NMR Spectroscopy analysis of phosphorus metabolites and the effect of adriamycin on these metabolite levels in an adriamycin-sensitive and -resistant human small cell lung carcinoma cell line*

S. de Jong¹, N.H. Mulder¹, E.G.E. de Vries¹ & G.T. Robillard²

¹Departments of Medical Oncology and ²Physical Chemistry, State University of Groningen, The Netherlands.

Summary 31P nuclear magnetic resonance (NMR) spectra of cells and of cell extracts revealed high levels of phosphoricholine (PC) and phosphocreatine (PCr) in an adriamycin-resistant human small lung carcinoma cell line (GLC4/ADR) and the adriamycin-sensitive parental cell line (GLC4). PCR levels in extracts of GLC4/ADR were increased compared to extracts of GLC4. We estimated that 11% of the total intracellular ATP is not bound to Mg²⁺ in both cell lines. This value corresponded to an intracellular free Mg²⁺ of 0.30 mM. The effects of different adriamycin concentrations, 0.05, 1 and 30 µM for GLC4 and 1, 30 and 200 µM for GLC4/ADR, on the phosphorus metabolite levels in continuously perfused cells were monitored. Significant differences between GLC4 and GLC4/ADR included: (a) a strong increase in the ATP level in the presence of 30 µM adriamycin in GLC4, only followed by a fast decrease after 5 h of perfusion. (b) a less dramatic increase in the PC level in GLC4/ADR and an unchanged ATP level in the presence of increasing adriamycin concentrations. (c) an increased GPC level in GLC4/ADR in the presence of adriamycin. The changes in PC and GPC levels in the presence of adriamycin suggested that the phospholipid turnover was increased in GLC4/ADR and could be stimulated in the presence of adriamycin. In both cell lines, PCR levels decreased faster than the ATP levels after adriamycin treatment. Thus, biochemical markers for adriamycin resistance can be detected with NMR spectroscopy. However, more studies are necessary to obtain parameters to distinguish drug-sensitive from drug-resistant tumours in patients by NMR spectroscopy.

Changes in energy-metabolism may be involved in resistance. Cell lines selected in vitro for resistance to adriamycin, Vinca alkaloid or colchicine, exhibit the multidrug (MDR) phenotype. In these resistant cells a M, 170,000 kD P-glycoprotein is overexpressed (Riordan & Ling, 1985; Pastan & Gottesman, 1987). This P-glycoprotein functions as a energy-dependent efflux pump to different types of antitumour drugs (Riordan & Ling, 1985; Horio et al., 1988). Increased free radical detoxification could also play a role in adriamycin-resistance of these cells (Batist et al., 1986; Sinha et al., 1987). Since both mechanisms are associated with energy-dependent processes, expressing the MDR phenotype may involve changes in energy requirements and energy metabolism. These changes have actually been observed in an adriamycin-resistant human breast cancer cell line (Cohen et al., 1986; Yeh et al., 1987; Lyon et al., 1988). Cell lines resistant to epipodophyllotoxins, ellipticine and m-AMSA have also been established which do not overexpress the P-glycoprotein (Gisson et al., 1986; Estey et al., 1987; Pommier et al., 1986; Beck et al., 1987; Fergusen et al., 1988).

Cross resistance to other drugs is still observed and sometimes drug accumulation is decreased. It is unknown whether this so-called atypical MDR (Beck et al., 1987) is accompanied by changes in energy requirements and energy metabolism.

In order to obtain biochemical characteristics for the atypical MDR phenotype we focussed on metabolites of both energy metabolism (PCr, ATP and Pi) and phospholipid metabolism (GPC, PE and PC) in the adriamycin-sensitive small cell lung carcinoma cell line (GLC4), and an adriamycin-resistant subline (GLC4/ADR), which exhibits the atypical MDR phenotype (Zijlstra et al., 1987a; De Jong et al., 1990). Phosphorus metabolite levels in living cells can be monitored by 31P NMR spectroscopy. So far, studies on the effect of adriamycin exposure on energy and lipid metabolism using 31P NMR were only done in in vivo models of murine mammary 16/C and murine mammary 17/C adenocarcinomas (Evanochko et al., 1983; Evelhoch et al., 1987). In vitro studies on cells using NMR spectroscopy necessitate trapping a dense cell suspension in a small volume. We have used the method described by Cohen et al. (1986) in which cells were embedded in agarose gel threads (Foxall & Cohen, 1983; Knop et al., 1984). They have applied their technique using 31P and 13C NMR spectroscopy to various cell lines (Cohen et al., 1986; Lyon et al., 1988; Daly et al., 1987). In their studies with small cell lung cancers the signal intensities of PCR did not change relative to the ATP signal intensities for over 24 h (Knop et al., 1987).

