Response-specific Adriamycin Sensitivity Markers Provided by in Vivo 31P Nuclear Magnetic Resonance Spectroscopy in Murine Mammary Adenocarcinomas

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ABSTRACT

The in vivo phosphorus-31 nuclear magnetic resonance (NMR) spectra of Adriamycin (ADR)-sensitive murine mammary adenocarcinomas (17/A) and an ADR-resistant subline of this tumor which has been isolated in vivo (17/A/ADR) were compared both before and after i.v. administration of 12 mg/kg ADR. Significant differences between ADR-sensitive and -resistant tumors for the changes observed 1 day after treatment (prior to significant decreases in tumor size) included: (a) the pH increased to greater than 7.3 in response to treatment (or pH remained elevated) in ADR-sensitive tumors only; (b) the inorganic phosphate to nucleoside triphosphates peak height ratio decreased to less than 1 in response to treatment only in ADR-sensitive tumors; (c) glycero-phosphocholine to nucleoside triphosphates peak height ratio decreased in response to treatment in ADR-sensitive tumors only; and (d) the phosphocholine to nucleoside triphosphates peak height ratio decreased in response to treatment in ADR-sensitive tumors only. These differences are evidence in support of the hypothesis that in vivo 31P-NMR provides response-specific markers of ADR sensitivity. Because 31P-NMR can be applied to humans, these differences may be of prognostic value in the clinical management of human breast cancer if they are present after treatment with lower, nontoxic doses of ADR.

INTRODUCTION

The response of human malignancies to chemotherapy varies markedly even for patients with tumors of the same tissue of origin (e.g., mammary adenocarcinomas). The effects of therapy on model tumor systems (i.e., in vitro mammalian cells and in vivo rodent tumors) are evidence that a number of tumor characteristics, intrinsic both to the tumor cell and to its local environment, contribute to this variation in therapeutic response. Unfortunately, many of the techniques used to monitor these characteristics in model tumor systems require invasive and often destructive sampling and either cannot be applied to human malignancies or are applied with significant difficulty and discomfort. Consequently, it is difficult to implement such techniques to provide predictors of tumor responsiveness to chemotherapeutic agents. The lack of reliable response predictors (i.e., pretreatment indicators of therapeutic response or indices of therapeutic response to a "test" dose of a fraction of the maximum tolerated dosage) results in some patients with nonresponsive tumors needlessly enduring the side effects and toxicities of a full course of therapy. Availability of predictive markers of chemotherapeutic response would allow selection of a more effective, individualized therapy.

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3 The abbreviations used are: NMR, nuclear magnetic resonance; PCr, phosphocreatine; NTP, nucleoside triphosphates; GPC, glycerophosphocholine; PC, phosphocholine; PE, phosphoethanolamine; pHmax, pH determined by 31P-NMR; ADR, Adriamycin; CV, cyclophosphamide.

MATERIALS AND METHODS

Murine Tumor Systems. The tumor lines were maintained in serial passage in the mouse strain of origin (C3H/He). The animals necessary to begin an experiment were pooled and implanted s.c. with 30- to 60-mg tumor fragments by trocar on the side, between the axillary and the inguinal regions (slightly closer to the axilla). When tumors reached 500 ± 100 mg in size, tumor-bearing mice were randomly distributed into the treatment and control groups. After examination by 31P-NMR (day 0), mice in the treatment group received an i.v. injection of 12...
mg/kg ADR. Individual body weights for all mice in each experiment were within 4 g and all mice were over 20 g at the time of tumor implantation. Mice were obtained from The Jackson Laboratories (Bar Harbor, ME) or The National Cancer Institute (Frederick, MD) and were supplied with food and water ad libitum.

Therapeutic Response. Tumor response was measured by the tumor growth delay ($T - C$), where $T$ is the median time (in days) required for the treatment group tumors to reach 1000 mg and $C$ is the median time (in days) for the control group tumors to reach the same size. Tumors were measured with a caliper three times each week until growth or regrowth exceeded 2000 mg at which time the mice were sacrificed. Tumor weights were estimated from two-dimensional measurements:

$$\text{Tumor weight (mg)} = (a \times b^2)/2$$

(A)

where $a$ is the longest dimension and $b$ is orthogonal to $a$ (both in mm).

$$\log_{10} \text{cell kill} = \frac{T - C}{3.32 \times T_a}$$

(B)

where $T - C$ is the tumor growth delay (in days) as described above and $T_a$ is the tumor volume doubling time (in days) estimated from the best fit straight line from a log-linear plot of the control group tumors in exponential growth (100–800 mg). The conversion of $T - C$ values to $\log_{10} \text{cell kill}$ is possible because the $T_a$ value of tumors regrowing posttreatment approximates the $T_a$ value of the tumors in untreated control mice.

