.3 ml) and 10% FBS (50 ml). Cells were grown as a suspension in F-120 flasks at 37°C under 5% CO_2 for 5–6 days before splitting. They were harvested by centrifugation at 150*g* for 10 min after removal from the glass by the addition of trypsin solution (10 ml of 2.5% trypsin solution to 90 ml of buffer solution consisting of 6 g NaCl, 6.6 g trisodium citrate and 0.01 g Phenol Red in 1 l of re-distilled water). Cell viability was checked by harvesting the cells as above and resuspending in sterile physiological saline at 0.5×10^6 cells in 3 ml. To 2 µl of this suspension was added 0.2 ml of Trypan Blue stain and the percentage of live cells counted using a haemocytometer. The cells were found to remain viable in physiological saline for at least 5 h (>90% viability) at room temperature.

High resolution NMR spectroscopy measurements

The standard sample size was approx. 10^8

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cells in a total volume of 0.5 ml. A standard narrow-bore Bruker WM 250 spectrometer (^{1}H operating frequency 250 MHz) was used to record all spectra. The spin-echo spectra were obtained using the Hahn sequence (90°-*t*-180°-*t*) with a delay time (*t*) of 60 ms. A presaturation pulse was applied at the water resonance frequency prior to accumulation to suppress the water signal. Data for the spin-echo spectra are as follows: acquisition time, 0.684 s; spectral width 3000 Hz. The 90° pulse was generated with a 4.5 μs pulse width. The free induction decay was collected in 4 K of data points zero filled to 16 K. A 1.0 Hz line broadening function was applied during Fourier transformation. The spin-echo NMR method used in this study is well documented for the study of erythrocyte biochemistry [10, 11] and to a lesser extent for cultured cancer cells [3, 7–9].

Imaging experiments

The imaging experiments were conducted on cell suspensions to which glucose (20 mg) was added then packed. The excess surface solution was drawn off with a long pasteur pipette and replaced by a concentrated solution of doxorubicin (200 μg/100 μl) applied carefully to the surface of the culture. The column of cells used was sufficiently long ($\sim 10^9$ cells) for the top of the culture to be outside the coil area. Thus, when the NMR tube was placed in the spinner the doxorubicin solution and the layer of cells exposed to it were NMR silent. Only after some time (hours) will the toxin diffuse sufficiently to reach the cells.

The imaging method employed is based on the classic studies of Lauterbaur *et al.* [12, 13] and Mansfield *et al.* [14, 15], where a rudimentary image can be produced by introducing a static field gradient along one of the main axes. However, in this study, rather than produce an image, we require only to localize the signal, arising from the lactate, on the *z*-axis of the NMR tube, thus allowing a perception of the kinetics of penetration of compounds into the matrix. Thus the NMR tube is allowed to spin during these experiments to average out the signal in the *x*–*y* plane.

The system was pre-calibrated using an NMR tube which had been compartmentalized by Teflon plugs to provide three spatially distinct magnetic environments. An offset in the *z*-axis was chosen which provided a fixed spatial calibration of 100 Hz per 30 mm. However, with the broadening effects encountered with cellular suspensions due to field inhomogeneities in the spin-echo mode, this value can only be regarded as a rough guide. A constant spatial aspect is created.

Spectra were recorded using the Hahn spin-echo pulse sequence as described above. The acquisition time was 0.171 s, with the data being collected in 1 K of Data points zero filled to 16 K. The broad, almost square wave, water resonance was irradiated using a presaturation pulse sequence prior to accumulation. As a single irradiation frequency was incapable of suppressing the total signal from the broad water frequencies, a sequence of values comprised of short dwell times and distributed across the water resonance was employed. Although some distortion was introduced into the NMR spectrum across the water frequencies, this simple approach effectively removed the water signal without affecting the lactate region (0–2 ppm).

Results and Discussion

The ^{1}H NMR spectra of cultured cancer cells, generated using the Hahn spin-echo pulse sequence are relatively easy to interpret [3–9]. Central to this study is the presence of a lactate methyl-resonance superimposed on the broad low field triglyceride signal, which is easily removed from the cell during the washing cycle of the sample preparation, but quickly returns on the onset of glycolysis (Fig. 1).

Recent studies have shown that various cultured cancer cell lines can remain viable for some hours under the very simple conditions which prevail in these NMR experiments [7–9]. On treating these cultures with the cytotoxic agent doxorubicin, a marked increase in the lactate signal as compared to a simple control is observed, indicating increased anaerobic glycolysis [8, 9]. The imaging experiments employed here have a large signal dispersion and cannot discriminate between the narrow lactate lines and the broad triglyceride signals, and it was necessary to repeat the above experiments for the lung cell cultures to establish that any changes observed in the imaging experiment were correctly interpreted. On the addition of doxorubicin to the lung cancer cell line, elevated glycolytic behaviour is observed (Fig. 2).

