Table 4. Percentage of changed minimum cidal concentration of the essential oil of *Eucalyptus* spp. at adjusted pH levels

	Essential oils of *Eucalyptus* spp. tested at various pH levels								
	E. citriodora			*E. dalrympleana*			*E. laveopinea*		
Fungi[a]	4	7	9	4	7	9	4	7	9
Ef	–	–	–	–	–	–	–	–	–
Mg	–	–	–	–	–	–	–	–	–
Mn	11.1	–	33.3	20.2	–	0.0	–		
Tm	62.5	–	75.0	25.0	–	50.0	75.0	25.0	62.5
Tr	40.0	30.0	60.0	25.0	12.5	50.0	20.0	40.0	40.0
Tv	–	–	–	50.0	–	50.0	75.0	–	62.5

[a]*Ef, Epidermophyton floccosum*; *Mg, Microsporum gypseum*; *Mn, Microsporum nanum*; *Tm, Trichophyton mentagrophyteis*; *Tr, Trichophyton rubrum*; *Tv, Trichophyton violaceum*.
–, Remained static.

mammalian skin at their normal and alkaline pH. Since the oils showed increased antifungal activity at pH 9 during *in vitro* investigation, they were tested for their irritant activity at pH 9. The oils did not produce any irritation or adverse effect up to 5% concentrations. It can be concluded that ointments prepared from the oils may be most effective at pH 9 without causing any irritation on the human skin. Therefore, the present study clearly demonstrates that the oils of *Eucalyptus* spp. hold good promise as antidermatophytic agents which could be used in therapeutic remedy against dermatophytoses on account of their various fungitoxic properties, viz. antifungal, long shelf life, can withstand heavy inoculum density, efficacy at various pH levels, wide range of activity and absence of any adverse effect. These results can be interpreted with caution. Although the *in vitro* susceptibility testing shows promising activity against commonly encountered dermatophytes, its clinical usefulness should be established by further studies. Hence, the oils can be easily used as ointments for better results to the control of fungal infection in human beings.

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Two-dimensional NMR spectroscopic study of fibroblast and fibrosarcoma cell lines

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Two-dimensional NMR spectroscopy has been used to study a fibroblast (MRC-5) and human fibrosarcoma cell lines (HFS-9 and HT-1080). The cell lines were graded based on their tumorigenic characteristics in nude mice, and the synthetic phase fraction of their cell cycle. Analysis of the spectra from cells suggests an increase in the levels of lipids, metabolites and resonance patterns attributed to fucosylated antigens as a function of increasing tumorigenicity. The paper discusses the use of one- and two-dimensional ^{1}H NMR techniques in the gradation of fibrosarcoma cells and in differentiating them from the normal homologue, namely fibroblast cells.

SOFT tissue tumours/sarcomas are a heterogenous group of tumours that arise as soft tissue masses and usually exhibit the differentiated features of adult soft tissue, although in some cases there is no clearly defined normal tissue homologue. Soft tissue includes smooth and striated muscle, fat, fibrous tissue and the vessels that serve these tissues. These tumours can occur anywhere in the body and at any age although the distribution varies according to the histological type. Benign tumours are at least 100

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times more common than their malignant counterparts and represent the most common group of human neoplasms[1]. Among the cases diagnosed annually at the Cancer Institute, leiomyosarcomas, fibrosarcomas and malignant fibrous histiocytomas are the most common, while in children rhabdomyosarcomas are the most commonly detected soft tissue tumours. The overall survival of the treated non-metastatic patients is approximately 50%. The benefits that would be achieved if there were early means of detection are many. Mutilating surgery and mortality could be avoided. A non-invasive method for detection would be of even greater advantage.

This study aims to use NMR spectroscopy to investigate soft tissue tumours. As with any other study, the usefulness of the technique has been first demonstrated in cells and then in tissues. Soft tissue tumours such as fibrosarcomas arise from fibroblastic cells and their benign counterpart is the fibroma. This paper discusses the use of one- and two-dimensional purged correlated spectroscopy on human fibroblasts and fibrosarcoma cells.