In the present study, 1P and 31H NMR spectroscopy was employed to monitor levels of energy and phospholipid metabolism in GLC4 and GLC4/ADR cells. The effect of adriamycin on these levels were monitored in continuously perfused cells using 31P NMR spectroscopy. The presence of phosphorus metabolites characteristic for atypical MDR and the presence of response-specific markers of adriamycin-sensitivity and -resistance are discussed.

Materials and methods

Materials

RPMI 1640 medium was purchased from Gibco (Paisley, Scotland). Dulbecco’s Modified Eagles’s medium (DME), F12 medium and foetal calf serum (FCS) were obtained from Flow Lab (Irvice, Scotland), low melting agarose from FMC (Rockland, ME) and adriamycin from Farmitalia Carlo Erba (Milano, Italy).

Cell lines

GLC4, a human small cell lung carcinoma cell line, was derived from a pleural effusion in our laboratory and kept in continuous culture in RPMI 1640 medium supplemented with 10% FCS. GLC4/ADR, a subline of the parental line, was made resistant by stepwise increasing concentrations of adriamycin, until the cells were growing at a continuous drug level of 1.18 µM. GLC4/ADR was 44-fold more resistant to adriamycin than GLC4 after a 1 h exposure in the clonogenic assay (Zijlstra et al., 1987a). GLC4/ADR exhibited cross-resistance to several other drugs (Zijlstra et al., 1987a; De
Cells (1–1.5 × 10⁷) were resuspended in DME/F12 medium (pH 7.4) and 10% FCS. The perfusion system was prepared as described previously (Foxall & Cohen, 1983; Knop et al., 1984) with some modifications. Low-gelling agarose (0.8 ml in 0.9% NaCl) was added at 37°C to 1.6 ml of a cell suspension. Agarose strands were extruded under light pressure through a teflon capillary tube (0.5 mm inside diameter) immersed in an ice/water bath into an Wilmad MRS tube (10 mm inside diameter). The gel threads were perfused by aspiration (1.4 ml min⁻¹). A low perfusion rate was used in this study, since the stability of the threads decreased in the presence of adriamycin. Consequently, the number of cells embedded in the threads had to be decreased to prevent partial acidification of the cells. A number of layers of nylon gauzes were used instead of a piece of sponge to restrain the threads, resulting in a higher perfusion capacity. The perfusate from a 200 ml reservoir consisted of DME/F12 and 10% FCS supplemented with penicillin (125 U/ml) and streptomycin (125 U/ml⁻¹). Oxygen (95%) and CO₂ (5%) were bubbled through the perfusate in the reservoir.

**Cell extracts**

Cells were obtained from 1–2 × 10⁶ cells. The extraction procedure was performed at 4°C. Ice-cold perchloric acid (10%) was added to the pellet and the cell mixture was vortexed at the beginning and the end of a 20 min period. The extracts were neutralised with KOH, centrifuged to remove the KClO₄ precipitate, freeze-dried and redissolved in D₂O (Evans & Kaplan, 1977).

**¹H and ³¹P NMR spectroscopy**

³¹P NMR spectra (121.4 MHz) of perfused cells at 37°C were obtained on a Varian VXR-300 spectrometer equipped with a VXR 5000 data station. Spectra were usually obtained from 1500 transients with a spectral window of ± 4000 Hz, 4K data points, a 65° pulse angle, a repetition rate of 2.25 s and a line broadening of 20 Hz. All ¹H chemical shifts in the spectra were set relative to PCl by setting the PCl signal to 0.00 ppm.

¹H NMR spectra of cell extracts were obtained from 1000 scans at 10°C with 90° pulse angle and a repetition time of 3.4 s under HDO decoupled conditions. ³¹P NMR spectra of cell extracts were obtained from 2000 scans at 10°C with a 55° pulse angle and a repetition time of 40 s under proton decoupled conditions. EDTA and diphenylphosphate were added to a final concentration of 10 mM and 0.15 mM, respectively.

