Identification of magnetic resonance detectable metabolic changes associated with inhibition of phosphoinositide 3-kinase signaling in human breast cancer cells

Mounia Beloueche-Babari,1 L. Elizabeth Jackson,1 Nada M.S. Al-Saffar,1 Suzanne A. Eccles,2 Florence I. Raynaud,2 Paul Workman,2 Martin O. Leach,1 and Sabrina M. Ronen1

1Cancer Research UK Clinical Magnetic Resonance Research Group, The Institute of Cancer Research and The Royal Marsden NHS Foundation Trust; 2Cancer Research UK Centre for Cancer Therapeutics, The Institute of Cancer Research, Sutton, Surrey, United Kingdom

Abstract

Phosphoinositide 3-kinase (PI3K) is an attractive target for novel mechanism-based anticancer treatment. We used magnetic resonance (MR) spectroscopy (MRS) to detect biomarkers of PI3K signaling inhibition in human breast cancer cells. MDA-MB-231, MCF-7, and Hs578T cells were treated with the prototype PI3K inhibitor LY294002, and the 31P MR spectra of cell extracts were monitored. In every case, LY294002 treatment was associated with a significant decrease in phosphocholine levels by up to 2-fold (P < 0.05). In addition, a significant increase in glycerophosphocholine levels by up to 5-fold was also observed (P < 0.01). The content of glycerophosphoethanolamine, when detectable, did not change significantly. Nucleotide triphosphate levels did not change significantly in MCF-7 and MDA-MB-231 cells but decreased by ~1.3-fold in Hs578T cells (P = 0.01). The changes in phosphocholine and glycerophosphocholine levels seen in cell extracts were also detectable in the 31P MR spectra of intact MDA-MB-231 cells following exposure to LY294002. When treated with another PI3K inhibitor, wortmannin, MDA-MB-231 cells also showed a significant decrease in phosphocholine content by ~1.25-fold relative to the control (P < 0.05), whereas the levels of the remaining metabolites did not change significantly. Our results indicate that PI3K inhibition in human breast cancer cells by LY294002 and wortmannin is associated with a decrease in phosphocholine levels. [Mol Cancer Ther 2006;5(1):187–96]

Introduction

The phosphoinositide 3-kinase (PI3K) pathway is a signaling cascade that is activated following association of PI3K with activated Ras proteins, stimulated growth factor receptors, and G protein-coupled receptors as well as via chemokine receptors and adhesion molecules (1, 2). PI3Ks are a family of lipid kinases that catalyze the formation of phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-triphosphate; these products selectively bind to many signaling proteins, including phosphoinositide-dependent kinase 1, protein kinase B/Akt, and Rac, leading to activation of downstream signaling pathways (1, 2). The PI3K family comprises three main classes: I, II, and III (3, 4); its members mediate many key cellular processes, including growth and survival, cell cycle entry, adhesion, and migration (5).

Growing evidence suggests that aberrant PI3K signaling could play a crucial role in the development of many human cancers (3–7). Ras proteins are activated in ~30% of all human cancers (8). Moreover, activation of Akt has been reported in a range of human tumors (7, 9). Amplification and mutation of the PIK3CA gene, which encodes the PI3K catalytic subunit p110α, has been identified in several types of cancer, including ovarian, cervical, breast, and brain tumors (10–13). Furthermore, deletion or reduced expression of the tumor suppressor PTEN, which encodes a lipid phosphatase that reverses PI3K activity, is very common and leads to sustained activation of PI3K signaling (3, 4, 7, 14). Based on these observations, it is now believed that PI3K signaling is an attractive target for mechanism-based anticancer treatment (15, 16).

Detecting biomarkers of target inhibition is critical for assessing the efficacy of treatment and for correlating antitumor effects with target suppression and is playing an increasingly important part in the clinical evaluation of novel molecular therapeutics (17, 18). Current techniques for measuring proof-of-concept, pharmacodynamic, or response biomarkers are surgically invasive. Thus, noninvasive end points would be extremely valuable (17).
Magnetic resonance spectroscopy (MRS) is a powerful technique for studying tissue biochemistry, as it allows the detection of several metabolites simultaneously and noninvasively (19). MRS is now increasingly used for monitoring tumor cell metabolism and alterations in response to therapy in cultured cells, animal models, and patients (20–22).

Typical in vivo $^{31}$P MR spectra of human tumors show a higher content of phosphomonoesters composed of phosphoethanolamine and phosphocholine as well as increased levels of phosphodiester composed of glycerophosphocholine and glycerophosphoethanolamine relative to normal tissues (20). In vitro MRS studies have revealed metabolic alterations that could be related to cellular transformation. In human mammary epithelial cells, malignancy was associated with increased levels of phosphocholine, total choline-containing metabolites, and the phosphocholine/glycerophosphocholine ratio (23). In rat Schwann cells, immortalization and transformation were associated with an increase in phosphocholine and a decrease in glycerophosphocholine levels (24). These alterations in phospholipid metabolites have been attributed to increased rates of choline transport and phosphorylation and changes in levels of phospholipid-metabolizing enzymes relative to noncancer cells (25–27).