The human fibrosarcoma cell lines HFS-9 and HT-1080 were obtained from National Facility for Animal Tissue and Cell Culture (NFATCC), Pune. The three cell lines were cultured under identical conditions in DMEM supplemented with 10% fetal bovine serum (Sigma, USA) and antibiotics: penicillin 10,000 units/ml, and streptomycin 10 mg/ml grown in a 10% CO_2 environment. Flow cytometry was done to characterize the grade of the two malignant cell lines based on their DNA content in the 'S' phase of the cell cycle (S phase fraction or SPF)[2], using the DNA cycle test plus kit from Becton Dickinson. The analysis was done on a Becton Dickinson FACScan flow cytometer. About $4–5 \times 10^6$ cells were injected subcutaneously in nude mice and the mice were kept under observation for growth of tumours for two months (6 mice per cell line) to test for tumorigenicity of the cell line. The cell cycle fractions and tumorigenic characteristics of the tumour cell lines used in this study are listed in Table 1. HT-1080, with a higher SPF and increased tumorigenicity (producing tumours within 8 days) has been classified as high grade relative to HFS-9. The cell lines were also analysed for their whole cell protein, lipid, triglyceride, phospholipid, cholesterol and cholesteryl ester content by standard biochemical procedures.

All the nuclear magnetic resonance spectroscopy experiments on cells were carried out on a JEOL-GSX-FT 400 MHz NMR spectrometer. A standard 5 mm probe head was used with the sample spinning at 15 Hz. For each experiment 10^8 cells were counted on a Neubauer slide before centrifuging and suspending in 0.4 ml PBS/D_2O. The residual water signal was suppressed using Delays Alternating with Nutations for Tailored Excitation (DANTE) sequence. The spectra were acquired over a spectral width of 8000 Hz using 32 K data points with a pulse delay of 3.2 s. A 11.5 µs excitation pulse was applied and 256 transients were acquired. An exponential window function with a line broadening of 3 Hz was applied to the data prior to Fourier transformation. Tri silyl propione sulfonate (TSPS) was used as the reference.

The purged correlation spectroscopy technique was used on cells, one thousand data points were collected in the t_2 dimension and 256 data points in t_1 dimension over a spectral width of 4000 Hz. Total experimental time for a PCOSY experiment consisting of 256 t_1 increments with 32 transients each was found to be less than 4 h. PCOSY matrices were zero filled to 512 points in t_1. The sinebell window function was applied in the t_1 and t_2 domains and