Since the relative separations between the β and α, and the β and γ peaks of ATP are proportional to the amount of ATP bound to Mg²⁺, the fraction of total ATP that is not complexed to Mg²⁺ (O) can be calculated (Gupta & Moore, 1980a). The free Mg²⁺ concentration can then be calculated using the dissociation constant of MgATP (ATP complexed to magnesium) (Kd = 38 µM) and the formula [Mg²⁺] = Kd (O⁻¹−1) (Gupta & Moore, 1980a).

Spectra of perfused cells were obtained 3 h after the perfusion was started, when no major changes in the spectra occurred. Two spectra were collected to estimate peak areas and peak heights at t = 0. Peak intensities of the different metabolites in spectra of cell extracts and in perfused cells were estimated by peak areas determined from computer simulated spectra using the deconvolution routine in the VXR-5000 software. Changes in the levels of the phosphorus metabolites in the presence or absence of adriamycin were estimated from resolution-enhanced spectra by comparing peak heights of the particular metabolite at different times. Peak heights of a given metabolite were expressed as a percent of the averaged peak height of this metabolite in the two spectra at t = 0. At lower fields an underlying 'hump' was absent in the spectra, which allowed the reliable and reproducible measurement of peak intensities. Using peak areas from resolution-enhanced spectra to estimate changes in metabolites did not give significantly different results.

**Statistics**

All results were expressed as means ± s.d. Statistical significance was determined by use of the Student's t-test.

**Results**

³¹P and ¹H NMR spectra of cell extracts

Cell extracts were made from adriamycin-sensitive (GLC₄) and adriamycin-resistant (GLC₄/ADR) cells. Assignments were made on basis of data in the literature (Daly et al., 1987; Evans & Kaplan, 1977; Evanchock et al., 1984) and by adding standard compounds. Extracts of both cell lines showed high levels of PC (1 in Figure 1a and b). Additional unidentified resonances could be seen, possibly AMP and PE, on the low field side of the PC peaks. Low levels of probably GPC (4) and GPE (3) could be detected in the extracts. High levels of Pcr (5) were detected in both cell lines with highest Pcr levels in the GLC₄/ADR extracts (Table I). Expanding the spectra revealed another triphosphate near the β resonances of ATP (11) that accounted for 25 ± 4% (s.d., n = 3) of the total peak area in GLC₄/ADR extracts and for 19 ± 4% in GLC₄ extracts. This triphosphate could be UTP, GTP or CTP (Evans & Kaplan, 1977; Evanchock et al., 1984). High resolution ¹H NMR spectra were obtained from extracts of both cell lines in D₂O. Figure 2 shows the results for GLC₄. The identification of the peaks was made using previous assignments in tumour cell extracts and by adding standard compounds (Evanchock et al., 1984). The most intense resonances originated from choline, PC, Pcr and creatine; furthermore, lactate, acetate and amino acids (alanine, proline, glutamic acid and glutamine) were found. In the low field region of the ¹H NMR spectra resonances from adenosine derivatives and some uracil-, guanine- and cytosine-containing compounds predominated. No major differences between spectra of GLC₄ and GLC₄/ADR were found.

³¹P NMR spectra of perfused cells

Spectra of perfused GLC₄ and GLC₄/ADR cells at 37°C were recorded at a 2.25 s repetition rate and a 55° pulse angle to ensure almost complete spin relaxation of the metabolites (Figure 3). To estimate the relative levels of phosphorus metabolites in GLC₄ and GLC₄/ADR we determined peak areas of the various metabolites (Table II) in several spectra at t = 0 by computer simulation of the spectra using a deconvolution routine. Partially overlapping peaks could be separated using this program. The peak areas were expressed to that of the βATP (8) resonance. Peak areas of Pi (2) were not used, since Pi was also present in the medium we used. Peak area of γATP (5) was increased in GLC₄/ADR compared to GLC₄. Since the peak areas are expressed relative to the βATP peak area, the increased area of the γATP resonance in GLC₄/ADR must be due to some component other than ATP. Comparing the extracts we concluded that ADP was this component.

**Free ADP and Mg²⁺ in intact cells**

Mg²⁺ modifies the equilibrium constant for the reactions
**Figure 1** $^{31}$P NMR spectra (121.45 MHz) of the perchloric extracts of GLC$_4$ a, and GLC$_4$/ADR b. The pH (meter reading) was 7.8. Peak assignments are: 1, PC; 2, Pi; 3, GPE; 4, GPC; 5, PCR; 6, $\gamma$ATP; 7, $\beta$ADP; 8, $\alpha$ATP; 9, $\alpha$ATP; 10, NAD; 11, $\beta$ATP.