We have shown that oncogenic transformation with mutated Ras in murine fibroblasts is associated with increased phosphocholine levels that are reversed following inhibition of Ras signaling (28). Work from our laboratory has also shown that the heat shock protein 90 inhibitor 17-allylamino-17-demethoxygeldanamycin, which depletes many oncoproteins, including Raf-I and Akt, leads to modulation of choline metabolism in human breast and colon carcinoma cells (29, 30). More recently, we have shown that down-regulation of extracellular signal-regulated kinase (ERK1/2) mitogen-activated protein kinase (MAPK) signaling with the MAPK kinase inhibitor U0126 is associated with an increase in phosphocholine and a decrease in glycerophosphocholine levels (31). This suggests that the effect on phosphocholine is downstream of both PI3K and Raf-MEK-ERK signaling pathways. In addition to this finding, the results suggest that MRS monitoring of phospholipid changes could be potentially useful in the noninvasive measurement of the effects of therapeutic modulators of both signaling pathways.

**Materials and Methods**

**Cell Lines**

For this study, three human breast cancer cell lines with increased expression of the epidermal growth factor receptor and activating mutation or overexpression in the three main Ras gene variants were used. These were MCF-7 with amplified N-Ras, MDA-MB-231 with activated K-Ras, and Hs578T with activated H-Ras. Other differences in genetic abnormalities included the status of the tumor suppressor p53, which was wild type in MCF-7 cells but mutated in MDA-MB-231 and Hs578T cells.

**Cell Culture**

MCF-7 and MDA-MB-231 cells were grown in DMEM containing 10% heat-inactivated FCS, 100 units/mL penicillin, and 100 μg/mL streptomycin. Hs578T cells (kindly donated by Dr. K. Colston, St. George’s Hospital, London, United Kingdom) were grown in RPMI 1640 containing 10% FCS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L l-glutamine. Cells were maintained at 37°C in a humidified 5% CO$_2$ atmosphere, and growth medium was replenished every 48 hours. All tissue culture materials were purchased from Life Technologies (Paisley, United Kingdom).

**Growth Inhibition Assay**

The effect of drug treatment on cell proliferation was measured using the sulforhodamine B assay (35). Briefly, cells growing in 96-well microtiter plates were treated with varying concentrations of LY294002 (Calbiochem, Nottingham, United Kingdom) or wortmannin (Sigma, Dorset, United Kingdom) for 96 hours. At the end of the treatments, cultures were fixed in 150 μL cold trichloroacetic acid for 30 minutes and then stained overnight with 0.4% (w/v) sulforhodamine B made up in 1% acetic acid. Plates were washed in 1% acetic acid to remove excess dye, treated with 150 μL of 10 mmol/L Tris to dissolve protein-bound sulforhodamine B, and then read on a Molecular Devices (Sunnyvale, CA) plate reader at 570 nm.

**PI3K Signaling Inhibition**

The three breast cell lines were treated with the PI3K inhibitor LY294002 at a concentration and exposure time that achieved ~50% drop in cell number per flask as well as suppression of PI3K signaling as measured by Akt phosphorylation at the Ser$^{473}$ residue (see below). MDA-MB-231 and MCF-7 cells were thus treated for 40 hours with 50 μmol/L LY294002, and Hs578T cells were treated for 30 hours with 100 μmol/L LY294002. In addition,
MDA-MB-231 cells were also treated for 24 hours with 225 μmol/L wortmannin, another PI3K inhibitor. The effect of PI3K inhibition on cell counts was assessed by determining the number of attached cells in each flask using a hemocytometer and trypan blue exclusion of dead cells.