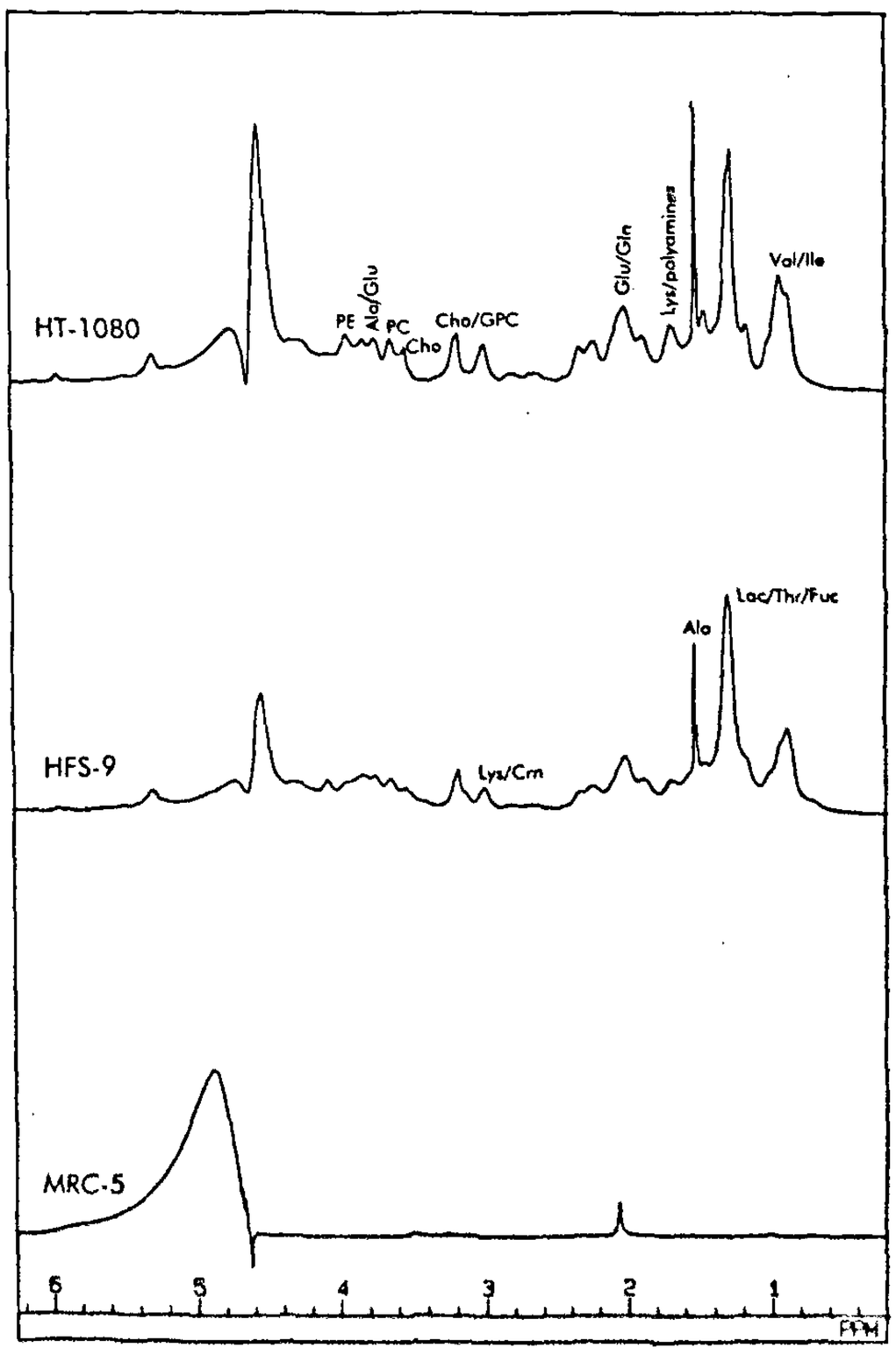

Figure 1. One-dimensional proton NMR spectra of 10^8 cells in 400 µl PBS in D_2O at 37°C. 256 transients were accumulated in the FID. An exponential window function with a line broadening of 3 Hz was applied prior to Fourier transformation. The spectra are plotted on a constant vertical scale (absolute intensity mode).

Table 1. Cell cycle phase and tumorigenic characteristics

Cell line	%G_1	%S	%G_2 + M	G_1CV	Tumorigenicity
HFS-9	69.22	16.69	14.09	4.7	Moderate (20 days, 5/6)
HT-1080	65.3	25.3	9.4	12.3	Strong (8 days, 6/6)

G_1, G_1 phase of cell cycle; S, Synthetic phase of cell cycle; M, Mitotic phase of cell cycle; CV, coefficient of variation.

Fourier transformed. The one-dimensional spectra were collected before and after the 2D spectra to check for sample stability. The spectra shown are representative of the three cell lines. One-dimensional and two-dimensional experiments were carried out on each cell three times and results were drawn only after the three spectra were found to be identical.

The proton 1D NMR spectra of the three cell lines are presented in Figure 1. The spectra of the fibroblast and fibrosarcoma cell lines are dominated by signals from fatty acid chains of lipid with resonances from methyl groups ($-CH_3-$) appearing at 0.9 ppm, methylene ($-CH_2-$) at 1.3, 1.6, 2.0 and 2.3 ppm. The olefinic group (–CH=) resonances are at 5.3 ppm. The resonance at 3.2 ppm is from the $N(CH_3)_3$ group of choline, phosphocholine and glycerophosphocholine[3]. Contributions from the resonances of carbohydrates, amino acids and phospholipid precursors are in the region 3.5–4.5 ppm of the spectrum. Qualitative differences are seen in the 1D spectra of the three cell lines, with a noticeable increase in the intensity of resonances in the spectra from bottom to top, i.e. from the fibroblast cell line to the high grade HT-1080. The resonances near 0.9 ppm and 1.3 ppm increase in intensity. The resonances at 1.5, 2.05, 2.2 and 2.8 ppm are seen to increase in the ascending order of the series. The abbreviation Fuc stands for contributions from fucose and Crn from creatine.

