Magnetic Resonance Spectroscopic Pharmacodynamic Markers of the Heat Shock Protein 90 Inhibitor 17-Allylamino,17-Demethoxygeldanamycin (17AAG) in Human Colon Cancer Models

Yuen-Li Chung, Helen Troy, Udaib Banerji, Laura E. Jackson, Mike I. Walton, Marion Stubbs, John R. Griffiths, Ian R. Judson, Martin O. Leach, Paul Workman, Sabrina M. Ronen

Background: 17-Allylamino,17-demethoxygeldanamycin (17AAG) is a novel anticancer drug that inhibits heat shock protein 90 (Hsp90), resulting in proteosomal degradation of several oncogenic proteins. We used phosphorus magnetic resonance spectroscopy (31P-MRS) to determine whether 17AAG treatment leads to alterations in phospholipids that could serve as pharmacodynamic markers for tumor response to 17AAG. Methods: HCT116, HT29, and SW620 colon cancer cells were treated with 17AAG, and extracts were examined by 31P-MRS. HT29 cells were also treated with the active metabolite of 17AAG, 17-amino,17-demethoxygeldanamycin (17AG), or the inactive 17AAG analog NSC683666. Mouse nude mice carrying HT29 xenografts were examined using in vivo 31P-MRS before and after 17AAG treatment; xenograft tumor extracts were examined by 31P-MRS and proton MRS (1H-MRS). Hsp90 client protein expression was determined by using western blots. Two-tailed t tests were used to compare metabolite concentrations and ratios, and a Mann–Whitney U test was used to compare proportions. All statistical tests were two-sided. Results: 17AAG treatment led to statistically significantly increased phosphocholine levels in all three cell lines (P = 0.02). 17AG treatment also increased phosphocholine levels in HT29 cells, whereas NSC683666 had no effect. The phosphononoester/phosphodiester ratio was statistically significantly increased in the HT29 xenografts after 17AAG treatment relative to the pretreatment ratio (P = 0.02), whereas no statistically significant change was observed after vehicle treatment (P = 0.62). Statistically significant increases in phosphocholine, phosphoethanolamine, and valine levels were also observed in tumor extracts treated with 17AAG. Conclusions: Inhibition of Hsp90 by 17AAG resulted in altered phospholipid metabolism in cultured tumor cells and in tumor xenografts. The increases observed in phosphocholine and phosphonoester levels suggest that these metabolites may have the potential to act as noninvasive pharmacodynamic markers for analyzing tumor response to treatment with 17AAG or other Hsp90 inhibitors. [J Natl Cancer Inst 2003;95:1624–33]

A number of signaling molecules have been successfully targeted by new anticancer agents, including the antibody trastuzumab (Herceptin), which is directed against ErbB-2; tyrosine kinase inhibitors, such as imatinib (Gleevec), which inhibits Bcr-Abl, Kit, and platelet-derived growth factor receptor; and farnesyl transferase inhibitors, which inhibit the functioning of Ras and other signaling molecules (1,2). However, because of redundancy in signaling pathways and the considerable degree of cross-talk between them, inhibition of any one signaling molecule is likely to have only limited impact, especially against common solid tumors in which multiple genetic abnormalities are known to exist (3). In contrast, inhibition of the heat shock protein 90 (Hsp90) molecular chaperone offers the possibility of simultaneous inhibition of a number of signaling pathways and may therefore prove more effective as a cancer therapy (4,5). Hsp90 is a chaperone protein that, together with Hsp70 and other co-chaperones, ensures the correct folding of several oncogenic proteins involved in malignant progression, including c-Raf-1, Cdk4, ErbB-2, Met, Akt, and mutant p53. Inhibition of Hsp90 results in degradation of oncogenic client proteins via the ubiquitin proteasome pathway (6).

17-Allylamino,17-demethoxygeldanamycin (17AAG), a novel anticancer drug that inhibits Hsp90 (4,5.7), is a member of the benzoquinone ansamycin antibiotic family, which includes the geldanamycins and herbimycins. 17AAG has lower hepatic toxicity than its parent compound, geldanamycin, but the same potent anticancer activity (8–10). 17AAG is now in phase I clinical trials as a potential anticancer drug; the absence of antiproliferative-related toxicity observed to date suggests that 17AAG and related drugs may be combined with cytotoxic agents (4,5).

In previous studies (11,12), human colon cancer cells treated with 17AAG showed depletion of Hsp90 client proteins c-Raf-1 and Akt and inhibition of signal transduction, leading to cell cycle arrest and apoptosis. Depletion of these client proteins and elevation of Hsp70 expression (both mRNA and protein) represent a molecular signature that is diagnostic of Hsp90 inhibition (4,11). However, molecular analysis requires biopsies before and after treatment. Hence, a noninvasive surrogate marker for Hsp90 inhibition and, potentially, for analysis of treatment re-

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See “Notes” following “References.”

