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In Vivo ^{31}P Nuclear Magnetic Resonance Spectroscopy of Rat 9L Gliosarcoma Treated with BCNU: Dose Response of Spectral Changes

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The 9L gliosarcoma, grown subcutaneously in juvenile Fischer 344 rats, was studied by *in vivo* ^{31}P NMR spectroscopy following treatment with 1,3-bis(2-chloroethyl)-1-nitrosourea. Dose-dependent increases in the proportion of high-energy phosphates were observed for doses between 10 and 36 mg/kg (from 80% of the LD_{10} to greater than the LD_{50}). These doses reduced clonogenic cell survival in a dose-dependent fashion by as much as 3 log orders and resulted in up to 16 days of growth delay (to pretreatment tumor volume). Increases in high-energy phosphates (relative to P_i) in the tumor were greater at higher doses despite the higher levels of clonogenic cell killing and the substantial host systemic toxicity.

INTRODUCTION

The potential utility of *in vivo* NMR spectroscopy as a noninvasive method for detecting and quantifying tumor response to therapy is being examined in both the laboratory (*1–6*) and the clinic (*4–7*). Validation of this method requires correlation of spectral changes induced by the therapeutic agent with accepted measures of therapeutic response. These issues are best addressed in experimental tumor models.

We have recently demonstrated that treatment of the subcutaneously (sc) implanted rat 9L gliosarcoma with the antineoplastic agent 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) results in a reversal of the spectral changes accompanying untreated growth of this tumor (*8*). During untreated growth this gliosarcoma exhibits a progressive decrease in bioenergetic status, which is reflected in a relative decrease in levels of the high-energy phosphates— nucleoside triphosphates (NTP) and phosphocreatine (PCr)—and an increase in P_i. Chemotherapy with BCNU results in a dramatic increase in the levels of NTP and PCr (relative to P_i) and a significant decrease in the ratio of phosphate monoesters (PME) to NTP. This earlier study was conducted at a single dose, 10 mg/kg (about 80% of the LD_{10}), and therapeutic response was indicated only by a delay in tumor growth. Here we have extended our

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initial investigations to higher doses and monitored therapeutic response by tumor volume measurements and by clonogenic assay, which measures the survival of those tumor cells with long-term ability to replicate (*9*). The dose–response extends over 3 to 4 log orders of cell kill and permits an evaluation of the relationship between therapeutic response and drug-induced spectral changes.

MATERIALS AND METHODS

Tumor implantation. To establish sc 9L tumors, 10-day-old Fischer 344 rats were inoculated in the right flank with 10^6 9L cells in 0.2 ml of growth medium. Young rats were used because of size restrictions imposed by the bore of the magnet.

Chemotherapy. BCNU was provided by the Drug Synthesis and Chemistry Branch of the Division of Cancer Treatment (National Cancer Institute). Twelve to thirteen days after tumor implantation, rats were treated with one of three doses of BCNU (10, 25, or 36 mg/kg) in 4% ethanol in normal saline or were sham-treated with the carrier solution. Injections were intraperitoneal (ip), and NMR spectra were collected 1 and 4 days following treatment.

In vivo NMR spectroscopy of tumors. Rats were anesthetized by ip injection of a mixture of ketamine and xylazine (ketamine HCl, 50 mg/kg; xylazine, 5 mg/kg; in 14% ethanol in normal saline) and examined by NMR 20 min after the onset of anesthesia.

Phosphorus-31 NMR spectra were obtained with a Bruker AM 360-WB multinuclear spectrometer (8.5 T/8.9-cm bore) interfaced to an Aspect 3000 computer. Homebuilt probes were equipped with three-turn solenoidal coils doubly tuned to ^{31}P and ^{1}H (*10*). Faraday shields were employed to eliminate signals from the animal body wall (*11*). The ^{31}P NMR spectral parameters were resonance frequency, 145.8 MHz; 7 μs pulse width (60° magnetization flip); 12 kHz bandwidth; 1K data points; 3 s recycle time; 10 min acquisition time; and number of scans required to produce a signal to noise ratio greater than 20:1 for the largest resonance. No corrections were applied for saturation effects, but these effects are negligible for NTP and small for PME, P_i, and PCr (*12*). Preliminary experiments indicated that the extent of partial saturation was not significantly changed by treatment with BCNU. Resolution was enhanced by the convolution-difference method (*13*) using 20 and 1000 Hz line broadening. Resonances were resolved and integrated with a Lorentzian line-fitting program (GLINFIT; Bruker Instruments, Inc.). Mean resonance area ratios were calculated for each dose level of BCNU and for the untreated control. The dose dependences of resonance area ratios were analyzed by linear regression.