The PCOSY spectra of the three cell lines are shown in Figure 2 *a–c*. Chemical shift assignments of the cross peaks from triglyceride were made based on the assignment of plasma membrane triglyceride and triolein in $CDCl_3$ by Holmes and Mountford[4]. Those of phospholipid metabolites choline, phosphocholine (PC), phosphoethanolamine (PE) and glycerophosphocholine (GPC),

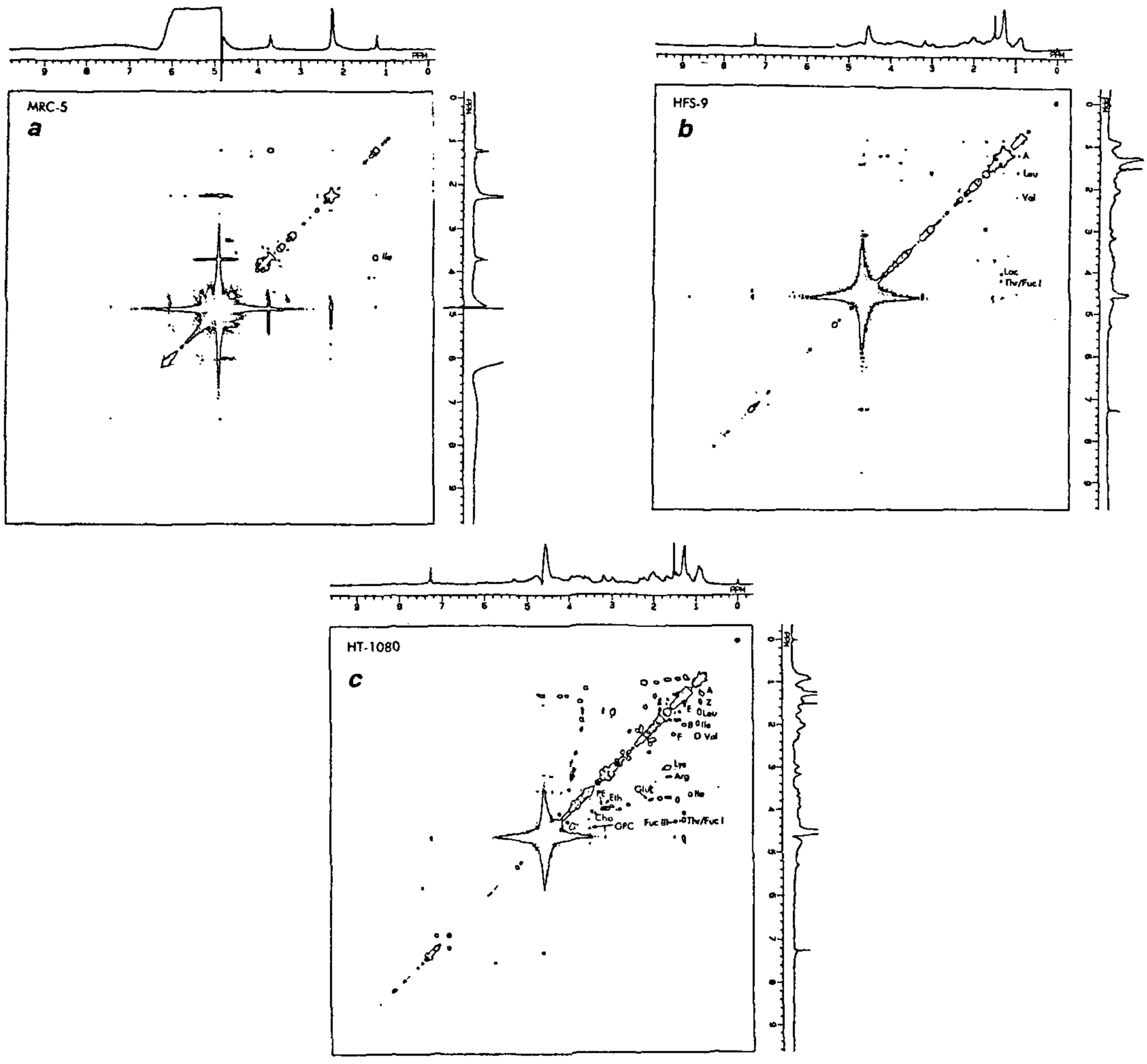

Figure 2. 400 MHz ^1H PCOSY spectra of 10^6 cells in 400 µl PBS in D_2O at 37°C. *a*, MRC-5; *b*, HFS-9; *c*, HT-1080. A sine bell window function was applied in the t_1 and t_2 domains prior to Fourier transformation. The contour plots use the same absolute intensity scaling and the same contour levels.