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sponse would be a more desirable endpoint to use both in clinical trials and eventually in patient management (13).

In vivo phosphorus magnetic resonance spectroscopy (31P-MRS) provides noninvasive biochemical information on both healthy and diseased tissues (14–16). This technique has also been used to study the biochemistry and physiology of tumor cells and solid cancers and to assess tumor response following therapies in both human and animal models (17–24). Markers for tissue bioenergetics, such as nucleoside triphosphate (NTP), inorganic phosphate (Pi), and intracellular pH, as well as various phosphorus-containing components of phospholipid membrane turnover, such as phosphomonoesters (PMEs) and phosphodiester (PDEs), are readily observed using 31P-MRS.

To identify a noninvasive and robust surrogate marker for Hsp90 inhibition and tumor response to 17AAG treatment, we performed a comprehensive 31P-MRS study in human colon carcinoma models. First, we used 31P-MRS on extracts of three human colon cancer cell lines (SW620, HCT116, and HT29) to determine whether any MR spectral changes are associated with Hsp90 inhibition after 17AAG treatment at concentrations that inhibit cell proliferation. Second, we examined the effect of 17AAG on an MF-1 nude mouse HT29 xenograft model to determine efficacy, to determine whether 31P-MRS changes observed in the cell studies were reproducible in vivo, and to determine whether 31P-MRS could provide a noninvasive pharmacodynamic marker for tumor response in clinical trials. The effect of 17AAG treatment on levels of Hsp90 client proteins and the Hsp70 co-chaperone was also determined to correlate these changes in protein expression with changes in the MR spectrum.

**Materials and Methods**

**Materials**

17AAG (powder or dissolved [25 mg/mL] in dimethyl sulfoxide [DMSO]), 17- amino,17-demethoxygeldanamycin (17AG), NSC683666, and egg phospholipids (vehicle) were provided by Dr. P. Ivy at the National Cancer Institute (Bethesda, MD). The powder form of 17AAG was used in the cell experiments, and the dissolved form of 17AAG was used in the xenograft experiments. Dulbecco’s modified Eagle medium, McCoy’s medium, fetal calf serum, penicillin, and streptomycin were purchased from Gibco (Paisley, U.K.). Perchloric acid (PCA) and potassium hydroxide were purchased from Merck (Poole, U.K.). Hypnorm was purchased from Jansen Pharmaceuticals (Buckinghamshire, U.K.), and Hypnovel was purchased from Roche (Welwyn Garden City, U.K.). Bicinchoninic acid and enhanced chemiluminescence reagents were purchased from Pierce (Rockford, IL). Tris–glycine gels and Tropifluor polyvinylidene fluoride membranes were purchased from Invitrogen (The Netherlands) and Immobilon (Bedford, MA), respectively. Primary antibodies SC-133 and SC-260 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), whereas primary antibodies SPA-810 and MAB-371 were purchased from Stressgen Biotechnologies (Victoria, British Columbia, Canada) and Chemicon International (Temecula, CA), respectively. Secondary anti-rabbit (cat. No. NA9340) and anti-mouse (cat. No. NA931V) antibodies were purchased from Amersham Pharmacia Biotechnology (Buckinghamshire, U.K.). All other chemicals were purchased from Sigma (Poole, U.K.).

**Cell Culture and Treatment**

All cell lines (HCT116, HT29, and SW620) (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal calf serum, 80 U/mL penicillin, and 80 µg/mL streptomycin at 37 °C in 5% CO2. Treatment with 17AAG, aimed at achieving comparable inhibition of cell growth for all three cell lines, was as follows: HCT116 and HT29 cells were treated with 1.36 µM 17AAG for 24 hours at 37 °C, and SW620 cells were treated with 1.36 µM 17AAG for 48 hours at 37 °C. The cells then underwent tetrypsinization (11,12) and trypsin blue exclusion assay (11,12). The effect of 17AAG treatment on cell proliferation was monitored by counting the cells in a vehicle (DMSO, 1:5000)-treated control flask and comparing that number with the number of cells in a 17AAG-treated flask. The effect of 17AAG treatment on the cell lines was further monitored by assessing Hsp70 and Hsp90 client protein levels using western blots as described below.

HT29 cells were also treated with either 1.36 µM 17AG (the active metabolite of 17AAG) or 1.36 µM NSC683666 (an inactive benzoquinone ansamycin analog) for 24 hours at 37 °C. The effects of these compounds on cell proliferation (using the method described above) and Hsp70 and Hsp90 protein expression (using western blots) were determined. HT29 cells were then treated with 10 µM doxorubicin for 24 hours at 37 °C to confirm that the effect of 17AAG and 17AG treatment on cell density and phosphorus metabolites was not due to cell death.