**Table I** Phosphorus metabolite levels in extracts of GLC$_4$ and GLC$_4$/ADR (n = 3)

|        | GLC$_4$   | GLC$_4$/ADR |
|--------|-----------|-------------|
| PC     | 1.83 ± 0.44$^a$ | 1.41 ± 0.11 |
| GPC    | 0.10 ± 0.05  | 0.13 ± 0.04 |
| PCR    | 0.71 ± 0.18  | 1.11 ± 0.16$^d$ |
| $\gamma$ATP$^b$ | 1.09 ± 0.14  | 1.20 ± 0.07 |
| $\alpha$ATP$^c$ | 1.56 ± 0.11  | 1.66 ± 0.12 |
| $\beta$ATP$^c$ | 1.00        | 1.00        |

$^a$Peak areas were obtained from spectra using a deconvolution routine (see Materials and methods) and were expressed relatively to the peak area of PATP (± s.d.). $^b$ADP, NAD and some other triphosphates might be present. $^c$Some other triphosphates might be present. $^d$P < 0.025, GLC$_4$ vs GLC$_4$/ADR.

**Figure 2** $^1$H NMR spectrum (300 MHz) of the perchloric extract of GLC$_4$/ADR. The pH was 7.4. The amplitude of the peaks in the high field region (5.5–9.5 ppm) is eight times that of the peaks in the low field region (0–4 ppm). A, adenine; G, guanine; C, cytosine; U, uracil; PCR, phosphocreatine; Cr, creatine; PC + C, phosphorylcholine and choline; Pro, proline; Glu, glutamic acid; Gln, glutamine; Ala, alanine; Lac, lactate.

**Figure 3** $^{31}$P NMR spectra (121.45 MHz) of perfused GLC$_4$ a, and GLC$_4$/ADR b, cells at 37°C. Peak assignments are: 1, PC; 2, Pi; 3, GPC; 4, PCR; 5, $\gamma$ATP; 6, $\alpha$ATP; 7, NAD; 8, $\beta$ATP.

**Table II** Phosphorus metabolite levels in perfused GLC$_4$ and GLC$_4$/ADR cells (n = 6)

|        | GLC$_4$   | GLC$_4$/ADR |
|--------|-----------|-------------|
| PC     | 1.96 ± 0.35 | 1.94 ± 0.29 |
| GPC    | 0.08 ± 0.09  | 0.09 ± 0.13 |
| PCR    | 0.64 ± 0.18  | 0.54 ± 0.14 |
| $\gamma$ATP$^b$ | 1.03 ± 0.15  | 1.27 ± 0.21$^e$ |
| $\alpha$ATP$^c$ | 1.50 ± 0.26  | 1.76 ± 0.34 |
| $\beta$ATP$^c$ | 1.00        | 1.00        |

$^a$Peak areas were obtained from spectra at t = 0 using a deconvolution routine (see Materials and methods) and were expressed relatively to the peak area of $\beta$ATP (± s.d.). $^b$ADP, NAD and some other triphosphates might be present. $^c$Some other triphosphates might be present. $^d$P < 0.025, GLC$_4$ vs GLC$_4$/ADR.
Perfused cells were added to the perfusate. Levels were obtained from peak heights of the metabolite in the $^{31}$P NMR spectra, as described in Materials and methods. The spectra of perfused cells were used to quantify relative catalysed by creatine kinase and adenylate kinase that are important for energy metabolism (Lawson & Veech, 1979). The spectra of perfused cells we had used to quantify relative levels of metabolites were also used to estimate the fraction of total ATP not bound to Mg$^{2+}$. This fraction was $0.11 \pm 0.01$ in GLC$_4$/ADR and $0.11 \pm 0.02$ in GLC$_4$ cells. The free intracellular Mg$^{2+}$ concentrations were calculated from these fractions as described in Materials and methods and were $0.32 \pm 0.03$ mM and $0.30 \pm 0.06$ mM in GLC$_4$/ADR and GLC$_4$. The total ADP concentration (MgADP and free ADP) can be calculated from ATP, PCr and Cr concentrations in extracts, the intracellular pH and the assumed equilibrium constant $K_a$ of the creatine kinase reaction at this Mg$^{2+}$ concentration according to Lawson et al. (1979). The ATP concentration in extracts of both cell lines was 6 nmol/10$^6$ cells (De Jong et al., manuscript in preparation). The Cr/PCr ratio was calculated from $^1$H NMR spectra of extracts. For GLC$_4$ and GLC$_4$/ADR these ratios were 1.33 $\pm$ 0.12 (s.d., n = 3) and 1.39 $\pm$ 0.37, respectively. Assuming that the intracellular and extracellular pH are equal (pH 7.3), the calculated total ADP concentration was $0.15 \pm 0.01$ nmol/10$^6$ cells for GLC$_4$ and GLC$_4$/ADR.

