Use of radiolabelled choline as a pharmacodynamic marker for the signal transduction inhibitor geldanamycin

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There is an urgent need to develop non-invasive pharmacodynamic endpoints for the evaluation of new molecular therapeutics that inhibit signal transduction. We hypothesised that, when labelled appropriately, changes in choline kinetics could be used to assess geldanamycin pharmacodynamics, which involves inhibition of the HSP90 molecular chaperone→Raf1→Mitogenic Extracellular Kinase→Extracellular Signal-Regulated Kinase 1 and 2 signal transduction pathway. Towards identifying a potential pharmacodynamic marker response, we have studied radiolabelled choline metabolism in HT29 human colon carcinoma cells following treatment with geldanamycin. We studied the effects of geldanamycin, on net cellular accumulation of (methyl-14C)choline and (methyl-14C)phosphocholine production. In parallel experiments, the effects of geldanamycin on extracellular signal-regulated kinase 1 and 2 phosphorylation and cell viability were also assessed. Additional validation studies were carried out with the mitogenic extracellular kinase inhibitor U0126 as a positive control; a cyclin-dependent kinase-2 inhibitor roscovitine and the phosphatidylinositol 3-kinase inhibitor LY294002 as negative controls. Hemicholinium-3, an inhibitor of choline transport and choline kinase activity was included as an additional control. In exponentially growing HT29 cells, geldanamycin inhibited extracellular signal-regulated kinase 1 and 2 phosphorylation in a concentration- and time-dependent manner. These changes were associated with a reduction in (methyl-14C)choline uptake, (methyl-14C)phosphocholine production and cell viability. Brief exposure to U0126, suppressed phosphocholine production to the same extent as Hemicholinium-3. In contrast to geldanamycin and U0126, which act upstream of extracellular signal-regulated kinase 1 and 2, roscovitine and LY294002 failed to suppress phosphocholine production. Our results suggest that when labelled with carbon-11 isotope, (methyl-11C)choline may be a useful pharmacodynamic marker for the non-invasive evaluation of geldanamycin analogues.

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There has been a recent shift in the paradigm for anticancer drug development, with a movement away from cytotoxic compounds towards agents that target specific gene products which create and drive the malignant phenotype (Garrett and Workman, 1999; Gelmon et al, 1999; Gibbs, 2000). Although some of these agents may result in reduction in tumour size, and may still be adequately evaluated by standard criteria based on tumour shrinkage or time to progression, most of these novel therapies may not be clinically assessable by conventional approaches (Garrett and Workman, 1999; Gelmon et al, 1999; Hudes, 1999).

There is, therefore, an urgent need to replace traditional response endpoints with more specific and relevant ones (Gelmon et al, 1999). Endpoints that measure effects on particular cell pathways, rather than general markers of tumour size or proliferation, may be particularly informative. However, developing such endpoints represents a significant challenge. Molecular events such as changes in phosphorylation and protein–protein interactions, are difficult to measure in the intact animal and patient.

The extracellular signal-regulated kinase (ERK) pathway operates downstream of activated receptor kinases and Ras, and regulates cell growth. This process involves activation of the cytoplasmic serine-threonine kinase Raf-1, which phosphorylates and activates mitogen activated kinase kinase mitogenic extracellular kinase (MEK), which in turn phosphorylates and activates ERK1/2 (p44/p42) on tyrosine and threonine residues. Phosphorylated ERKs shuttle into the nucleus where they activate transcription factors (Daum et al, 1994). To date, several inhibitors of the ERK cascade have been discovered (Gibbs, 2000), of particular interest to us. Gelandanamycin is a benzoquinoid ansamycin antibiotic related to herbimycin A. Gelandanamycin binds to the ATP binding domain of the heat shock protein 90 (HSP90), in a complex with various co-chaperones, leading to degradation of oncogenic client proteins such as Raf-1, p185erbB2, cyclin-dependent kinase 4, some hormone receptors and also mutant p53 (Schulte et al, 1996, 1997; An et al, 1997; Grenert et al, 1997; Prodromou et al, 1997; Mayer and Bukau, 1999). Experiments with NIH3T3 cells have shown that Raf-1, for instance, exists in a native heterocomplex with HSP90, cdc37 and other proteins (Grenert et al, 1997). Inhibition of this complex by gelandanamycin markedly decreases the cellular half-life of the Raf-1 protein, and this subsequently results in Raf-1 depletion and blockage of Raf-1-MEK-ERK signalling.
Currently, a geldanamycin analogue, 17- (allylamino)-17-demethoxygeldanamycin (17AAG) is undergoing Phase 1 clinical trials; therefore there is a need to develop a pharmacodynamic endpoint to monitor the effect of such agents (Egorin et al., 1998; Kelland et al., 1999; Losiewicz et al., 1999; Clarke et al., 2000; Hostein et al., 2001).