$^{31}$P-NMR Spectroscopy. $^{31}$P-NMR spectra were obtained at 121.5 MHz on a General Electric GN-300 spectrometer. $^{31}$P-NMR data were acquired: (a) before treatment (of the treatment group, day 0); (b) 24 h after treatment (day 1); and (c) every 48 h thereafter until tumors were either too small (<200 mg) or too large (>1000 mg) for NMR observation (days 3 and 5). For NMR observation, mice were anesthetized by i.p. injection of 60 mg/kg pentobarbital and immobilized in a plastic tube with the tumor isolated from the body by sliding the connecting skin through a 4-mm slot until the tumor rested on the outside of a 6-mm diameter hole. This arrangement facilitated positioning the animal within the NMR probe while both immobilizing and isolating the tumor. A two-turn surface coil $^{31}$P antenna of diameter slightly less than that of the tumor was used. This geometrical arrangement ensured negligible signal contribution from normal tissue adjacent to the tumor (11, 12). Typical acquisition parameters, chosen to ensure the observation volume is essentially the same for all metabolites (11) and to optimize sensitivity, were as follows: 9.0-μs pulse length at 100 W; 10-KHz spectral width; 1024 data points; 5-s recycle time. Data were acquired in four consecutive, 60-scan (5-min) blocks for a total acquisition time of 20 min. The difference spectra of the first and last blocks were examined to verify tumor metabolic stability and, consequently, the absence of significant blood flow effects due to either anesthesia (13) or the mouse support/tumor isolation system. The magnetic field homogeneity was optimized by observing the $^{1}$H signal from tumor H$_2$O with the $^{31}$P surface coil antenna (14). Typical 1H$_2$O linewidths were 0.25 to 0.35 ppm.

The pH$_{nmr}$ values were calculated from the chemical shift of P$_i$ using the Henderson-Hasselbalch equation and the values for pK$_z$ and limiting chemical shifts reported by Ng et al. (15). Chemical shifts were measured from resolution-enhanced, baseline-corrected spectra produced by linewidth stripping and Gaussian apodization (16) of the free induction decays and subtraction from the spectra of the third order polynomial fit of 10–15 points in the spectral baseline (standard G.E./Nicolet software). This facilitated the definition of the P$_i$, PCr, GPC, PC, and PE resonances. Chemical shifts were referenced to that of the a-phosphate peak of NTP. Using the standard G.E./Nicolet software, in our hands, peak intensity ratios calculated using peak heights determined from resolution-enhanced spectra [P(eak)P(ick) command] are more accurate than those calculated using peak areas determined from spectra with 0.2 ppm exponential line broadening [GEM(N)CAP deconvolution program]. The accuracy of calculated peak intensity ratios was evaluated using "simulated" spectra containing the $^{31}$P metabolite peaks at known relative intensities, with preapodization linewidths ranging from 0.2 to 0.5 ppm and typical in vivo noise levels. These "simulated" spectra, generated by weighted summation of free induction decays obtained from each of the metabolites individually, were analyzed by both methods and the results compared to the known ratios.

Statistical Analysis. The two-tailed Student's t test (independent samples) was used to determine if any significant differences in either the NMR metabolic characteristics or the ADR-induced changes in those characteristics were present between the ADR-sensitive and -resistant tumors. The two-tailed Student's t test (paired samples) was used to determine if any of the ADR-induced changes in the NMR metabolic characteristics were significant.

RESULTS

Representative in vivo $^{31}$P-NMR spectra of an ADR-sensitive tumor (mammary 17/A) and an ADR-resistant tumor (mammary 17/A/ADR) are shown in Figs. 1A and 2A, respectively. The average initial $^{31}$P NMR metabolic characteristics (pH$_{nmr}$ and the levels of P$_i$, PCr, GPC, PC, and PE relative to NTP) of both ADR-sensitive and -resistant tumors are summarized in Table 1 (data include both treatment and control groups). In these 400- to 600-mg tumors, the peak height ratios of GPC:NTP, PC:NTP, and PE:NTP were higher in ADR-sensitive tumors.