**Western Blotting**

The effect of signaling inhibition on target protein levels was analyzed by Western blotting for phosphorylated Akt (P-Akt) following LY294002 and wortmannin treatments. For this, cells were treated with lysis buffer (150 mmol/L NaCl, 28.2 mmol/L Tris-HCl, 1.1 mmol/L Tris base, 0.2% SDS, 1% NP40, 10% glycerol, 0.5 mmol/L sodium orthovanadate, 10 mmol/L sodium pyrophosphate, 0.1 mol/L NaCl, and 1 mmol/L EDTA) and equal amounts of protein, measured using the Bio-Rad (Herford, United Kingdom) assay method and bovine serum albumin as a standard, were loaded onto 10% polyacrylamide gels and protein was transferred onto Immobilon-P membranes (Millipore, Bedford, MA). Blots were blocked in 5% nonfat milk for 2 hours and then incubated with primary anti-P-Akt Ser473 antibody or anti-total Akt antibody (Cell Signaling, Hertfordshire, United Kingdom) in 2% bovine serum albumin overnight. This was followed by incubating the membranes with horseradish peroxidase–conjugated anti-rabbit secondary antibody (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) for 1 hour. Equal gel loading was verified by incubating the blots with an anti-glyceraldehyde-3-phosphate dehydrogenase antibody (Chemicon, Hampshire, United Kingdom) followed by an anti-mouse secondary antibody in 5% nonfat milk for 1 hour. Specific binding of the above antibodies to their target antigens was detected using Enhanced Chemiluminescence Plus reagents (Amersham Biosciences, Buckinghamshire, United Kingdom) and exposure to X-OMAT Kodak autoradiography film (Rochester, NY).

**Cell Cycle Analysis**

Flow cytometry was used to determine the effect of PI3K signaling inhibition on cell cycle distributions. This was achieved by fixing ~2 × 10^6 trypsinized cells in 70% cold ethanol and then staining with a PBS solution containing 40 μg/mL propidium iodide and 100 μg/mL RNase A (Sigma). Cells were sorted using an Elite ESP Beckman Coulter (High Wycombe, United Kingdom) cell sorter at 488 nm, and data were analyzed using WinMDI computer software version 2.7 (Scripps Institute, La Jolla, CA) and Cylchrome computer software version 1.0.2 (College of Medicine, University of Wales, Wales, United Kingdom).

**Cell Extracts for 31P MRS Measurements**

These were done on 1 × 10^8 to 2 × 10^8 cells growing in the logarithmic phase using the dual-phase extraction method (28). Lyophilized samples of the water-soluble fraction were reconstituted in 700 μL of a D_2O solution containing 10 mmol/L EDTA and 0.86 mmol/L methyl-enediphosphonic acid (as internal reference) at pH 8.2.

**Intact Cell Experiments**

MDA-MB-231 cells were treated in culture with 50 μmol/L LY294002 for 40 hours as described above. At the end of the treatment, cells (~3 × 10^7–4 × 10^7 in total) were collected by trypsinization and washed with D_2O-saline, resuspended in a total volume of 500 μL D_2O-saline containing 2 mmol/L freshly made up phosphocreatine, and then transferred into a 5-mm nuclear magnetic resonance tube. The total time of cell handling before the commencement of the nuclear magnetic resonance measurement was ~25 minutes. At the end of each experiment, cell viability was monitored using trypan blue staining. Cell morphology was also assessed using confocal microscopy and staining for nuclear material and actin filaments using ToPro-3 and phalloidin, respectively.

**31P MRS Acquisition and Analysis**

31P MR spectra were acquired at room temperature on a 500-MHz Bruker spectrometer using composite pulse 1H decoupling, a 30° flip angle, a 2-second repetition time, a spectral width of 100 ppm, and 32K data points. Total acquisition times were in the region of 3 hours for cell extracts and 7 minutes for intact cells. A 1-Hz line broadening was applied for the spectra from cell extracts and 10 Hz for the spectra from intact cells and all spectra were analyzed using MestRe-C version 2.3 (University of Santiago de Compostela, Santiago de Compostela, Spain).

The absolute metabolite content of each cell in the extract experiments was determined by peak integration normalized relative to the internal reference and corrected for cell number per sample and for saturation. In the intact cell measurements, the chemical shift of the MR spectra was referenced relative to the glycero-phosphocholine signal at 0.49 ppm. As the spectra of MDA-MB-231 cell extracts showed no intracellular phosphocreatine signal (Fig. 3), the phosphocreatine signal in the intact cell spectra was taken to represent exogenously added phosphocreatine and was used as a semiquantitation standard. No phosphocreatine signal was recorded in the spectra of intact MDA-MB-231 cells resuspended in D_2O-saline alone (data not shown), further confirming that phosphocreatine was mostly extracellular. Preliminary measurements showed little change in the intensity of the phosphocreatine signal in our samples within the period of the experiment.

**Statistical Analysis**

Two-tailed unpaired Student’s t test was used to verify the statistical significance of the results, with a P ≤ 0.05 considered to be significant. All results are expressed as mean ± SD.

**Results**

**Effect of PI3K Signaling Inhibition with LY294002 on Human Breast Cancer Cell Extracts**

Treatment with the PI3K inhibitor LY294002 caused a significant antiproliferative effect on the three cell lines as measured by the sulforhodamine B assay with IC_{50} values for a 96-hour exposure of 1.7 ± 0.3, 6.5 ± 0.4, and 5.3 ± 0.4 μmol/L for MCF-7, MDA-MB-231, and HS578T cells, respectively (n ≥ 3). In all subsequent experiments,
LY294002 was used at a concentration and exposure time that achieved a ~50% drop in cell number per flask as well as blockade of PI3K signaling indicated by the reduction in P-Akt levels (Fig. 1).