**Cell Cycle Analysis**

Cell cycle analysis of control and treated cells by flow cytometry was performed on cells (1 × 10^6) fixed in 70% ethanol, which were then treated with 100 µg/mL RNase A in phosphate-buffered saline and stained with 4 µg/mL propidium iodide (12), using an Elite Enhanced System Performance cell sorter (Beckman Coulter, High Wycombe, U.K.) at 488 nm. The cytometry data were analyzed using WinMDI and CyChed software (University of Wales College of Medicine, Cardiff, U.K.).
**In Vitro** 31P-MRS of Cell Extracts

To obtain an MR spectrum, 2 x 10^7 to 4 x 10^7 cells in logarithmic phase were extracted as previously described (20,26). Briefly, cells were rinsed with ice-cold saline and fixed in 6 mL of ice-cold methanol. Cells were then scraped off the surface of the culture flask, collected into tubes, and vortexed for 30 seconds at room temperature to optimize phospholipid metabolite extraction from the ruptured cells. Chloroform (6 mL) was then added to each tube, followed by an equal volume of de-ionized water. Following phase separation and solvent removal (20,26), samples were stored at -80 °C until analysis. Prior to acquisition of the MRS spectra, the water-soluble metabolites were resuspended in deuterium oxide with 10 mM EDTA (pH 8.2). Proton (1H)-decoupled 31P-MRS spectra were acquired at room temperature on a 500-MHz Bruker spectrometer (Bruker Biospin, Coventry, U.K.). Metabolite contents were determined by peak integration, normalized relative to the peak area (Bruker Biospin, Coventry, U.K.). Metabolite concentrations from the resulting spectra. As a result, the signal intensities observed in the in vivo 31P-MRS spectra are expressed as ratios of metabolites (30).

**In Vitro** 1H- and 31P-MRS of Tumor Extracts

The freeze-clamped tumors were divided into two groups. Half of the tumors were extracted in 6% PCA as previously described (31), and the rest were used for western blot analysis (described below). Neutralized extracts were freeze-dried and reconstituted in 1 mL of deuterium oxide, and the extracts (0.5 mL) were placed in 5-mm NMR tubes. For 1H-MRS, the water resonance was suppressed by using gated irradiation centered on the water frequency. Sodium 3-trimethylsilyl-2,2,3,3-tetadeuteropropionate (50 µL, 5 mM) was added to the samples for chemical shift calibration and quantitation. Immediately before the MRS analysis, the pH of the samples was readjusted to 7 with PCA or potassium hydroxide. Metabolite concentrations of betaine, lactate, alanine, β-hydroxybutyrate, creatine, phosphocholine (PC; with a contribution from phosphoethanolamine [PE]), glycero phosphocholine (GPC), valine, glutamate, taurine, and glycine were quantified from the 1H-MR spectra (32). For 31P-MRS, which was carried out after the 1H-MRS study, EDTA (50 µL, 60 mM) was added to each sample for chelation of metals ions, and methylene diphosphonic acid (50 µL, 5 mM) was added to each sample for chemical shift calibration and quantititation. The extract spectra for both the control and the treated animals were acquired under identical conditions.

**Protein Extraction and Western Blot Analysis**

Cells and xenograft tissue were lysed in 50 µL and 1 mL of lysis buffer (containing 0.1% Nonidet P-40, 50 mM HEPES [pH 7.4], 250 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 µg/mL aprotinin, 20 µM leupeptin, 1 mM dithiothreitol, 1 mM EDTA, 1 mM NaF, 10 mM β-glycerophosphate, and 0.1 mM sodium orthovanadate), respectively. The protein supernatant was collected after centrifugation at 18 000g for 10 minutes at 4 °C. Protein concentrations were determined by bicinchoninic acid reagents (Pierce). Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 4%–20% precast Tris–glycine gels and transferred electrophoretically to 0.45 µM polyvinylidene fluoride membranes. The membranes were then blocked in 0.5% casein blocking buffer (10 mM Tris–HCl [pH 7.6], 155 mM NaCl, 0.1% Tween-20, and 0.02% thimerosal) and incubated overnight at 4 °C with primary antibodies, followed by a 1-hour incubation with horseradish peroxidase-conjugated secondary antibodies at room temperature. The membranes were then washed with enhanced chemiluminescence reagent for 1 minute and exposed to hyperfilm (Amersham Pharmacia Biotechnology, Buckinghamshire, U.K.), which was then developed on a Konica SRX-101A automatic developer (Konica, Tokyo, Japan). Band intensities were assessed by visual examination. The primary antibodies used and their dilutions were as follows: c-Raf-1, 1 : 250 (SC-133); Hsp70, 1 : 2000 (SPA-810); Cdk4, 1 : 1000 (SC-260); and glyceraldehyde-3-phosphate dehydrogenase, 1 : 5000 (MAB-371). Secondary anti-rabbit (NA9340) and anti-mouse (NA931V) antibodies were used at a 1 : 1000 dilution (11,12).