Figs. 1B and 2B show the in vivo $^{31}$P-NMR spectra, observed 1 day after i.v. injection of 12 mg/kg ADR, of the tumors represented in Figs. 1A and 2A, respectively. In contrast to the substantial changes apparent in the spectral characteristics of the ADR-sensitive tumor (Fig. 1), no major changes were apparent in the spectral characteristics of the ADR-resistant tumor (Fig. 2). Treatment with 12 mg/kg ADR i.v. resulted in a tumor growth delay ($T - C$) of 19 days (1.6 $\log_{10}$ cell kill) for ADR-sensitive tumors and 0 days for ADR-resistant tumors. Table 2 summarizes the average changes observed in the NMR metabolic characteristics of individual ADR-sensitive and -resistant tumors within 1 day after i.v. injection of 12 mg/kg ADR. ADR produced several significant changes in the metabolic characteristics of the ADR-sensitive tumors, within 1 day after treatment, which were absent in the ADR-resistant tumors. These changes include: (a) pH$_{nmr}$ increased; (b) P$_i$:NTP peak height ratio decreased; (c) PC:NTP peak height ratio decreased; and (d) GPC:NTP peak height ratio decreased. The only significant change observed by 1 day after treatment in ADR-resistant tumors was an increase in the PE:NTP peak height ratio.

The average $^{31}$P-NMR metabolic characteristics of ADR-sensitive and -resistant tumors observed prior to and up to 5 days after treatment are presented in Fig. 3, A–F. In ADR-sensitive tumors, the metabolic characteristics that changed significantly by 1 day after treatment either continued changing until day 3 (pH$_{nmr}$: P < 0.05 for change from day 1 to 3, and GPC:NTP; P < 0.05 for change from day 1 to 3), after which they remained unchanged, or did not change from posttreatment (day 1) levels for up to 5 days after treatment (P$_i$:NTP and PC:NTP). Over that time period, the metabolic characteristics of ADR-sensitive tumors that did not change significantly

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* J. L. Evelhoch and N. A. Keller, unpublished data.
by 1 day after treatment (PCr:NTP and PE:NTP) remained at pretreatment (day 0) levels. As treated ADR-resistant tumors progressed, no significant changes occurred between days 1 and 3. However, general trends toward acidic pH and increases in the peak height ratios of P:\textsubscript{1}:NTP, GPC:NTP, PC:NTP, and PE:NTP were evident. The increase in the P\textsubscript{1}:NTP peak height ratio between days 0 and 3 was significant (P < 0.05). By 1 day after treatment, among the differences between the metabolic characteristics of ADR-sensitive and -resistant tumors present prior to treatment only the GPC:NTP peak height ratio remained higher in ADR-sensitive tumors (P < 0.001). By 3 days after treatment, there were no significant differences between ADR-sensitive and -resistant tumors.

The NMR metabolic characteristics of untreated ADR-sensitive tumors (control group; N = 4) exhibited no statistically significant changes as the tumors progressed. However, the data indicate a general trend towards increased peak height ratios for P\textsubscript{1}:NTP (+0.91 ± 1.38 change from day 0 to 1),

Table 1 Initial \textsuperscript{31}P-NMR metabolic characteristics of murine mammary adenocarcinomas 17/A and 17/A/ADR

<table>
<thead>
<tr>
<th>Metabolic characteristics</th>
<th>Mamm 17/A</th>
<th>Mamm 17/A/ADR</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH\textsubscript{mP} \textsuperscript{*}</td>
<td>7.15 ± 0.04</td>
<td>7.12 ± 0.05</td>
</tr>
<tr>
<td>(6.88 to 7.33) \textsuperscript{p}</td>
<td>(6.76 to 7.31)</td>
<td></td>
</tr>
<tr>
<td>P\textsubscript{1}:NTP</td>
<td>1.60 ± 0.26</td>
<td>1.65 ± 0.20</td>
</tr>
<tr>
<td>(0.90 to 3.92) \textsuperscript{p}</td>
<td>(1.01 to 3.59)</td>
<td></td>
</tr>
<tr>
<td>GPC:NTP</td>
<td>0.49 ± 0.06</td>
<td>0.65 ± 0.14</td>
</tr>
<tr>
<td>(0.27 to 1.20) \textsuperscript{p}</td>
<td>(0.31 to 2.39)</td>
<td></td>
</tr>
<tr>
<td>PCr:NTP</td>
<td>1.74 ± 0.17</td>
<td>0.72 ± 0.06</td>
</tr>
<tr>
<td>(0.79 to 3.06) \textsuperscript{p}</td>
<td>(0.24 to 1.19)</td>
<td></td>
</tr>
<tr>
<td>PE:NTP</td>
<td>1.52 ± 0.12</td>
<td>1.07 ± 0.06</td>
</tr>
<tr>
<td>(0.69 to 2.24) \textsuperscript{p}</td>
<td>(0.62 to 1.25)</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{*} Values in parentheses, mean ± SE of the respective characteristics and the range of values observed.