The effect of LY294002 on cell cycling was also investigated in the three breast lines by flow cytometry. Figure 2 shows an example of DNA histograms acquired from control and LY294002-treated MCF-7 and MDA-MB-231 cells. The effect of LY294002 on cell cycle distribution was cell line dependent. In MCF-7 cells, the inhibitor caused a significant increase in the G1-phase population (53 ± 3 to 81 ± 2%; n = 6; P = 0.00001) and a decrease in the fraction of cells in S phase (38 ± 4 to 14 ± 2%; P = 0.00001) and G2 phase (9 ± 3 to 5 ± 2%; P = 0.01; Fig. 2A). In Hs578T cells, LY294002 also caused a drop in the S-phase fraction (24 ± 2 to 18 ± 1%; n = 3; P = 0.02). However, no significant effect on cell cycle distribution was seen in MDA-MB-231 cells following exposure to growth inhibitory concentrations (P > 0.2; Fig. 2B).

We next set out to correlate the molecular and cellular response to LY294002 with the 31P MRS of treated cells to detect the metabolic alterations, if any, associated with PI3K signaling inhibition. A typical spectrum acquired from the aqueous fraction of MDA-MB-231 cell extracts is illustrated in Fig. 3, and data obtained from LY294002 treatment of all three cell lines are summarized in Table 1. The most consistent and significant alterations observed following treatment with LY294002 were a reduction in phosphocholine levels and an increase in glycerophosphocholine levels. Cellular phosphocholine levels decreased from 42 ± 2 to 32 ± 1 fmol/cell in MDA-MB-231 cells (P = 0.002), from 7.7 ± 0.7 to 4 ± 1 fmol/cell in Hs578T cells (P = 0.03), and from 16 ± 3 to 11 ± 3 fmol/cell in MCF-7 cells (P = 0.04). LY294002 treatment caused glycerophosphocholine content to increase from 6 ± 2 to 12 ± 1 fmol/cell in MDA-MB-231 cells (P = 0.01), from 18 ± 7 to 29 ± 8 fmol/cell in MCF-7 cells (P = 0.05), and from 1.0 ± 0.3 to 5.5 ± 0.5 fmol/cell in Hs578T cells (P = 0.0009). Phosphoethanolamine was only detectable in MCF-7 cells and its levels increased from 6 ± 2 to 12 ± 3 fmol/cell (P = 0.01) following LY294002 treatment. Nucleotide triphosphate levels did not change significantly in MDA-MB-231 and MCF-7 cells but dropped from 18 ± 1 to 14 ± 1 fmol/cell in Hs578T cells (P = 0.01) following inhibitor treatment. Glycerophosphoethanolamine was only detectable in MCF-7 and MDA-MB-231 cells and its levels also increased up to 2-fold in these cells, but this did not reach statistical significance.

Following LY294002 treatment, the glycerophosphocholine/nucleotide triphosphate ratio increased significantly to 247 ± 46% (P = 0.01) in MDA-MB-231 cells, 210 ± 45% (P = 0.003) in MCF-7 cells, and 820 ± 250% (P = 0.05) in Hs578T cells. Furthermore, the phosphocholine/glycerophosphocholine ratio decreased significantly to 42 ± 7% (P < 0.0001), 40 ± 13% (P = 0.004), and 10 ± 5% (P = 0.0007) relative to control in MCF-7, MDA-MB-231, and Hs578T cells, respectively.

Effect of PI3K Inhibition with LY294002 in Live MDA-MB-231 Cells

31P MRS measurements were also done on intact MDA-MB-231 cells following treatment with LY294002 and the spectra are illustrated in Fig. 4. The method used did not compromise normal cell morphology (data not shown) or viability (consistently in the region of 95–99%). In addition, it allowed rapid probing of cellular 31P MRS-detectable metabolites, as all the signals detectable in the spectra from cell extracts were also present in the spectra from the intact cells with the exception of inorganic phosphate, presumably because of its absence from the extracellular milieu. The metabolite ratios of phosphocholine/glycerophosphocholine and phosphocholine/nucleotide triphosphate were also comparable in extracts and intact cell measurements (i.e., 7 ± 3 versus 6 ± 1 and 4 ± 1 versus 5 ± 3, respectively).