**Statistical Analysis**

Data are presented as the mean and 95% confidence intervals (CIs). For comparison of metabolite concentrations and ratios, t tests were used, with a P value of <.05 considered to be statistically significant. The Mann–Whitney U test was performed for comparison of proportions, with a P value of <.05 considered to be statistically significant. Spearman’s rank correlation was used to correlate changes in tumor size with PME/PDE ratios. All statistical tests were two-sided.

**RESULTS**

**Cellular and Molecular Effects of 17AAG Treatment**

SW620, HCT116, and HT29 cells were treated with doses of 17AAG designed to achieve comparable inhibition of cell proliferation for all three cell lines. After 24-hour (HCT116 and
HT29 cells) or 48-hour (SW620 cells) incubation, the number of cells per flask was reduced substantially in each cell line to approximately 60% of the number of vehicle-treated control cells (66%, 95% CI = 62% to 70% in SW620; 58%, 95% CI = 39% to 77% in HCT116; and 57%, 95% CI = 56% to 58% in HT29) (data not shown), consistent with decreased proliferation.

17AAG treatment was also associated with depleted levels of the Hsp90 client proteins c-Raf-1 and Cdk4 and with increased Hsp70 levels (Fig. 1, A). These results provide molecular evidence that Hsp90 was inhibited in the 17AAG-treated cells.

To further characterize the cellular effects of 17AAG, cell cycle distribution following 17AAG treatment was determined by flow cytometry. For HT29 cells, the percentage of cells in G1 phase decreased statistically significantly from 61% to 46% (difference = −15%, 95% CI = −23% to −7%; P = .02), whereas the percentage of cells in G2 phase increased statistically significantly from 12% to 30% (difference = 18%, 95% CI = 11% to 25%; P = .02). For HCT116 cells, the percentage of cells in G1 phase increased statistically significantly from 36% to 61% (difference = 25%, 95% CI = 13% to 36%; P = .03), whereas the percentage of cells in S phase decreased from 42% to 17% (difference = −25%, 95% CI = −35% to −16%; P = .04). For SW620 cells, the percentage of cells in G1 phase increased statistically significantly from 59% to 75% (difference = 16%, 95% CI = 13% to 19%; P = .001), and the percentage of cells in the G2 phase also increased statistically significantly from 5% to 15% (difference = 10%, 95% CI = 5% to 13%; P = .02). The percentage of SW620 cells in S phase was decreased statistically significantly from 36% to 10% (difference = −26%, 95% CI = −28% to −23%; P = .001).

In Vitro 31P-MRS of Cell Extracts

To identify potential noninvasive markers of Hsp90 inhibition, we determined the 31P-MR spectrum of colon cancer cells treated in vitro with 17AAG. Fig. 2 illustrates 31P-MR spectra of control and 17AAG-treated HT29 cells, demonstrating an increase in PC and GPC levels following treatment. A statistically significant doubling of PC levels (relative to those in vehicle-treated cells) was detected in HT29 and HCT116 cells, and a statistically significant 20-fold increase (again, relative to those in vehicle-treated cells) in these levels was detected in SW620 cells. Increased GPC levels were also detected in all three cell lines; the increase was statistically significant for both HCT116 (in which GPC was below detection in controls) and HT29 cells. Changes in NTP levels were not statistically significant. Glycero-phosphoethanolamine (GPE) was not detectable in HCT116 cells and did not change (at 0.3 fmol/cell, 95% CI = 0.0 to 0.3 fmol/cell) following 17AAG treatment in SW620 cells but showed a statistically significant increase from 2.0 to 5.0 fmol/cell (difference = 3.0 fmol/cell, 95% CI = 1.1 to 4.9 fmol/cell; P = .01) in HT29 cells. PE levels in cell extracts remained below detection level in all three cell lines (data not shown).