Effect of adriamycin on energy metabolite levels of perfused GLC$_4$ and GLC$_4$/ADR cells

Perfused cells were continuously exposed to 0.05 $\mu M$, 1 $\mu M$ and 30 $\mu M$ adriamycin (GLC$_4$) and to 1 $\mu M$, 30 $\mu M$ and 200 $\mu M$ adriamycin (GLC$_4$/ADR) while the time course of the phosphorus metabolite levels was followed. Each $^{31}$P NMR spectrum was obtained by accumulating 1500 scans which took 1 h. Only significant changes are indicated.

PCr levels in the control (untreated GLC$_4$ cells) increased to 130% of the initial value ($t = 2-12$ h, $P < 0.05$ vs $t = 0$) (Figure 4a). In the presence of 30 $\mu M$ adriamycin, PCr levels decreased rapidly after 4 h ($t = 5-15$ h, $P < 0.01$ vs control) and were almost undetectable at 15 h. In untreated GLC$_4$/ADR cells PCr levels did not increase significantly of the initial value, while with a high concentration of adriamycin (200 $\mu M$) PCr levels decreased after 4 h ($t = 5-15$ h, $P < 0.005$ vs control) and became undetectable after 13 h (Figure 4b). The standard deviations of PCr levels were rather large in adriamycin treated cells of both cell lines because the peak intensity was low and therefore more susceptible to noise.

Since the $\gamma$ATP and $\alpha$ATP resonances might contain some contributions from ADP, changes in height of the $\beta$ATP resonance were used to determine the influence of adriamycin on ATP. ATP levels in untreated GLC$_4$ cells increased to 140% of the initial value ($t = 4-15$ h, $P < 0.05$ vs $t = 0$) (Figure 5a). In the presence of 30 $\mu M$ adriamycin an increase to 175% of the initial ATP level was seen within 5 to 6 h ($t = 2-7$ h, $P < 0.025$ vs control), which subsequently decreased to 20% at 15 h (Figure 5a and 6a). In untreated GLC$_4$/ADR cells ATP levels increased to 125% of the initial value ($t = 3-15$ h, $P < 0.05$ vs $t = 0$) (Figure 5b). With 200 $\mu M$ adriamycin ATP dropped to undetectable levels at 15 h ($t = 6-15$ h, $P < 0.01$ vs control). Changes in $\gamma$ATP and $\alpha$ATP intensities in untreated and adriamycin treated GLC$_4$

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Figure 4 Effect of different adriamycin concentrations on PCr levels in perfused GLC$_4$ a, and GLC$_4$/ADR b, cells at 37°C. After the $^{31}$P NMR spectra at $t = 0$ were obtained, adriamycin was added to the perfusate. Levels were obtained from peak heights of the metabolite in the $^{31}$P NMR spectra, as described in Materials and methods. a, control (---), 0.05 $\mu M$ (---), 1 $\mu M$ (---) and 30 $\mu M$ (----) adriamycin, b, control (---), 1 $\mu M$ (---), 30 $\mu M$ (----) and 200 $\mu M$ (----) adriamycin. Points, mean of three experiments, bars, s.d.

Figure 5 Effect of different adriamycin concentrations on $\beta$ATP levels in perfused GLC$_4$ a, and GLC$_4$/ADR b, cells at 37°C. After the $^{31}$P NMR spectra at $t = 0$ were obtained, adriamycin was added to the perfusate. Levels were obtained from peak heights of the metabolite in the $^{31}$P NMR spectra, as described in Materials and methods. a, control (---), 0.05 $\mu M$ (---), 1 $\mu M$ (---) and 30 $\mu M$ (---) adriamycin, b, control (---), 1 $\mu M$ (---), 30 $\mu M$ (---) and 200 $\mu M$ (---) adriamycin. Points, mean of three experiments, bars, s.d.
and GLC₄/ADR cells were almost identical to changes in βATP intensity (results not shown). Neither the percentage of unbound ATP nor the intracellular Mg²⁺ concentration changed in the presence of various concentrations of adriamycin.