31P MRS analysis of intact MDA-MB-231 cells indicated that LY294002 treatment was associated with a significant decrease in phosphocholine levels to 68 ± 8% (n = 4; P = 0.006) and an elevation in glycerophosphocholine

**Figure 2.** Effect of inhibition with LY294002 on cell cycle distributions in MCF-7 and MDA-MB-231 human breast cancer cells. DNA histograms generated from flow cytometry analysis showing an increase in the G1 fraction and the drop in the S and G2 fractions in MCF-7 cells but no alterations in cell cycle distribution in MDA-MB-231 cells following treatment with 50 μmol/L LY294002 for 40 h.

**Figure 1.** Effect of LY294002 on PI3K signaling in MDA-MB-231, MCF-7, and Hs578T cells. Western blots showing depletion of P-Akt (Ser473) in MCF-7 and MDA-MB-231 cells treated with 50 μmol/L LY294002 for 40 h and Hs578T cells treated with 100 μmol/L LY294002 for 30 h. Total Akt is also shown together with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control.
levels to 145 ± 23% (P = 0.03) relative to the control, whereas the cellular content of glycerophosphoethanolamine and nucleotide triphosphate remained unchanged within experimental uncertainty (equal to 130 ± 50%; P = 0.4 and 90 ± 35%; P = 0.7, respectively). Furthermore, a substantial decrease in the phosphocholine/glycerophosphocholine ratio to 49 ± 13% (P = 0.006) was also recorded.

Effect of PI3K Inhibition with Wortmannin in MDA-MB-231 Cell Extracts

Treatment of MDA-MB-231 cells with wortmannin was associated with a significant reduction in cell proliferation with an IC_{50} following a 96-hour exposure to the agent of 50 μmol/L (n = 2). In addition, a substantial decrease in P-Akt levels (Fig. 5A) was also detected, thereby confirming blockade of PI3K signaling following exposure to wortmannin. This treatment led to significant cell cycle distribution changes (Fig. 5B): an increase in the G_1 phase (48 ± 3 to 66 ± 5%; n = 3; P = 0.01) and a decrease in the S phase (38 ± 2 to 21 ± 3%; n = 3; P = 0.003) cell populations, whereas no significant change was detected in the G_2-phase cell population (16 ± 3% versus 13 ± 5%; P = 0.44).

As with LY294002, 31P MRS analysis of wortmannin-treated cells showed a significant reduction in cellular levels of phosphocholine to 80 ± 4% (n = 3; P = 0.02) relative to the control (Fig. 3). However, any changes in the remaining 31P-detectable metabolites (i.e., glycerophosphocholine, glycerophosphoethanolamine, and nucleotide triphosphate) were not statistically significant (95 ± 2%; P = 0.08; 93 ± 17%; P = 0.6; and 89 ± 4%; P = 0.06, respectively).

Table 1. Effect of PI3K signaling inhibition with LY294002 on 31P MRS-detectable metabolites of control and treated MDA-MB-231, MCF-7, and Hs578T cells

<table>
<thead>
<tr>
<th>Metabolites* (fmol/cell)</th>
<th>Treatment</th>
<th>MDA-MB-231 (n = 4)</th>
<th>MCF-7 (n = 6)</th>
<th>Hs578T (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphocholine</td>
<td>Control</td>
<td>42 ± 2</td>
<td>16 ± 3</td>
<td>7.7 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>LY294002</td>
<td>32 ± 1</td>
<td>11 ± 3</td>
<td>4 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P = 0.002</td>
<td>P = 0.04</td>
<td>P = 0.03</td>
</tr>
<tr>
<td>Glycerophosphocholine</td>
<td>Control</td>
<td>6 ± 2</td>
<td>18 ± 7</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>LY294002</td>
<td>12 ± 1</td>
<td>29 ± 8</td>
<td>5.5 ± 0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P = 0.01</td>
<td>P = 0.05</td>
<td>P = 0.0009</td>
</tr>
<tr>
<td>Nucleotide triphosphate</td>
<td>Control</td>
<td>10 ± 3</td>
<td>11 ± 3</td>
<td>18 ± 1</td>
</tr>
<tr>
<td></td>
<td>LY294002</td>
<td>9 ± 1</td>
<td>8 ± 2</td>
<td>14 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P = 0.8</td>
<td>P = 0.2</td>
<td>P = 0.01</td>
</tr>
</tbody>
</table>

*Metabolite concentrations represent the content present in each control or LY294002-treated cell line.

Discussion

There is growing evidence that the PI3K signaling pathway, a well-characterized downstream effector of important cell membrane activities, could be involved in the development of a variety of human cancers (3, 4, 7, 10–13). Thus, blocking PI3K signaling could prove to be a useful approach for targeted anticancer therapy. Inhibitors of PI3K are now undergoing development (15, 16). Biomarkers of target inhibition are an essential feature of the development of novel molecular therapeutics, allowing a “pharmacologic audit trail” to be constructed that relates pharmacokinetic and pharmacodynamic end points to treatment response (17, 18, 36–38). Monitoring the molecular changes induced by PI3K inhibitors will facilitate the clinical evaluation of this novel type of therapy, as blockade of the intended target can be monitored and treatment outcome can be assessed. In contrast to most molecular end points, a minimally invasive method would have the advantage that human tissue biopsies would not be needed (17, 36).