HT29 cells were also treated with 17AG, the active metabolite of 17AAG, or with an inactive benzoquinone ansamycin analog, NSC683666, to verify that the 31P-MRS changes were due to Hsp90 inhibition. Western blots (Fig. 1, A) confirmed that 17AG treatment led to upregulation and depletion, respectively, of the Hsp70 and Hsp90 client proteins c-Raf-1 and Cdk4; however, NSC683666 treatment had no effect on levels of the proteins. Representative 31P-MR spectra of HT29 cells after treatment with 17AAG, 17AG, NSC683666, and vehicle are shown in Fig. 2, and the changes in PC, GPC, and NTP concentrations and cell density after treatment with 17AAG, 17AG, NSC683666, and vehicle are summarized in Fig. 3. After 24-hour incubation, cell counts were 57% (95% CI = 56% to 58%) of that of controls following 17AAG treatment and 67% (95% CI = 57% to 77%) of that of controls following 17AG treatment but were comparable to controls following NSC683666 treatment (106%, 95% CI = 87% to 125%). Treatment of HT29 cells with 17AAG induced a statistically significant increase in PC levels from 16.2 fmol/cell (vehicle-treated) to 29.7 fmol/cell (difference = 13.5 fmol/cell, 95% CI = 4.2 to 22.6 fmol/cell; P = .02) (Table 1). No statistically significant change in PC level was observed in HT29 cells following NSC683666 treatment (PC level after treatment = 17.9 fmol/cell; difference = 1.7 fmol/cell, 95% CI = −1.3 to 4.7 fmol/cell; P = .19); however,
following 17AG treatment, PC levels increased statistically significantly to 24.0 fmol/cell (difference = 7.8 fmol/cell, 95% CI = 1.6 to 13.9 fmol/cell; \( P = .02 \)). A similar trend was observed for GPC levels. GPC levels increased statistically significantly from 4.8 fmol/cell to 10.9 fmol/cell (difference = 6.1 fmol/cell, 95% CI = 3.9 to 8.3 fmol/cell; \( P = .002 \)) following 17AAG treatment. GPC levels were essentially unchanged following NSC683666 treatment (5.0 fmol/cell; difference = 0.2 fmol/cell, **Table 1.** Effect of 17-allylamino, 17-demethoxygeldanamycin (17AAG) treatment on cell density and phosphorus metabolite levels from cell extracts by *in vitro* phosphorus magnetic resonance spectroscopy (\(^{31}\)P-MRS)*

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Control-treated (N = 3)</th>
<th>17AAG-treated (N = 3)</th>
<th>Difference† (95% CI)</th>
<th>( P )‡</th>
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</thead>
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<tr>
<td>SW620</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC</td>
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<td>2.1</td>
<td>2.0 (1.4 to 2.7)</td>
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<tr>
<td>GPC</td>
<td>1.3</td>
<td>2.3</td>
<td>1.0 (−0.5 to 2.4)</td>
<td>.14</td>
</tr>
<tr>
<td>NTP</td>
<td>5.8</td>
<td>7.2</td>
<td>1.4 (−2.5 to 5.2)</td>
<td>.43</td>
</tr>
<tr>
<td>HCT116</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>6.5</td>
<td>11.6</td>
<td>5.1 (2.5 to 7.8)</td>
<td>.006</td>
</tr>
<tr>
<td>GPC</td>
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<td>1.3</td>
<td>1.3 (0.9 to 1.8)</td>
<td>.001</td>
</tr>
<tr>
<td>NTP</td>
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<td>7.6</td>
<td>1.9 (−2.7 to 6.4)</td>
<td>.33</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>PC</td>
<td>16.2</td>
<td>29.7</td>
<td>13.5 (4.2 to 22.6)</td>
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<tr>
<td>GPC</td>
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<td>10.9</td>
<td>6.1 (3.9 to 8.3)</td>
<td>.002</td>
</tr>
<tr>
<td>NTP</td>
<td>8.9</td>
<td>15.1</td>
<td>6.2 (0.6 to 11.7)</td>
<td>.10</td>
</tr>
</tbody>
</table>

*Data are expressed as the mean. CI = confidence interval; PC = phosphocholine; GPC = glycerophosphocholine; NTP = nucleoside triphosphate.
†Difference in metabolite levels was calculated by subtracting the value from the control-treated (i.e., vehicle using dimethyl sulfoxide) group from the value from the 17AAG-treated group.
‡Two-tailed unpaired \( t \) test was used to compare changes between the control- and 17AAG-treated groups.
§Below detection level by MRS, i.e., <0.1 fmol/cell.
95% CI = -2.1 to 2.4 fmol/cell; \( P = .88 \)), and GPC levels increased statistically significantly to 10.3 fmol/cell with 17AG treatment (difference = 5.5 fmol/cell, 95% CI = 4.4 to 6.5 fmol/cell; \( P < .001 \)). Changes in NTP levels with 17AAG, 17AG, or NSC683666 treatment were not statistically significant. Overall, these results (i.e., the similar effects of 17AAG and 17AG on metabolite levels) are consistent with the hypothesis that the effects of 17AAG and 17AG on \(^{31}\text{P}-\text{MRS} \) metabolites are due to their inhibitory effects on Hsp90.

HT29 cells were treated with the cytotoxic chemotherapeutic agent doxorubicin to confirm that the effects of 17AAG and 17AG treatment on cell density and phosphorus metabolites were not due to cell death. Treatment with doxorubicin decreased cell counts to 55% (95% CI = 44% to 66%) of controls but had no statistically significant effect on either PC or GPC levels (data not shown). Thus, these findings suggest that the effects of 17AAG and 17AG treatment on \(^{31}\text{P}-\text{MRS} \) metabolites are not due simply to cell death.