Tumor volume measurement. Tumor volume was estimated by measuring tumor dimensions along the long axis of the tumor and across the tumor at the widest point. Tumor volume was calculated by the equation, Volume = 0.5 (length $\times$ width^2).

Clonogenic assays. In vitro clonogenic cell survival assays of tumors treated *in vivo* were adapted from Rosenblum *et al.* (*14*). One day following BCNU treatment tumors were minced, digested with a mixture of collagenase, pronase, and DNase (*15*), washed, and counted. Cells were plated at appropriate densities in Eagle's minimum essential medium with 15% fetal bovine serum. Plates were incubated for 2 weeks at 37°C, after which they were stained with crystal violet. Colonies of more than 50 cells were counted.

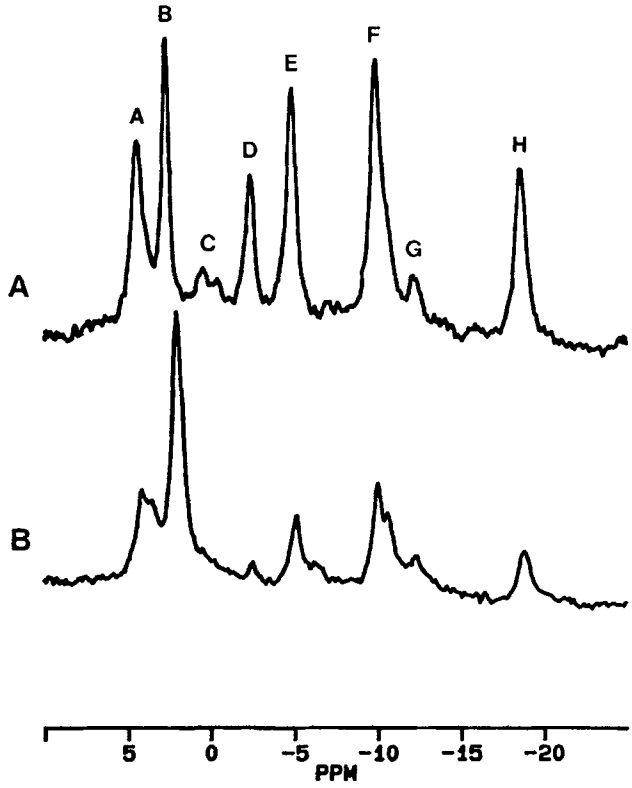

FIG. 1. *In vivo* spectra of 4–5 cm^3 sc implanted 9L tumors at Day 13 postimplantation. Spectrum A was obtained 1 day following treatment of the host with BCNU (10 mg/kg), while spectrum B is from an age- and size-matched sham-treated control. The assignments are: Peak A, phosphate monoesters (PME) consisting principally of phosphoethanolamine and phosphocholine; Peak B, P_i; Peak C, phosphate diesters (PDE) consisting principally of glycerophosphorylethanolamine and glycerophosphorylcholine; Peak D, PCr; Peak E, γ-NTP and β-nucleoside diphosphates (β-NDP); Peak F, α-NTP, α-NDP, and pyridine nucleotides; Peak G, unassigned diphosphodiester, possibly uridine diphosphoglycoside; and Peak H, β-NTP.

RESULTS

In vivo NMR spectroscopy of tumors. Figure 1A shows a typical spectrum of an sc 9L gliosarcoma 13 days following implantation of 10^6 cells and 1 day after treatment with 10 mg/kg of BCNU. The spectrum exhibits well-resolved resonances, which have previously been assigned (see figure caption) (*8*). Figure 1B shows the spectrum of a size- and age-matched sham-treated control. The levels of high-energy phosphates, NTP and PCr, compared to relative P_i are substantially elevated following treatment with the antineoplastic agent.