In the present study, we used a noninvasive technique (i.e., MRS) to explore potential biomarkers associated with PI3K inhibition by the prototype inhibitors LY294002 and wortmannin (39) in human breast cancer cells. All three cancer cell lines used had increased activation of growth factor signaling and they each harbored an
activating mutation or overexpression in a different Ras variant. The choice of this model was based on previous observations showing that the different Ras isoforms can differ in their ability to activate downstream signaling pathways, such as the Raf-MEK-ERK1/2 MAPK, PI3K, and Rac pathways (40, 41), with PI3K being more efficiently activated by H-Ras than by K-Ras (42). Thus, our selection of cell lines allowed us also to assess whether similar effects might be observed in the presence of different activated Ras isoforms. In addition, our experimental model provided a means for assessing the effect of PI3K signaling inhibition on cellular metabolism under conditions of diverse basal enzyme activity. For example, the basal activity of PI3K is >3-fold higher in MDA-MB-231 cells relative to MCF-7 cells (43).

Preliminary studies were done to establish concentrations and exposure times that achieved ~50% decrease in cell counts as well as blockade of Akt phosphorylation. 31P MR spectra obtained from the three breast lines, exposed to treatment conditions that caused a similar marked decrease in Akt phosphorylation and cell number per flask, showed that inhibition with LY294002 was associated with a decrease in cellular phosphocholine content and an increase in the levels of cellular glycerophosphocholine. Phosphocholine decreased by up to 50%, whereas glycerophosphocholine increased up to 5-fold relative to the control. The changes were readily detectable and statistically significant in every case and were also seen in intact cells as well as in extracts of treated cells, indicating that they are alterations occurring within living cells.

To determine whether these metabolic alterations would also be triggered following exposure to another PI3K inhibitor, MDA-MB-231 cells were treated with the structurally dissimilar PI3K inhibitor wortmannin. Following exposure of MDA-MB-231 cells to wortmannin at a concentration and exposure time that caused a similar decrease in Akt phosphorylation and cell counts as was seen with LY294002, a significant decrease in phosphocholine content was observed. In contrast, wortmannin-treated MDA-MB-231 cells showed no significant alterations in glycerophosphocholine levels. In addition, there was no significant change in the cellular levels of glycerophosphoethanolamine or nucleotide triphosphate. Comparison of results obtained from experiments with LY294002 and wortmannin indicated that the drop in phosphocholine was the common metabolic marker of PI3K signaling down-regulation by the two inhibitors. Thus, it is likely that this effect on phosphocholine is related to the action of the two inhibitors on their common target PI3K. The increase in glycerophosphocholine, however, was associated with LY294002 treatment rather than PI3K inhibition. These results are consistent, robust, and reproducible in the three breast cancer lines and the reduction in phosphocholine is seen with both PI3K inhibitors, a finding that has not been reported previously.

The elevation in glycerophosphocholine that was observed with LY294002 but not wortmannin is of interest, because glycerophosphocholine levels also increase in human breast cancer cells following exposure to some chemotherapeutic agents (44). The basis for the difference in effect on glycerophosphocholine levels between LY294002 and wortmannin is unclear at present. However, this is not surprising given that the two compounds are structurally dissimilar and interact with different cellular targets in addition to PI3K (39, 45). Recently, a study has reported that LY294002 and its inactive analogue LY303511, but not wortmannin, potentiate the antiproliferative effect of chemotherapeutic agents via a non-PI3K-dependent pathway (46). Thus, it may be that the increase in glycerophosphocholine seen with LY294002 could reflect other effects exerted by this compound independently of the PI3K pathway. This hypothesis requires more detailed investigation.

Phosphocholine represents the first intermediate in the synthesis of the membrane phospholipid phosphatidylcholine via the Kennedy pathway and is formed following the phosphorylation of choline via choline kinase.

Figure 4. Effect of LY294002 treatment on the 31P MR spectra of intact MDA-MB-231 cells. 31P MR spectrum obtained from intact MDA-MB-231 cells. Inset, expansion of the PME and PDE regions (−1 to 6 ppm) in control and LY294002-treated cells. PCr, phosphocreatine.