The energy status of a cell could be described by the PCr/βATP ratio. We averaged the results from 1 to 5 h, 6 to 10 h and 11 to 15 h for the different adriamycin concentrations used (Figure 7). From this figure it could be concluded that the PCr level decreased faster than the βATP level in both cell lines in response to high adriamycin concentrations, 30 μM for GLC₄ (t = 5–15 h, P < 0.01 vs control) and 200 μM for GLC₄/ADR (t = 10–15 h, P < 0.025 vs control). With 0.05 μM adriamycin the PCr level decreased faster than the βATP level in GLC₄ cells (t = 10–15 h, P < 0.05 vs control).

**Figure 6** ³¹P NMR spectra (121.45 MHz) of perfused GLC₄, a, and GLC₄/ADR, b, cells at different intervals in the presence of 30 μM adriamycin. Adriamycin was added to the perfusate directly after the spectra at t = 0 h were obtained. Peak assignments are as described in Figure 3.

**Effect of adriamycin on phospholipid metabolite levels of perfused GLC₄ and GLC₄/ADR cells**

PC levels in untreated GLC₄ cells increased to 170% of the initial value (t = 2–15 h, P < 0.05 vs control) (Figure 8a). In the presence of 30 μM adriamycin the level slowly dropped to 40% of the initial value (t = 5–15 h, P < 0.005 vs control) (Figures 6a and 8a). In untreated GLC₄/ADR cells PC levels increased to 150% (t = 3–15 h, P < 0.05 vs t = 0) (Figure 8b). In the presence of increasing concentrations of adriamycin PC levels increased less compared to levels in untreated cells (30 μM adriamycin, t = 7–15 h, P < 0.01 vs control) and even decreased in the presence of 200 μM adriamycin (t = 3–15 h, P < 0.025 vs control). The low intensity made the level of GPC difficult to estimate; consequently the results were averaged. Levels of GPC were expressed as percentage of the initial peak height of βATP at 0 h, since GPC levels were sometimes undetectable in the 0 h spectra. GPC peaks were almost undetectable in GLC₄ and did not change in the presence of adriamycin (Figures 3a and 6a). GPC levels in GLC₄/ADR increased from 37 ± 9% (s.d.) in untreated cells to 63 ± 17% (P < 0.05 vs control) in 1 μM adriamycin treated cells and to 76 ± 29% (P < 0.05 vs control) in 30 μM treated cells (Figures 3b and 6b). In the presence of 200 μM adriamycin GPC levels increased to 62 ± 6% (P < 0.005 vs control) and after 8 h decreased to control values.

**Extracts of adriamycin treated cells**

Extracts were made of cells treated with adriamycin to examine the possibility that changes in components as estimated in intact cells spectra were actually due to the appearance of new components. After treatment of GLC₄ cells and GLC₄/ADR cells with 1 and 30 μM adriamycin for 5 h similar results were obtained in extracts as in perfused cells, while no new components were detected in the spectra (results not shown). Extracts were made from control GLC₄ cells and from cells continuously incubated with 0.05 μM and 1 μM adriamycin for 15 h. Two unassigned components, probably PE and AMP, were clearly visible to the low field of PC (l) in control GLC₄ cell spectra (Figure 9a), that disappeared after treatment with 1 μM adriamycin (results not shown). No new components were detected in extract spectra of control GLC₄/ADR cells (Figure 9b) and extract spectra from cells after treatment with 1 and 30 μM adriamycin for 15 h. °H NMR spectra of these extracts showed no changes at all in the presence of adriamycin (results not shown).
PC was observed in cell extracts as well as intact cells (Figures 1–3). The PE resonance was not present in the spectra, since these cells were grown in media without ethanolamine. However, these cells can still produce phosphatidylethanolamine by decarboxylation of phosphatidylserine (Daly et al., 1987; Ansell & Spanner, 1982). Phospholipid analysis of our cell lines indeed revealed the presence of phosphatidylethanolamine (Zijlstra et al., 1987b). The presence of PC and PE in tumour cells might be of diagnostic value, since in vivo human tumours showed elevated levels of PC and PE compared to the tissue of origin (Daly & Cohen, 1989).