To determine whether the apparent metabolic activation observed at a low dose of BCNU is observed at higher doses, we examined dose-dependent changes in tumor NMR spectra. Ratios of some metabolites differed between treated and control tumors in a dose-dependent manner at both 1 and 4 days following chemotherapy

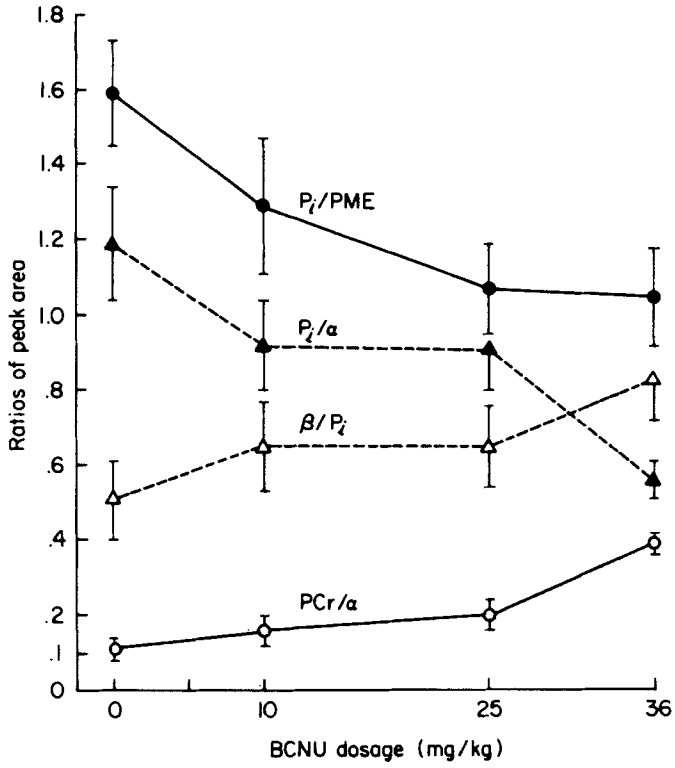

FIG. 2. Metabolite resonance area ratios (mean ± SE) 1 day after treatment of the host with one of the dose levels of BCNU. The linear regression lines are not shown for the sake of clarity, but the total number of data points used in the linear regressions was $n = 44$, and sample size varied for each dose as follows: 0 mg/kg, $n = 13$; 10 mg/kg, $n = 12$; 25 mg/kg, $n = 10$; and 36 mg/kg, $n = 9$. Higher dose levels of BCNU produced higher animal mortality, resulting in smaller sample sizes.

(Figs. 2 and 3). The PME/α-NTP, P_i/PCr, β-NTP/α-NTP, and β-NTP/γ-NTP ratios did not vary significantly with dose on Day 1 following chemotherapy. One day following chemotherapy linear regressions of resonance area ratios with dose showed a significant decrease in the P_i/α-NTP ratio ($r = -0.46$; $n = 44$; $P < 0.01$) and in the P_i/PME ratio ($r = -0.40$; $n = 44$; $P < 0.01$). Similar regressions showed a significant increase in the β-NTP/P_i ratio ($r = 0.28$; $n = 44$; $P < 0.05$) and in the PCr/α-NTP ratio ($r = 0.65$; $n = 44$; $P < 0.001$).

Four days following chemotherapy with BCNU, linear regression analysis showed a significant decrease in the P_i/α-NTP ($r = -0.60$; $n = 27$; $P < 0.001$), P_i/PME ($r = -0.40$; $n = 27$; $P < 0.05$), and PME/α-NTP ratios ($r = -0.64$; $n = 27$; $P < 0.001$). Significant increases were found in the β-NTP/P_i ($r = 0.50$; $n = 27$; $P < 0.01$) and PCr/α-NTP ratios ($r = 0.36$; $n = 27$; $P < 0.05$).

Tumor pH, measured by the chemical shift of the inorganic phosphate resonance, was not found to be significantly affected by any treatment. Data from treated and control tumors were pooled, and average tumor pH (6.90 ± 0.17) was calculated from the chemical-shift difference between PCr and P_i (*16*).