Figure 5. Effect of wortmannin on PI3K signaling and cell cycle distribution in MDA-MB-231 cells. A, Western blots showing depletion of P-Akt (Ser473) levels in MDA-MB-231 following a 24-h exposure to 225 μmol/L wortmannin. Also included are total Akt and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. B, DNA histograms showing an increase in the G1 phase and a reduction in the S-phase cell populations following wortmannin treatment in MDA-MB-231 cells.
involvement of particular cell cycle changes in the observed cause cell cycle arrest (52), it was necessary to rule out the and that both LY294002 and wortmannin are known to progression by up-regulating cyclin D1 and degrading p27 shown to be higher in rapidly proliferating cells (49, 50) the cell cycle (48). Levels of phosphocholine have been the turnover of phospholipids is subject to regulation by (47). Phosphocholine can also be produced from phosphatidylcholine hydrolysis via phospholipase C (PLC; ref. 47). The precise mechanism linking the metabolic cycle of phosphocholine to the mitogenic signal transduction machinery needs to be investigated in more detail. However, it is established that growth factor stimulation and Ras activation lead to modulation of phospholipid metabolism (54, 55). More specifically, the PI3K pathway has been shown to participate in the activation of choline kinase as well as PLD (56, 57). Thus, possible explanations for the decrease in phosphocholine that follows treatment with LY294002 and wortmannin could be (a) inhibition of PLD activity causing a reduction in the free choline pool available for phosphor-ylation of choline by choline kinase. However, we cannot rule out the involvement of other choline-metabolizing enzymes, such as PLA and PLC, and CTP phosphocholine cytidylyl-transferase, which can also be modulated by Ras signaling (59–62), in the observed effect on phosphocholine.

Although a comprehensive review of the literature is beyond the scope of this discussion, Fig. 6 provides a decrease in phosphocholine levels. For this, we analyzed the cell cycle distribution of control and treated cells. Following exposure to LY294002, the percentage of cells in the G1 phase remained unaltered in MDA-MB-231 and Hs578T cells but increased in MCF-7 cells. In addition, a decrease in S-phase cell population was also recorded in MCF-7 and Hs578T cells but not in MDA-MB-231 cells. The basis for these differences is not clear. It is possible that genetic and biochemical differences (including Ras and p53 status) that exist between the three cell lines may be involved in defining the cellular response to LY294002. Because the drop in phosphocholine levels was common to all three cell lines, we concluded that the MRS changes induced by LY294002 could not simply be a result of alterations in cell cycle distribution. Furthermore, whereas LY294002 and wortmannin had differing effects on cell cycle distribution in MDA-MB-231 cells, they both caused a reduction in phosphocholine, further corroborating our conclusion that this change was not merely a consequence of cell cycle arrest. The roles played by phosphocholine within cells are multiple and diverse and include biosynthesis of lipid membranes and cell signaling (47). Hence, it is likely that its cellular content could be regulated by several factors, including (a) the availability of its precursor choline, (b) the levels and activity of choline-metabolizing enzymes, and (c) the extent to which signal transduction pathways may be involved in the regulation of choline metabolism. Treatment of the three breast lines used in this study as well as the HCT116 human colon carcinoma cell line with the MEK inhibitor U0126 also led to a significant decrease in cellular phosphocholine (31). This suggests that phosphocholine content could be regulated by signaling downstream of both PI3K and ERK1/2 MAPK pathways. Alternatively, it could involve the cross-talk that can occur between these two pathways at the level of Raf-1 and Akt (53).

The precise mechanism linking the metabolic cycle of phosphocholine to the mitogenic signal transduction pathways is beyond the scope of this discussion, Fig. 6 provides a detailed view of the regulatory pathways involved in phosphocholine metabolism. The figure illustrates the interactions between Ras-Raf-MEK-ERK1/2 pathway inhibitor U0126, and phospholipase C (PLC), as well as the HCT116 human colon carcinoma cell line with MEK inhibitor U0126. Metabolites: CDP-Chol, CDP choline; DAG, diacylglycerol; FAs, fatty acids; G3P, glycerol 3-phosphate; Lyso-PtdCho, 1-acyl- or 2-acyl-phosphatidylcholine; PA, phosphatidic acid; PtdCho, phosphatidylcholine. Black circles, metabolic enzymes; white rectangles, phospholipid metabolism. The roles played by phosphocholine within cells are multiple and diverse and include biosynthesis of lipid membranes and cell signaling (47). Hence, it is likely that its cellular content could be regulated by several factors, including (a) the availability of its precursor choline, (b) the levels and activity of choline-metabolizing enzymes, and (c) the extent to which signal transduction pathways may be involved in the regulation of choline metabolism. Treatment of the three breast lines used in this study as well as the HCT116 human colon carcinoma cell line with the MEK inhibitor U0126 also led to a significant decrease in cellular phosphocholine (31). This suggests that phosphocholine content could be regulated by signaling downstream of both PI3K and ERK1/2 MAPK pathways. Alternatively, it could involve the cross-talk that can occur between these two pathways at the level of Raf-1 and Akt (53).
MRS Detects Metabolic Markers of PI3K Inhibition