In adriamycin-resistant MCF-7 breast cancer cells, PC levels were increased, while PC, GPC, GPE and diphosphodiester levels were decreased compared to the ATP level (in the original report the PC and PE peaks were assigned to sugar phosphates) (Cohen et al., 1986). NMR studies of other cell lines indicated that differences observed in metabolite levels did not correlate specifically with drug-resistance (Evelhoch et al., 1987). Decreased GPC, PC and PE levels were also observed in in vivo adriamycin-resistant 17/A adenocarcinomas, but as the untreated tumours progressed, the differences between the adriamycin-sensitive and -resistant tumours disappeared (Evelhoch et al., 1987). Therefore, it is uncertain whether differences in phosphorus metabolite levels are related to the MDR or the atypical MDR phenotype.

Both GLC₄ and GLC₄/ADR are anchorage-independent cell lines. Since an increase in PC could be related to an

Discussion

³¹P NMR spectra from perfused cells and extracts showed the same resonances. Several cellular compartments have been described such as mitochondria that might influence the peak intensity of βATP (Gupta & Yushok, 1980b). However, phosphorus metabolite content in cellular extracts as determined by ³¹P NMR and biochemical analysis are in agreement (Desmoulin et al., 1986). Furthermore, since spectral resolution was enhanced and complete relaxation of the phosphorus resonances spectra was obtained in our extracts, the significance of differences observed in peak areas relative to the βATP peak area is better indicated by comparing cell extracts. The relative PCr concentration was higher in extracts of GLC₄/ADR compared to GLC₄, however the ratio PCr/Cr was not changed. The percentage of unbound ATP and the intracellular Mg²⁺ concentration were similar in intact GLC₄ and GLC₄/ADR cells. These results indicate that the equilibrium constant for the creatine kinase reaction and the equilibrium of this reaction are equal for both cell lines. The calculated intracellular Mg²⁺ concentration of 0.3 mM was comparable with the intracellular Mg²⁺ concentration of 0.4 mM in Ehrlich ascites tumour cells (Gupta & Yushok, 1980b). The relatively higher PCr level in GLC₄/ADR could increase the capacity of these cells to maintain the ATP pool. In a previous report, high levels of PCr and low levels of diphosphodiester were observed in variant SCLC cell lines compared to classic SCLC cell lines (Knop et al., 1987). Thus, the presence of high levels of PCr and the absence of diphosphodiester support our earlier characterisation of these two cell lines as variant SCLC cell lines (Zijlstra et al., 1987a).

Figure 7: Effect of different adriamycin concentrations on the PCr/βATP ratio in perfused GLC₄ a, and GLC₄/ADR b, cells at 37°C. After the ³¹P NMR spectra at t = 0 h were obtained, adriamycin was added to the perfusate. Ratios were obtained from peak heights of the metabolites in the ³¹P NMR spectra, as described in Materials and methods and Results.

Figure 8: Effect of different adriamycin concentrations on PC levels in perfused GLC₄ a, and GLC₄/ADR b, cells at 37°C. After the ³¹P NMR spectra at t = 0 h were obtained, adriamycin was added to the perfusate. Levels were obtained from peak heights of the metabolite in the ³¹P NMR spectra as described in Materials and methods, a, Control (---O---), 0.5 μM (---●---), 1 μM (---■---) and 30 μM (---▲---) adriamycin, b, Control (---O---), 1 μM (---■---), 30 μM (---▲---) and 200 μM (---△---) adriamycin. Points, mean of three experiments, bars, s.d.
increased cell growth (Daly et al., 1987), the significant increase in PC and ATP in the control experiments with continuous perfused cells was probably due to cell growth. Furthermore, the perfusion experiments showed that several phosphorus metabolites were response-specific biochemical markers of adriamycin sensitivity and resistance. When GLC2 cells were treated with 30 μM adriamycin ATP levels increased faster than levels in untreated cells. This increase could either be due to a decreased energy consumption or an increased energy production. Even treatment with 200 μM adriamycin did not result in a strong increase of the ATP level in GLC2/ADR, although the level dropped to undetectable during the experiment in a way similar to ATP levels in GLC2 cells treated with 30 μM adriamycin. In both cell lines the PCR/ATP ratio decreased in response to high adriamycin concentrations. PCR was probably used to maintain the ATP pool at a stable level via creatine kinase as described in muscle (Bessman & Carpenter, 1985).