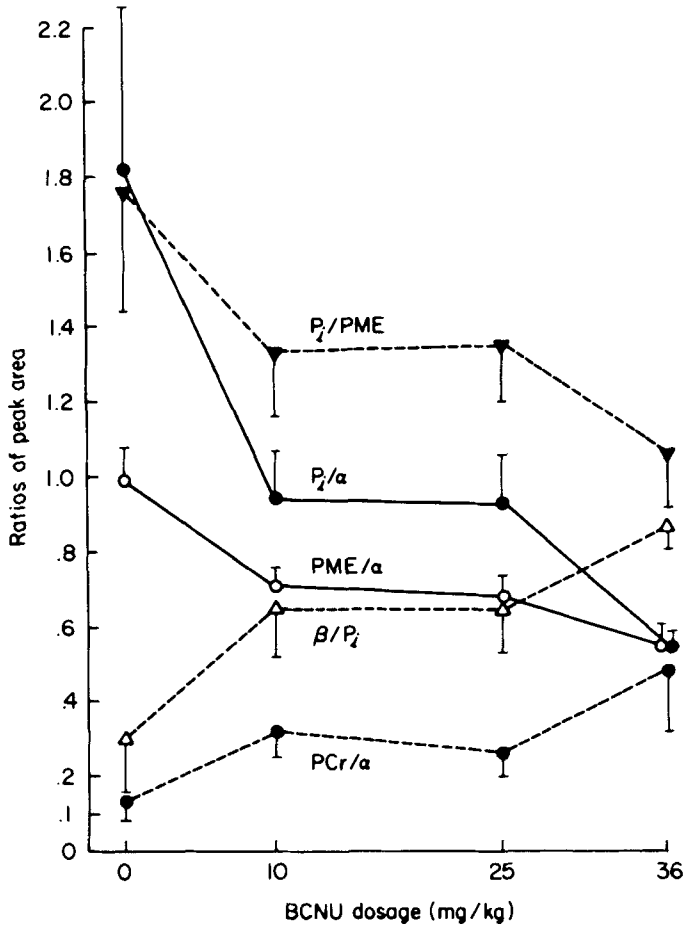

FIG. 3. Metabolite resonance area ratios (mean ± SE) 4 days after treatment of the host with one of the dose levels of BCNU. The linear regression lines are not shown for the sake of clarity, but the total number of data points used in the linear regressions was $n = 27$. The sample size varied for each dose as follows: 0 mg/kg, $n = 5$; 10 mg/kg, $n = 6$; 25 mg/kg, $n = 9$; and 36 mg/kg, $n = 7$. Toxicity associated with BCNU produced higher animal mortality at Day 4 than at Day 1, and higher mortality at the higher dosage levels of BCNU.

The sample size of NMR data for animals which received the highest dose of BCNU (36 mg/kg) is small because all the animals treated with this dose died within 9 days. Shortly after treatment rats became lethargic when undisturbed and irritable upon disturbance. Alopecia was common by the second day following treatment.

Tumor volume measurements. The sham-treated tumor showed an approximate doubling time (T_d) of 2.7 days (Fig. 4). Treatment of the tumor with BCNU (10 mg/kg) produced a growth delay of about 13 days (measured in logarithmic range of the growth curves of treated and control tumors). The surviving fraction following treatment with 10 mg/kg BCNU was calculated employing the formula (*17*)