speculative model based on the currently available data. This model suggests more experiments that can be carried out to elucidate further details on the effects of molecular therapeutics on phosphocholine and other metabolites, including the use of RNA interference to knockdown particular components of the pathways shown. With respect to this model, it is increasingly apparent that activation of more than one downstream Ras effector pathway (e.g., Raf-1 and RafGDS) is necessary for the up-regulation of enzymes, such as choline kinase and PLD (56, 57, 63). Thus, abrogation of signaling via any one of the various effector pathways may well be sufficient for the effect on phosphocholine to become manifest as seen with the Raf-MEK-ERK1/2 pathway inhibitor U0126 (31). Interestingly, treatment of human breast and colon cancer cells with the heat shock protein 90 inhibitor 17-allylamino-17-demethoxygeldanamycin, an agent that is known to simultaneously block Raf-MEK-ERK1/2 and PI3K signaling (64), was associated with an elevation in levels of phosphocholine and glycerophosphocholine (29, 30). However, given the number of downstream pathways simultaneously modulated by this agent, these metabolic changes are likely to reflect the balance of interplay between the multiple molecular effects and consequently may not necessarily be associated with down-regulation of Raf-MEK-ERK1/2 or PI3K signaling exclusively.

We have shown that the common marker associated with PI3K signaling inhibition by LY294002 and wortmannin is a decrease in phosphocholine levels. Interestingly, this decrease in phosphocholine was also seen following inhibition of the ERK1/2 MAPK pathway (31) which suggests that phosphocholine modulation could involve many cellular players. More work is required to elucidate the basis for the observed changes in relation to signaling downstream of the PI3K and Raf-MEK-ERK1/2 pathways.

Although not specific to PI3K, the change in phosphocholine is robust, reproducible, and consistent across three cell lines and seen in both extracts and intact cells. It could therefore be a useful indicator of inhibition of both PI3K and Raf-MEK-ERK1/2 pathways, which are of major interest in modern cancer drug development. In this context, phosphocholine could be classified as a biomarker (i.e., a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic response to a therapeutic intervention) as distinct from a surrogate marker that is expected to predict clinical benefit (65). However, before the change in phosphocholine can be recommended as a biomarker for use with inhibitors of these pathways, it needs to be evaluated further, particularly in human tumor xenograft models.

The common drop in phosphocholine across all three cell lines regardless of the differences in oncogenic abnormalities present in each (e.g., Ras isoform activation and p53 status) suggests the potential value of phosphocholine as a biomarker for monitoring PI3K inhibition in a wide range of human cancers.

The broad utility of the change in phosphocholine as well as its noninvasive potential represent additional strengths. These are consistent with the need to develop biomarkers of target modulation, particularly those that are noninvasive, in modern drug development (18, 37). Although specific markers of pathway modulation are more desirable, validated generic markers of target modulation are also required in drug development. For example, proliferating cell nuclear antigen and Ki-67 staining are widely used proliferation markers for monitoring the effect of several types of anticancer treatments, including antihormonal agents and signaling inhibitors (66, 67). When validated in vivo, phosphocholine could potentially offer the added advantage of being a noninvasive biomarker of inhibition of the major oncogenic signal transduction pathways.

Progress in MRS methodology now allows for $^1$H decoupled $^{31}$P MRS to be done on clinical spectrometers, which facilitates the identification in patients of changes occurring in the individual components of the spectrum, including phosphocholine (68). This technical advance will be key in assessing the real value of MRS in providing biomarkers of response to PI3K inhibition in a clinical setting. Studies with isoform-selective PI3K inhibitors, when they become available, will also be important to further validate phosphocholine as a potential pharmacodynamic and/or response biomarker in vivo.

Acknowledgments

We thank Jenny Titley (Cancer Research UK Centre for Cancer Therapeutics) for assistance with flow cytometry analyses.

References


Mounia Beloueche-Babari, L. Elizabeth Jackson, Nada M.S. Al-Saffar, et al.

Identification of magnetic resonance detectable metabolic changes associated with inhibition of phosphoinositide 3-kinase signaling in human breast cancer cells

Updated version
Access the most recent version of this article at:
http://mct.aacrjournals.org/content/5/1/187

Cited articles
This article cites 64 articles, 28 of which you can access for free at:
http://mct.aacrjournals.org/content/5/1/187.full#ref-list-1

Citing articles
This article has been cited by 11 HighWire-hosted articles. Access the articles at:
http://mct.aacrjournals.org/content/5/1/187.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.