An interesting finding was the effect of adriamycin treatment on the PC and GPC level only in GLC2/ADR cells. We could not confirm the increase in GPC levels in extracts. There are two possibilities. First, by using the continuous perfusion system GLC2/ADR cells were physically stressed resulting in an increase in phospholipid turnover that was stimulated by adriamycin treatment. Secondly, the peak we saw, was not due to GPC, but to a phospholipid component also resulting from an increased phospholipid turnover. In phospholipid synthesis, choline is converted to PC and further converted to phosphatidylcholine (Ansell & Spanner, 1982). This phospholipid is degraded to GPC and then to choline by glycophosphocholine phosphodiesterase (EC 3.1.4.2) (Ansell & Spanner, 1982; Morash et al., 1988). The increased phospholipid turnover might be related to the reduced adriamycin accumulation in GLC2/ADR cells. The reduced drug accumulation was not due to the increased activity of the P-glycoprotein (Zijlstra et al., 1987a; De Jong et al., 1990).

In human and rat neuroectodermal tumours ATP levels decreased strongly within 6 to 12 h after cyclophosphamide, vincristine and methotrexate treatment, while PCR levels remained undetectable (Naruse et al., 1985). In MOPC 104E myeloma PCR/ATP ratio increased within 1 day after treatment with a curative dose of cyclophosphamide or 1,3-bis(2-chloroethyl)-1-nitrosourea, while PCR and ATP levels strongly reduced within 4 days (Ng et al., 1982). In this study it was concluded, that the changed PCR/ATP ratio must partially reflect the effect of the chemotheraphy on energy metabolism within the tumour cells. ATP/PI and PCR/PI ratios in adriamycin-sensitive mammary 17/A adenocarcinoma (Evelhoch et al., 1987), in the RIF-1 fibrosarcoma (Li et al., 1988) and in GL gliosarcoma (Steen et al., 1988) were increased after adriamycin, cyclophosphamide and 1,3-bis(2-chloroethyl)-1-nitrosourea treatment, respectively. The increase of these ratios after treatment was explained by reenergization of the tumour, while untreated control tumours in these studies showed declining ATP and PCR levels (Evelhoch et al., 1987; Li et al., 1988; Steen et al., 1988).

Untreated neuroectodermal tumours were still in an active stage which may explain the fast reduction in ATP levels 3 h after treatment with cyclophosphamide (300 mg kg⁻¹) (Naruse et al., 1985), while an opposite effect of cyclophosphamide (300 mg kg⁻¹) was seen in RIF-1 fibrosarcoma (Li et al., 1988). Untreated MOPC 104E myelomas were in a moderate active stage which may explain the slow decrease in ATP levels in 4 days (Ng et al., 1982).

In our in vitro experiments the continuous perfused cells were supplied with sufficient nutrients. Therefore, these results showed without any interference from reenergization that adriamycin treatment had an effect on the energy metabolism in the adriamycin-sensitive GLC2 tumour cells which resulted in an increase of cellular ATP. The strong decrease in ATP and PCR levels after treatment were comparable with the effects of chemotherapy in vivo tumours that were in a metabolic active stage (Ng et al., 1982; Naruse et al., 1985). The same adriamycin concentration had no effect on ATP and PCR levels in the adriamycin-resistant GLC2/ADR tumour cells compared to untreated GLC2/ADR cells. No differences in phosphorus metabolites were observed in adriamycin treated and untreated adriamycin-resistant mammary 17/A adenocarcinoma, while adriamycin had a large effect on the adriamycin-sensitive tumour (Evelhoch et al., 1987). To distinguish drug-sensitive from drug-resistant tumours in patients, it will be necessary to compare changes in phosphorus metabolite levels in tumours after treatment with an estimation of the changes in nucleoside triphosphates and PCR levels in this tumours that would occur without chemotherapeutic treatment, since the metabolic stage of a tumour probably determines the changes in phosphorus metabolites after chemotherapy. To obtain a reliable estimation, tissue heterogeneity, tumour size, type of tumour, the glycolytic rate of the tumour, tumour hypoxia and the degree of vascularisation of a tumour have to be determined. Till now only a few often preliminary data are available on human tumour bioenergetics and responses to chemotherapy observed by NMR (Steen, 1989). The continuous perfusion system can be used to study the relation between hypoxia and/or glucose deprivation and chemotherapeutic effectivity in in vitro experiments.
In conclusion, $^{31}$P NMR spectroscopy can be used in in vitro experiments to reveal biochemical markers for adriamycin-resistance and -sensitivity. However, much more in vitro and in vivo studies with drug-sensitive and drug-resistant cells are necessary to obtain parameters to distinguish drug-sensitive from drug-resistant tumours in patients after chemotherapy by NMR spectroscopy.

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