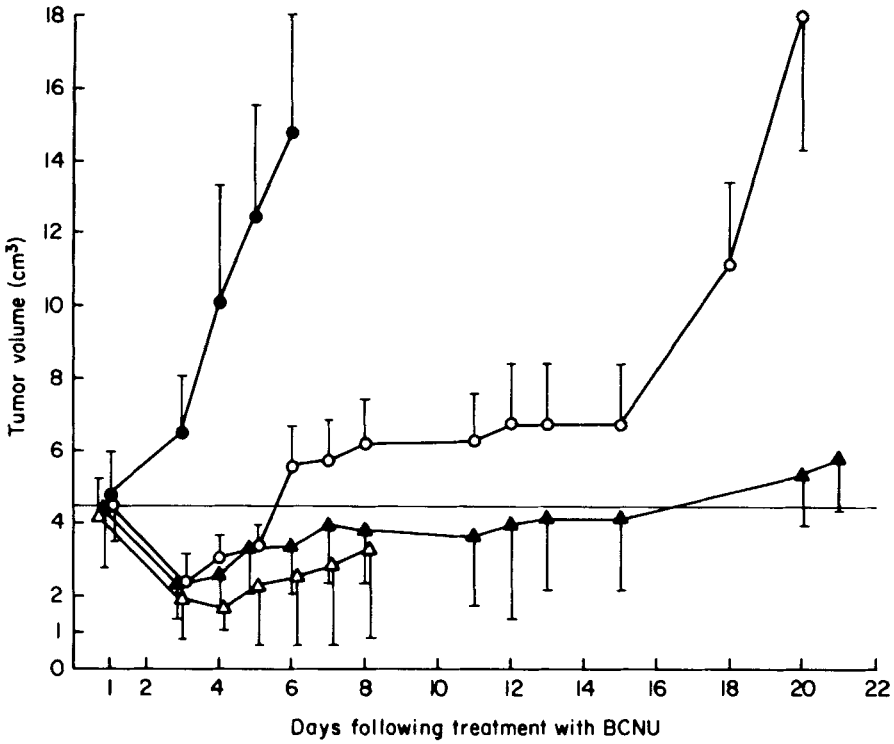

FIG. 4. Tumor volume growth measurements (mean ± SE) of sc tumors following treatment with one of the dose levels of BCNU. Volume was measured until the tumor underwent roughly two doublings or until the animals died. Sample size for the control and for each dose level of BCNU at Day 1 was $n = 4$ animals. Treatments were as follows: (●) 0 mg/kg BCNU; (○) 10 mg/kg BCNU; (▲) 25 mg/kg BCNU; (△) 36 mg/kg BCNU. The entire sample of animals receiving 36 mg/kg BCNU died within 9 days of treatment, while at least three of four animals in each other test group survived until the end of the measurement period.

$$\log_{10} \text{surviving fraction} = (\text{growth delay})/3.32T_d) = -1.45$$

$$\text{surviving fraction} = 3.55\%.$$

Treatment of the tumor with an intermediate dose of BCNU (25 mg/kg) produced a growth delay of at least 21 days (surviving fraction <0.2%).

Clonogenic cell survival assays. The plating efficiency of untreated 9L tumors was 45% under the conditions of this assay. Treatment of the tumor with 10 or 25 mg/kg BCNU produced 2 to 3 log orders of clonogenic cell kill (Fig. 5), while treatment with 36 mg/kg produced approximately 3 log orders of cell kill. Clonogenic cell survival of the untreated control and three treated groups was significantly correlated with BCNU dosage ($r = -0.63$; $n = 70$ plates; $P < 0.001$).

DISCUSSION

We have previously shown that growth of untreated 9L is associated with a significant decrease in the β-NTP/α-NTP ($P < 0.01$), and β-NTP/γ-NTP ($P < 0.001$)

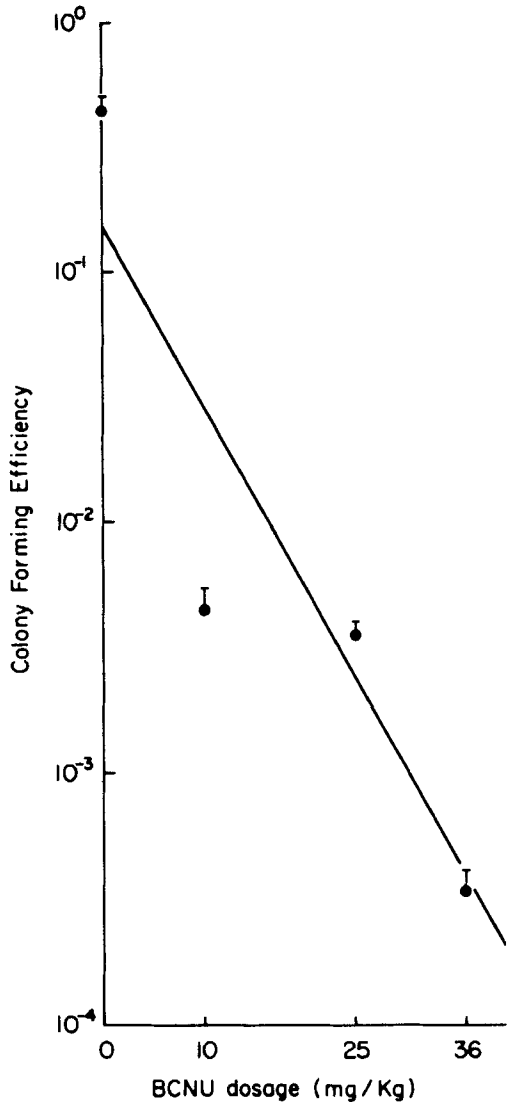

FIG. 5. *In vitro* colony forming efficiency (mean ± SE) 1 day following *in vivo* treatment with BCNU on Day 12 after tumor implantation.