The results of the imaging experiments are

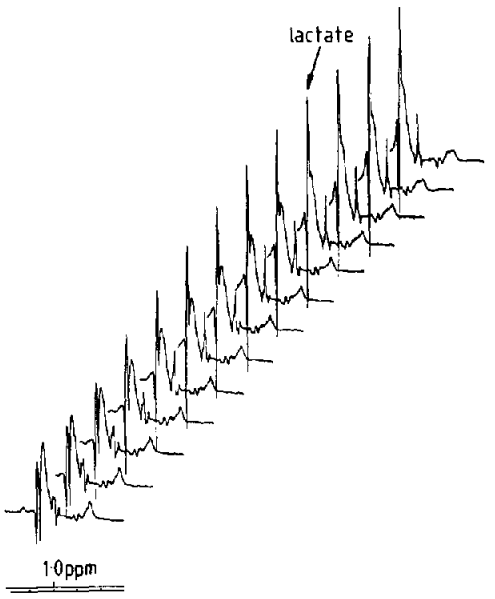

Figure 1
The time course for glycolysis in the culture derived from small cell carcinoma of the lung treated with glucose 1.0 mg shown as a series of spin-echo spectra from the high field region (0–2 ppm), with spectra recorded every 20 min. Lactate is identified as the prominent narrow line resonance with a marked positive and negative phasing on the broad low field triglyceride resonance, this latter signal remaining unchanged throughout.

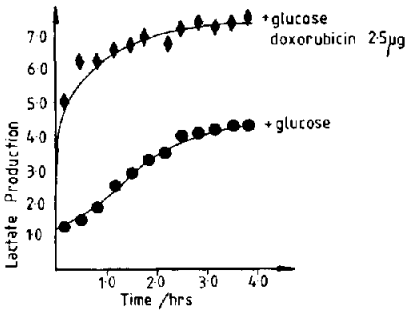

Figure 2
Plot of glycolytic behaviour (lactate production) in the lung cancer cultures on stressing with doxorubicin ◆ (2.5 μg/10^8 cells) as compared with the simple control with doxorubicin absent ●. The peak height (Fig. 1) is used to measure lactate production.

shown in Fig. 3. The control shows little change over the 12 h duration of the experiment, whereas in the cultures to which doxorubicin had been applied there is a peak in the high field region of the spectra which increases in size and shifts to lower field. Plotting these data provides a clear insight into the progress of stress in the tumour model. Initially there is no observed change as the drug–cell interface is outside the coil area. As the drug penetrates

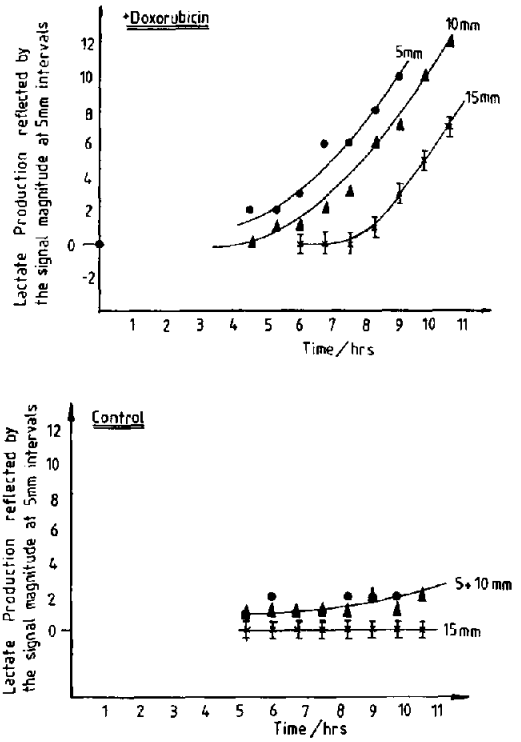

Figure 3
Plot of glycolytic behaviour as a function of distance down the z-axis of the NMR tube in the model tumour caused by the diffusion of the concentrated doxorubicin solution (above), as compared with the simple control with doxorubicin absent (below). (●, 5 mm; ▲, 10 mm; ⧗, 15 mm from the top of the NMR tube.)

the culture, stress is observed. Measuring this at three different locations (5, 10 and 15 mm) down the tube provides a set of spatially distinct stress curves. The rate of lactate production measured at the three points in the tube also show this progressive behaviour, the values having a sequential nature. These results are in marked contrast to the passive nature of the control in which there is little change.

Thus the two types of experiment provide complementary information about the cellular response to chemotherapeutic agents. The homogeneous field experiment is a plausible model for tumour tissue especially for those cells which comprise the surface layers. It is these cells which form the primary barrier to drug therapy and an understanding of their chemical and physical behaviour is required. The response illustrated is that of glycolytic stress. However, the approach also allows the assessment of cellular defense by other mechanisms such as glutathione metabolism [9] and changes in the membrane dynamics [16].

The imaging experiments contain the special spatial element which is required of any system which seeks to model solid tumour. We have not tried to influence the hypoxic nature of the cells, but this can be achieved relatively easily through control of the gaseous environment. The hypoxic nature of the cultures could be assessed in tandem with the imaging and spectroscopic investigations through the pH shifts which can be detected through the intracellular phosphate in ^{31}P NMR experiments such as those described for ischaemia and hypoxia by others [17].

The model does not seek to treat vascularized tissue, but this can also be approached by using semi-permeable canulae, with imaging in the x–y plane rather than the z-axis to study radial diffusion. We have chosen to employ spin-echo methods for the study of cell cultures in both the imaging and spectroscopy experiments. These are standard methods for the study of biological process in *in vivo* spectroscopy. It is hoped that these studies will provide a bridging experiment for cancer chemotherapeutics which link experiments in flasks with whole animal studies by providing a tumour model which can be based on a variety of cultures of human cancer cells from primary explants.

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