Previous studies (33–35) have shown that the levels of PMEs can be modulated by the availability of exogenous precursors. The increase observed may have due to a decrease in cell counts consistent with decreased proliferation, leading to a reduction in choline consumption following treatment. Therefore, it was necessary to rule out the possibility that the alterations observed in PC and GPC levels following 17AAG treatment were not a result of increased choline availability per cell. To eliminate this possibility, HT29 cells were grown at different cell densities, covering the range observed in control and treated flasks. No statistically significant difference in MR spectra was observed for cells grown at densities ranging from \( 1 \times 10^7 \) to \( 8 \times 10^7 \) cells per flask (data not shown). Thus, the changes in phosphorus metabolites following 17AAG and 17AG treatment are unlikely to be due to the effects of cell density.

**In Vivo \(^{31}\text{P}-\text{MRS} \) of HT29 Xenografts**

Previous studies (10) have shown statistically significant growth delays when HT29 human colon tumor xenografts are treated with 17AAG. Consistent with these results, 17AAG treatment for 4 days reduced tumor size to 97% of pretreatment volume, whereas control tumors increased in size to 120% of pretreatment volume (difference = 23%, 95% CI = 7% to 41%; \( P = .006 \)). Western blots of the excised tumors showed increased levels of Hsp70 in the 17AAG-treated group (Fig. 1, B). Those same studies also demonstrated client protein depletion in association with elevated Hsp70 levels in this tumor model.

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**In Vivo \(^{31}\text{P}-\text{MRS} \) of HT29 Xenografts**

Previous studies (10) have shown statistically significant growth delays when HT29 human colon tumor xenografts are treated with 17AAG. Consistent with these results, 17AAG treatment for 4 days reduced tumor size to 97% of pretreatment volume, whereas control tumors increased in size to 120% of pretreatment volume (difference = 23%, 95% CI = 7% to 41%; \( P = .006 \)). Western blots of the excised tumors showed increased levels of Hsp70 in the 17AAG-treated group (Fig. 1, B). Those same studies also demonstrated client protein depletion in association with elevated Hsp70 levels in this tumor model.
treated mouse are presented in Fig. 5, A; peaks were assigned as previously described (36). In vitro 31P-MRS of the extracts, in which the PME signal is better resolved than in in vivo experiments, showed statistically significantly elevated levels of PE (P = .02), PC (P = .07), and the (PE + PC)/(GPE + GPC) ratio (P = .04) (equivalent to the PME/PDE ratio in vivo) in the 17AAG-treated tumors relative to the vehicle-treated tumors (Table 3).

Expanded in vitro 1H-MRS spectra (i.e., 3 to 3.55 ppm) of the tumor extracts from a 17AAG-treated and a control (vehicle)-treated mouse are presented in Fig. 5, B, showing PC and GPC. Concentrations of betaine, lactate, alanine, β-hydroxybutyrate, creatine, PC (with a contribution from PE), GPC, valine, glutamate, taurine, and glycine in tumor extracts were also quantified. Of these various metabolites, only the levels of PC (with a contribution from PE) (P = .04) and valine (P = .003), together with the PC/GPC ratio (P = .001), were found to be statistically significantly increased in the 17AAG-treated tumor extracts in comparison with vehicle-treated tumor extracts (Table 3).

Table 3. In vitro phosphorus magnetic resonance spectroscopy (31P-MRS) and proton magnetic resonance spectroscopy (1H-MRS) of HT29 tumor extracts following vehicle (i.e., dimethyl sulfoxide) or 17AAG (17-allylamino, 17-demethoxygeldanamycin) treatment.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Vehicle (N = 9)</th>
<th>17AAG (N = 11)</th>
<th>Difference† (95% CI)</th>
<th>P‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>31P-MRS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PE</td>
<td>0.51</td>
<td>0.70</td>
<td>0.19 (0.04 to 0.35)</td>
<td>.02</td>
</tr>
<tr>
<td>PC</td>
<td>0.45</td>
<td>0.59</td>
<td>0.14 (–0.02 to 0.30)</td>
<td>.07</td>
</tr>
<tr>
<td>GPE</td>
<td>0.40</td>
<td>0.42</td>
<td>0.02 (–0.08 to 0.13)</td>
<td>.66</td>
</tr>
<tr>
<td>GPC</td>
<td>0.79</td>
<td>0.80</td>
<td>0.01 (–0.25 to 0.27)</td>
<td>.92</td>
</tr>
<tr>
<td>(PC + PE)/ (GPC + GPE)</td>
<td>0.81</td>
<td>1.12</td>
<td>0.31 (0.00 to 0.59)</td>
<td>.04§</td>
</tr>
<tr>
<td>1H-MRS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>1.23</td>
<td>1.66</td>
<td>0.43 (0.01 to 0.90)</td>
<td>.04</td>
</tr>
<tr>
<td>GPC</td>
<td>1.55</td>
<td>1.49</td>
<td>–0.06 (–0.57 to 0.50)</td>
<td>.90</td>
</tr>
<tr>
<td>Valine</td>
<td>0.14</td>
<td>0.24</td>
<td>0.10 (0.04 to 0.16)</td>
<td>.003</td>
</tr>
<tr>
<td>PC/GPC</td>
<td>0.81</td>
<td>1.15</td>
<td>0.34 (0.19 to 0.51)</td>
<td>.001§</td>
</tr>
</tbody>
</table>

†The difference in metabolite levels was calculated by subtracting the vehicle control group value from the 17AAG-treated group value.
‡Two-tailed unpaired t test was used to compare changes between the vehicle- and 17AAG-treated groups.
§Two-tailed Mann–Whitney U test was used to compare changes between the vehicle- and 17AAG-treated groups.