ratios and an increase in the P_i/α-NTP ($P < 0.01$) and PME/α-NTP ($P < 0.01$) ratios (*8*). A decrease in the NTP β/α and β/γ ratios reflects a decrease in the relative level of nucleoside triphosphates since the peaks designated as α-NTP overlap with α-nucleoside diphosphate (NDP), uridinediphosphoglycoside, and pyridine dinucleotide peaks. The γ-NTP peak overlaps with the β-NDP peak, whereas the β-NTP peak originates only from nucleoside triphosphates. The decrease in these ratios as well as the increase in the P_i/α-NTP ratio reflects a decrease in the bioenergetic status of the untreated tumor. A similar progressive decrease in bioenergetic status has been

observed for many tumors during untreated growth and has been attributed to the onset of vascular insufficiency (*1, 2, 4–6, 18, 19*).

Response of the 9L tumor to chemotherapy is accompanied by substantial decreases in tumor volume (Fig. 4); tumors were roughly halved in volume by Day 3 following each dose of BCNU. Although tumor volume has been shown to have a strong effect on NMR spectral parameters (*20*), the metabolic activation of 9L following chemotherapy is not simply a result of size-dependent changes in tumor metabolism. Our data show that NMR spectral parameters of the treated tumors are significantly different from the controls 1 day following chemotherapy (Fig. 2), even though the control tumors and all of the treated tumors had similar volumes at that time (Fig. 4).

Clonogenic cell kill at the lowest dose of BCNU (10 mg/kg) is about 99% (96.5% on the basis of growth delay), while at the highest dose of BCNU (36 mg/kg) clonogenic cell kill is greater than 99.9% (Fig. 5). Therefore the spectral changes cover a wide range of therapeutic response and indicate that the NMR method is capable of detecting a clinically significant response to chemotherapy.

It is surprising that NMR spectroscopy can detect differences between tumors subjected to 99 versus 99.9% clonogenic cell kill when such small differences might be expected to be outside the detection limit of the spectroscopic method. This probably reflects the fact that the NMR spectrum is not directly monitoring clonogenic cell kill, but rather some physiological or metabolic effect associated with therapeutic response. It is important to note that estimates of cell kill relate to genetic damage that impairs the replicative capacity of tumor cells but may not have an immediate effect on the metabolic status of the tumor. Cells that are genetically "doomed to die" may be metabolically energized. Dead tumor cells probably exhibit spectra similar to those of ischemic tissue (T. C. Ng, W. T. Evanochko, and J. D. Glickson, unpublished). Failure to observe evidence of dead cells in the tumor indicates efficient clearance of these cells from the tumor.

These observations are strikingly different from those reported by Naruse *et al.* (*21*) for cultured human neuroblastoma cells implanted in newborn hamsters. Following high doses of cyclophosphamide or vincristine, spectra from these tumors showed little or no high-energy phosphate and exhibited characteristics similar to those of dead or ischemic tissues (*21*). The highest dose of BCNU used in our experiments (36 mg/kg) was 2.7 times higher than an LD_{10} dose for adult rats (*22*), and was at least an LD_{50} dose for juvenile rats. Despite this high dose of BCNU the tumor appeared to have increased relative levels of high-energy phosphate following treatment. Our results show that metabolic activation of the 9L gliosarcoma can occur despite the substantial tumor cell kill and overwhelming systemic toxicity produced by high doses of BCNU.

Response of 9L gliosarcoma to doses of BCNU ranging from below the LD_{10} to above the LD_{50} results in significant changes in ^{31}P NMR spectral parameters which parallel therapeutic response over several log orders of cell kill as measured by growth delay and clonogenic cell survival assay. While the mechanism underlying these spectral changes remains to be elucidated, these observations suggest a useful clinical role for ^{31}P NMR spectroscopy as a noninvasive method for detection and quantification of tumor response to chemotherapy.