\textsuperscript{p} Difference between initial characteristic observed in 17/A and 17/A/ADR tumors is significant at the 99% confidence level (P < 0.01).
The principal aim of these studies was to determine whether in vivo 31P-NMR spectroscopy provides response-specific predictive markers of ADR sensitivity. Evidence for several predictors of ADR sensitivity is provided by the differences observed between ADR-sensitive and -resistant murine mammary adenocarcinomas in the ADR-induced changes in the 31P-NMR metabolic characteristics present within 1 day of treatment.

Predictive markers of tumor response to ADR were provided by the ADR-induced changes in both pHnmr and the P¡:NTP ratio. An alkaline shift in pHnmr and a decrease in the P¡:NTP ratio were observed within 1 day of treatment only in ADR-sensitive tumors. Similar drug-induced changes have been observed in a mammary 16/C tumor 27 h after treatment with 13 mg/kg ADR i.v. (8) and in RIF-1 fibrosarcomas 2 to 4 days after treatment with 150 mg/kg CY (18). The changes in tumor biochemistry that result in the observed 31P-NMR spectral changes are not known. The alkaline pHnmr shift may have been due to a change in intracellular pH or a redistribution of P¡ within the tumor and consequently a change in the weighting of pHnmr (i.e., the concentration and relaxation time of P¡ throughout the tumor may have shifted in response to the drug).

Because the height of P¡ decreases relative to PC, PE, and GPC as well as NTP, it appears that the decrease in the P¡:NTP ratio was primarily due to a decrease in the height of P¡. This decrease could have been due to: (a) removal of P¡ from the tumor; (b) relocation of P¡ within the tumor to an “NMR-invisible” location (e.g., immobilized on DNA, membranes or proteins); or (c) an increase in the T1 value of P¡. However, in consideration of the magnitude of the observed decrease, it is unlikely that T1 effects contributed significantly to the decreased P¡:NTP ratio. Irrespective of the causes of these spectral changes, their absence in ADR-resistant tumors is indicative of a relationship between these changes and the antineoplastic action of ADR. Whether they are directly related to the primary cytotoxic events of drug action or a secondary consequence of drug-induced cell death is unknown. However, because both ADR and CY inhibit DNA synthesis and the minimum in the rate of DNA synthesis in RIF-1 tumors treated with 150 mg/kg CY coincided with the minimum in the P¡:NTP ratio (18), there is most likely a direct relationship between these changes and drug-mediated effects on DNA synthesis.

The ADR-induced modulation of the lipid metabolite levels

![Fig. 3. Plots show the mean ± SE of the 31P-NMR metabolic characteristics of both Mamm 17/A (ADR-sensitive) and Mamm 17/A/ADR (induced ADR resistance) studied at 0 and 5 days after i.v. injection of 12 mg/kg ADR. For Mamm 17/A: days 0 and 1, N = 12; days 3 and 5, N = 8; day 5, N = 6. For Mamm 17/A/ADR: day 0 and 1, N = 10; day 3, N = 4. Metabolic characteristics: A, pH (determined by 31P-NMR); B, P¡:NTP peak height ratio; C, PCr:NTP peak height ratio; D, GPC:NTP peak height ratio; E, PE:NTP peak height ratio.](https://cancerres.aacrjournals.org/article-pdf/47/8/3389/2743474)
also provided markers of therapeutic response to ADR. For tumors treated with 12 mg/kg ADR i.v., the ratios of GPC:NTP and PC:NTP decreased within 1 day after treatment only in ADR-sensitive tumors. It is not known what changes in tumor biochemistry are responsible for the observed 31P-NMR spectral changes. Nor is it known whether these changes are directly related to ADR-induced cell death. Nevertheless, it is likely that the spectral changes are related to the antineoplastic action of ADR because they are absent in nonresponding (ADR-resistant) tumors. Previous in vivo 31P-NMR studies of chemotherapy-induced changes in murine tumors have not reported modulation of the lipid metabolites (5–7). Whether such modulations are unique to this tumor and/or chemotherapeutic agent is unknown. However, because ADR exerts direct effects on the cell membrane (19), it is expected that these lipid metabolite levels also may be modulated by ADR in other ADR-sensitive tumors.