DISCUSSION

Pharmacodynamic and tumor response biomarkers are increasingly important for the development of new molecular therapeutics (13,37). By inhibiting the Hsp90 molecular chaperone, 17AAG depletes oncogenic client proteins, inhibits tumor cell proliferation, and induces apoptosis (7,9–12). However, a noninvasive direct or surrogate marker of tumor response to this novel treatment would be useful (13). We investigated MRS for potential markers of tumor response in three human colon cancer cell lines (HT29, HCT116, and SW620) and in an HT29 xenograft mouse model. In all three cell lines, levels of PC and GPC statistically significantly increased following 17AAG treatment. In the HT29 xenograft model, we observed an MRS-detectable increase in the PME peak (with contributions from both PC and PE) but no changes in GPC and GPE peaks following 17AAG treatment. A statistically significant inverse correlation was found between the change in PME/PDE ratio and tumor size following 17AAG treatment (P = .02).

Inhibition of Hsp90 by 17AAG was demonstrated by induction of Hsp70 expression and reduction of client protein (c-Raf-1 and Cdk4) levels, which is consistent with our previous findings (10–12). The pattern of changes observed following Hsp90 inhibition was also seen in the HT29 xenografts. An antiproliferative effect on cell and tumor growth was demonstrated by cell counts in vitro and by tumor volume in vivo. Tumors from HT29-xenografted mice treated with 17AAG showed a cyto-static response over the treatment period coincident with the observed molecular changes. Our previous studies (10) with HT29 xenografts have shown that this response translates into a prolonged growth delay. It should be noted that the changes
observed in the phosphorus-containing metabolites were observed in the same in vitro cell cultures and in vivo HT29 xenografts in which the molecular signature of Hsp90 was also demonstrated. However, it should also be noted that our investigations were performed using a single 17AAG dose level and at a single time point both in vitro and in vivo. Hence, further studies will need to examine more closely the dose–response and time–response relationships of the changes in phospholipid levels in tumor cells and xenografts.

It is well established that 17AAG affects the cell cycle in a cell line–dependent manner (12). Indeed, similar differences in cell cycle effects between HT29 and HCT116 cells have been previously observed (12). These differences in cell cycle effects may relate to a differential response to depletion of Hsp90 client proteins involved in cell cycle control (4). Importantly, the same spectral alterations were observed in HT29 and HT116 cells regardless of the difference in cell cycle changes, indicating that the cell cycle effect is not responsible for the modulation in phospholipid metabolites following 17AAG treatment.

Because the TotP levels in the tumor were unlikely to change substantially during these experiments, the reduced β-NTP/TotP ratio following 17AAG treatment suggests that NTP levels in the HT29 tumor xenografts decreased following 17AAG treatment and that tumor bioenergetics were compromised. This finding was not observed in vitro, where absolute quantitation was possible, probably because cultured tumor cells are bathed in well-oxygenated medium, whereas in in vivo experiments, the structurally and functionally disturbed microcirculation impairs delivery of nutrients, including oxygen (38,39).

In vivo, the PME level, whether compared with TotP, PDE, or β-NTP levels, increased statistically significantly following 17AAG treatment from pretreatment (Table 2). The principal PME signals in tumors are PC and PE, precursors of phosphatidylcholine and phosphatidylethanolamine, which are major components of biologic membranes (40). In 31P-MRS of the tumor extracts, the PME level increased statistically significantly after 17AAG treatment (P = .02) and, although the PC level also increased with 17AAG treatment, the difference did not reach statistical significance (P = .07; Table 3). In the 1H-MR spectra of the tumor extracts, the PC plus PE level was statistically significantly higher in the 17AAG-treated group than in the vehicle-treated group (P = .04). The singlet (i.e., single peak) at 3.22 ppm in the 1H-MR spectrum arises mainly from nine equivalent protons from the PC molecule with a small (approximately 20%) contribution from the PE molecule, which has only two protons resonating at 3.22 ppm (41). Hence, the statistically significant increase in the peak at 3.22 ppm is likely due to an elevated level of PC.

Our in vivo findings confirm the statistically significant increase in PC levels following 17AAG treatment that we observed for all three colon cancer cell lines in vitro. In tumor cells, the association between increased PC and Hsp90 inhibition by 17AAG treatment was further confirmed by studies using the active 17AAG metabolite 17AG and the inactive analog NSC683666. Changes in the MR spectra similar to those observed with 17AAG treatment were also seen with 17AG treatment but not with NSC683666 treatment. In addition, we ruled out the possibility that the increase in PC levels was due to inhibition of cell growth by monitoring the effect of doxorubicin, a standard chemotherapeutic agent, on cell proliferation of HT29 cells. This treatment caused a comparable decrease in cell number but had no statistically significant effect on PC levels.