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REFERENCES

1. W. T. EVANOCHKO, T. C. NG, AND J. D. GLICKSON, *Magn. Reson. Med.* **1,** 508 (1984).
2. J. P. WEHRLE AND J. D. GLICKSON, *Cancer Biochem. Biophys.* **8,** 157 (1986).
3. J. R. GRIFFITHS, Z. BHUJWALLA, R. C. COOMBES, R. J. MAXWELL, C. J. MIDWOOD, R. J. MORGAN, A. H. N. NIAS, P. PERRY, M. PRIOR, R. A. PRYSOR-JONES, L. M. RODRIGUES, M. STUBBS, AND G. M. TOZER, *Ann. N. Y. Acad. Sci.* **508,** 183 (1987).
4. J. D. GLICKSON, W. T. EVANOCHKO, T. T. SAKAI, AND T. C. NG, *in* "NMR Spectroscopy of Cells and Organisms," Vol. 1, (R. K. Gupta, Ed.), p. 99, CRC Press, Boca Raton, FL, 1987.
5. J. P. WEHRLE, C. P. MARTIN, AND J. D. GLICKSON, *in,* "Innovations in Diagnostic Radiology" (J. H. Anderson, Ed.), Springer-Verlag, Heidelberg, in press.
6. J. D. GLICKSON, J. P. WEHRLE, S. S. RAJAN, S-J. LI, AND R. G. STEEN, *in* "NMR in Biomedical Research" (J. W. Pettegrew, Ed.) Springer-Verlag, New York, in press.
7. P. F. DALY AND J. S. COHEN, *Cancer Res.* **49,** 770 (1989).
8. R. G. STEEN, R. J. TAMARGO, K. A. MCGOVERN, S. S. RAJAN, H. BREM, J. P. WEHRLE, AND J. D. GLICKSON, *Cancer Res.* **48,** 676 (1988).
9. E. J. HALL, "Radiobiology for the Radiologist," 2nd ed., p. 227, Harper & Row, Philadelphia, 1978.
10. S. S. RAJAN, J. P. WEHRLE, AND J. D. GLICKSON, *J. Magn. Reson.* **74,** 147 (1987).
11. T. C. NG, W. T. EVANOCHKO, AND J. D. GLICKSON, *J. Magn. Reson.* **49,** 526 (1982).
12. P. OKUNIEFF, F. KALLINOWSKI, P. VAUPEL, AND L. J. NEURINGER, *J. Natl. Cancer Inst.* **80,** 745 (1988).
13. I. D. CAMPBELL, C. M. DOBSON, R. J. P. WILLIAMS, AND A. V. XAVIER, *J. Magn. Reson.* **11,** 172 (1973).
14. M. L. ROSENBLUM, K. D. KNEBEL, D. A. VASQUEZ, AND C. B. WILSON, *Cancer Res.* **36,** 3718 (1976).
15. P. R. TWENTYMAN, J. M. BROWN, J. W. GRAY, A. J. FRANKO, M. A. SCOLES, AND R. F. KALLMAN, *J. Natl. Cancer Inst.* **64,** 595 (1980).
16. T. C. NG, W. T. EVANOCHKO, R. N. HIRAMOTO, V. K. GHANTA, M. B. LILLY, A. J. LAWSON, T. H. CORBETT, J. R. DURANT, AND J. D. GLICKSON, *J. Magn. Reson.* **49,** 271 (1982).
17. T. H. CORBETT AND F. A. VALERIOTE, *in* "Rodent Tumor Models in Experimental Cancer Therapy" (R. F. Kallman, Ed.), p. 233, Pergamon Press, NY, 1987.
18. S-J. LI, J. P. WEHRLE, S. S. RAJAN, R. G. STEEN, J. D. GLICKSON, AND J. HILTON, *Cancer Res.* **48,** 4736 (1988).
19. J. L. EVELHOCH, S. A. SAPORETO, G. H. NUSSBAUM, AND J. J. H. ACKERMAN, *Radiat. Res.* **106,** 122 (1986).
20. P. G. OKUNIEFF, J. A. KOUTCHER, L. GERWECK, E. MCFARLAND, B. HITZIG, M. URANO, T. BRADY, L. NEURINGER, AND H. D. SUIT, *Int. J. Radiat. Oncol. Biol. Phys.* **12,** 793 (1986).
21. S. NARUSE, K. HIRAKAWA, Y. HORIKAWA, C. TANAKA, T. HIGUCHI, S. UEDA, H. NISHIKAWA, AND H. WATARI, *Cancer Res.* **45,** 2429 (1985).
22. D. E. BULLARD, S. H. BIGNER, AND D. D. BIGNER, *Cancer Res.* **45,** 5240 (1985).