Several of the differences between ADR-sensitive and -resistant MCF-7 human breast cancer cells observed in vitro (9) were also present before treatment in these in vivo ADR-sensitive and -resistant mammary adenocarcinomas. For untreated 400–600-mg tumors, the GPC:NTP ratio and the ratios of the phosphomonoesters (primarily PC and PE with minor contributions from fructose-6-phosphate and glucose-6-phosphate, respectively) to NTP were significantly higher in ADR-sensitive tumors than in ADR-resistant tumors. The observation of these differences in vivo is evidence in support of the hypothesis that they may provide markers of the development of ADR resistance during the course of treatment. However, because the ratios of GPC:NTP, PC:NTP, and PE:NTP both increased and became more scattered as untreated tumors progressed, they no longer differed in larger ADR-sensitive and -resistant tumors. Thus, the ratios of these lipid metabolites relative to NTP are unlikely to provide reliable markers of the development of ADR resistance in vivo. Whether the increased ratios were due to increases in the lipid metabolites or decreased NTP cannot be ascertained from the present data. If the changes were primarily due to decreased NTP, as might be expected in larger (more necrotic) tumors (5), then measurement of GPC, PC, and PE independently (20) may provide reliable markers of the development of ADR resistance during the course of treatment. However, although ADR treatment resulted in a decrease in the ratios of GPC:NTP and PC:NTP to essentially the same ratios observed in ADR-resistant tumors prior to treatment (when NTP levels were high), the fraction of cells resistant to ADR present in the mammary 17/A tumors after a single dose of 12 mg/kg ADR is much less than 1%. Therefore, the levels of GPC and PC may not be directly related to ADR resistance and the level of PE appears to be the only potential marker of ADR resistance. Recently Cohen et al. have extended their studies to examine MDA-MB231 and HBL100 human breast cancer cells in vitro and s.c. human lung and colon tumors in mice in vivo. Based on the results of these studies, they have also concluded that the differences observed between ADR-sensitive and -resistant MCF-7 cells do not appear to correlate specifically with drug resistance.5

The previously reported merit of the PCr:NTP ratio as either a pretreatment ADR-sensitivity marker (9) or an indicator of ADR-induced changes in tumor metabolism (8) was not corroborated by the present study. The PCr:NTP ratio was not significantly higher in ADR-resistant tumors as was observed in vitro for MCF-7 breast cancer cells with induced ADR resistance (9). This is primarily due to the large scatter observed for this ratio in the resistant tumors. This scatter is most likely the result of variability in the distribution of physiological environments present in solid tumors (21). The PCR:NTP ratio did not increase in response to ADR as was observed in a mammary 16/C treated with a slightly higher dose of ADR (13 mg/kg i.v.; 8). Because we did observe an increase in the PCR:NTP ratio in three out of 12 tumors examined and Evanochko et al. (8) reported the results for a single tumor, this difference is likely the consequence of variations in response between individual tumors and may not be significant.

Because 31P-NMR spectroscopy can be used to examine human tumors in situ (22–25), the ADR sensitivity markers identified in these studies may become clinically important. However, for these markers to be useful clinically, they must be present after treatment with lower “test doses” of ADR. The ability to observe these markers after lower doses of ADR is dependent upon both the fraction of the observed cells responding to treatment and whether the changes observed in the 31P-NMR spectral characteristics correspond to lethal or nonlethal changes in tumor metabolism. The dose used in these studies resulted in 1.6 log10 cell kill (i.e., ~97% of the cells were killed). Dose-response data for treatment of early stage mammary 17/A tumors with ADR indicate that a decrease in the dose by a factor of two (to 6 mg/kg) would result in a log10 cell kill of ~0.9 (i.e., 87% of cells killed).6 If the dose-response curve is similar for these advanced-stage 500-mg tumors (as is expected because the log10 cell kill from the present study falls on the best-fit line to a log-linear plot of the dose-response data from early stage tumors), the changes should be apparent whether or not they correspond to lethal changes in the tumor metabolism. Further studies are required to address this question. If 31P-NMR spectroscopy can provide noninvasive, nondestructive response predictors to a lower “test dose” of ADR, it could both allow patients with nonresponsive tumors to avoid the side effects and toxicities of a full-course of therapy and aid in the selection of patients for the clinical trials of new antitumor agents.

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