Interestingly, although both PC and PE peaks were observed in untreated tumor extracts, only the PC peak was observed in the cultured cell extracts. Similar findings (32) in HT29 tumor xenografts and cultured cells have been attributed to the greater availability of ethanolamine in vivo when compared with cells in culture, where ethanolamine is only present in added serum. The relative amounts of PC and PE, both of which contribute to the PME signal, appear to vary among both experimental models and human tumors in patients (42). Precursor availability may also have influenced the increased GPC levels that we detected in the cell extracts but not in the tumor extracts following 17AAG treatment.

Our finding that PC and PME levels increase during Hsp90 inhibition and arrest of cell growth following 17AAG treatment was unexpected. Indeed, in many previous preclinical and clinical studies (23,35,42–46), PME levels are high in rapidly proliferating tumors and decrease after tumor response to standard chemotherapy or radiotherapy treatment. The time scale in which these changes in PME levels were previously observed (45,46) is similar to the time scale of the changes seen in this study; hence, the increased PME levels that we found are unlikely to be a transient effect. Therefore, our findings with 17AAG treatment cannot be a general consequence of tumor growth inhibition. One possible explanation for the rise in PME levels during tumor growth is that they may indicate membrane synthesis in rapidly dividing cells (42) and that growth inhibition by conventional cytotoxic agents lowers the PC level by inhibiting proliferation. Another possible explanation involves the effects of oncogenic mutations, particularly activation of the Ras signaling pathways (47,48), on phospholipid metabolism. Our previous results (10–12) have shown that the Ras/Raf/mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (Ras/Raf/MEK/ERK) and phosphatidylinositol-3-kinase/threonine protein kinase (PI3K/AKT) pathways are blocked following inhibition of Hsp90 by 17AAG. Whatever the explanation, our finding that 17AAG treatment caused an increase in PC and PME levels is unusual and could have important implications for the understanding of the mechanism of action for 17AAG.

The elevated PC level we observed in vitro and the elevated PME level we observed in vivo could, in principle, arise in three possible ways: 1) increased uptake and phosphorylation of extracellular choline to form PC, 2) de novo synthesis of PC, or 3) mobilization of MR-invisible cholines to PC. Hypothesis 1 is unlikely in view of the study of Liu et al. (49), who showed that geldanamycin inhibits choline kinase and net cellular accumulation of (methyl-14C)choline and (methyl-14C)phosphocholine. Hypothesis 2 is also unlikely, because synthesis of PC in mammalian cells is thought to be confined to the liver. Therefore, hypothesis 3 is the most probable explanation for the elevated levels of PC in vitro and PME in vivo. Hence, the most likely occurrence is that 17AAG promotes the hydrolysis of an MR-invisible pool of phosphatidylcholine, causing an increase in PC both in cell extracts and in vivo. In addition, the increase in intracellular PC might also inhibit choline kinase and thus impede the uptake of extracellular choline, which could explain the results of Liu et al.

Activation of pathways downstream of tyrosine receptor kinases and Ras has been associated with several alterations in enzymes associated with choline metabolism and phosphatidyl-
choline synthesis (50–52). Choline kinase activation, inhibition of cytidine triphosphate–phosphocholine cytidylytransferase, and activation of phospholipases A2, C, and D have all been reported at different steps in the Ras/Raf/MEK/ERK, PI3K/AKT, and Ras–guanine nucleotide dissociation stimulator signaling pathways (50–55). By depleting proteins involved in those signaling pathways, 17AAG treatment probably affects the enzymes involved in choline metabolism and alters the balance between synthesis and hydrolysis of phosphorylcholine.

In conclusion, the present study has identified changes in phospholipid metabolism associated with inhibition of the Hsp90 molecular chaperone by 17AAG in human colon cancer cells. The effects of 17AAG treatment on phospholipid metabolites were found to occur both in cell lines treated in culture and in tumor xenografts in mice. The increased PME/PDE ratio was found to correlate with tumor response, indicating that it could act as a potential predictive marker. The treatment-induced changes were observed at 17AAG doses that caused tumor growth inhibition via inhibition of Hsp90, as demonstrated by changes in client protein and Hsp70 levels. Hence, monitoring the pharmacodynamic effects of 17AAG treatment and possibly of other Hsp90 inhibitors on phospholipid metabolism by MRS—using either 31P or the more sensitive 1H—may provide a noninvasive surrogate marker for Hsp90 inhibition and potentially for tumor response in solid tumors in clinical trials with this drug and other Hsp90 inhibitors.

REFERENCES


NOTES

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