Table 2. Whole cell chemical composition

Cell line	MRC-5	HFS-9	HT-1080
Total protein (mg/10^8 cells)	9.86 ± 0.2	11.1 ± 1.1	11.56 ± 1
Total lipid (mg/10^8 cells)	5.0 ± 1	6.1 ± 0.1	6.9 ± 1.2
Phospholipid (nmol/mg lipid)	210 ± 29	864 ± 27	1219 ± 41
Free cholesterol (nmol/mg lipid)	205 ± 24	611 ± 30	714 ± 15
Cholesteryl ester (nmol/mg lipid)	ND	31 ± 3	54.5 ± 7
Triacylglycerol (nmol/mg lipid)	111 ± 7	230 ± 20	299 ± 9

free amino acids and peptides were based on the assignments of Sze and Jardetzky[5] and fucose (Fuc) from our spectrum of fucose and the assignments of Lean *et al.*[6].

An increase in the number and size of off-diagonal cross peaks is observed in the PCOSY spectra of the cell lines with increasing tumorigenicity. Some of the cross peaks from triglyceride namely B, E and F are identifiable only in HT-1080, so also those of phospholipids such as, phosphoethanolamine (PE), ethanolamine (Eth), choline (Cho) and glycerophosphocholine (GPC). The spectrum from MRC-5 shows only a single cross peak from amino acid isoleucine. These results are of significance because they reflect the growth rate of the cells. Malignant cells are associated with increased glycolytic rate, hence lactate is seen only in the PCOSY spectra of the tumour cell lines. Even the most slowly growing tumours possess a glycolytic capacity far in excess of the cells from which they have arisen. A high capacity of glycolysis is apparently necessary to maintain high concentrations of metabolic intermediates that can be used as precursors for macromolecular synthesis. Besides increased lactate, increased lipids such as triglyceride and phospholipids have also been reported[7]. The chemical composition of the three cell lines is presented in Table 2, which also shows an increase in the levels of these lipids with increasing grade. There is a need for enhanced phospholipid metabolism to support cell division and signal transduction events associated with phospholipid hydrolysis and changes within the plasma membrane responsible for invasion, metastasis and expression of growth factor receptors. A large increase in phosphocholine has been shown to be one of the earliest responses of tumour cells to growth factors[7]. Phosphoethanolamine may play an important role in the modification of membrane shape in malignant cells. Substantial alterations to the plasma membrane also occur, with drastic changes in the quantity and type of surface glycolipids and glycoproteins[8]. In colorectal cell lines, levels of fucosylated antigens have been found to correlate with grade, and the αL (Fuc1 → 2) βDGal linkages common to these antigens were identified as a likely source for the H5–H6 couplings of fucose seen in the 2D spectra of colorectal cells[6]. In the fibrosarcoma cells too, HFS-9 has a single cross peak at 1.33, 4.27, while HT-1080 has two cross peaks in this region corresponding to FucI and FucIII (1.33, 4.27 and 1.41, 4.3 ppm, nomenclature of Lean *et al.*[6]). A similar finding in mouse fibrosarcoma cell lines has been reported earlier by us[9]. Literature reports on the presence of saccharide antigens in sarcomas are restricted to studies on experimental animal tumours. There are no reports on the accumulation of fucosylated glycolipids. It would be of interest to see if tumour tissue also presents a similar two-dimensional spectrum, and then whether these cells and tissues are over-reactive for antibodies to these fucolipids/fucogangliosides (such as the blood group antigens Le^a, Le^b and Le^y).

Thus, one- and two-dimensional PCOSY techniques have been able to offer a gradation between fibroblast cells, intermediate grade fibrosarcoma and high grade fibrosarcoma cell lines. The gradation has been based on the identification of phospholipid, triglyceride cross peaks to a lesser extent, and cross peaks from membrane fucosylated antigens, which relate to grade and tumorigenicity of the cell lines. Similar studies on colorectal and breast cancer cells are available in the literature[3,6,10]. This is the first such study on fibrosarcoma cell lines. The clinical implication of this study is that ^{1}H NMR techniques may be used as an adjunct to conventional histology in the identification of the type of lesion (normal vs tumour, different grades of tumour) non invasively in soft tissue sarcomas like fibrosarcoma.

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