Here we have studied the use of labelled-choline as a pharmacodynamic marker of the geldanamycin-inhibited ERK pathway in a human colon carcinoma model. Choline is a marker for evaluating malignant transformation and proliferation by non-invasive methods such as magnetic resonance spectroscopy (MRS) and positron emission tomography (PET) (Smith et al., 1991; Negendank, 1992; Hara et al., 1997, 1998; Aboagye and Bhujiwalla, 1999). After its transport into cells, choline is converted to phosphocholine by choline kinase in the presence of Mg2+ and ATP. This phosphorylation step commits extracellular choline to phosphatidylcholine biosynthesis and essentially 'traps' choline within the cell, making carbon-11 labelled choline an attractive PET probe. Phosphocholine and breakdown products of phosphatidylcholine are essential signalling molecules for cell growth, and phosphatidylcholine is the most abundant phospholipid constituent of the lipid bilayer in eukaryotic cells (Pelech and Vance, 1989; Cullis and Hope, 1991; Cuadrado et al., 1993; Exton, 1994). Despite its importance, little is known about the mechanisms that regulate choline kinase activity and hence phosphocholine production. A recent study on the yeast Saccharomyces cerevisiae revealed that choline kinase is activated through phosphorylation by the Ras-cyclic adenosine monophosphate (cAMP) pathway (Kim and Carman, 1999). Choline kinase has also been found to be a substrate for yeast protein kinase A (PKA) (Kim and Carman, 1999). Increased levels of phosphocholine are found in mouse fibroblast cell lines transformed by H-Ras, v-Src and Mos but not c-Fos (Hatnam and Kent, 1995; Hernandez-Alcoceba et al., 1997). Furthermore, transformation of benzo(a)pyrene-immortalised human mammary epithelial cells by ErbB2, led to increased phosphocholine levels (Aboagye and Bhujiwalla, 1999). These findings suggest that ErbB2, Src, Ras and Mos may regulate choline kinase through the ERK pathway in mammalian cells.

Our results demonstrated that inhibition of ERK1/2 phosphorylation by geldanamycin is associated with a dose- and time-dependent inhibition of phosphocholine production. This specificity of the effect was verified by using the inhibitors U0126, rosiglitazone and LY294002. U0126 (1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene) is a noncompetitive inhibitor of MEK with no other effects in the ERK cascade (Favata et al., 1998). Rosiglitazone is a potent and selective inhibitor of cyclin-dependent kinases, particularly cdk2 (Rudolph et al., 1996; Meijer et al., 1997). It acts by competing for the ATP binding domain of the cyclin-dependent kinases and has no significant effects on other kinases such as ERK1/2 at the concentrations used (Rudolph et al., 1996; Meijer et al., 1997). LY294002 (2-(4-morphinyl)-8-phenyl-4H-1-benzopyran-4-one) is a specific inhibitor of phosphatidylinositol 3-kinase (PI3K). It acts by competing for the ATP binding domain of the enzyme and has no effect on MAP kinase or ERK1/2 (Matter et al., 1994; Thomas et al., 1997; Muise-Hemericks et al., 1998). The results suggest that non-invasive imaging of phosphocholine formation may be a useful method for evaluating the effect of geldanamycin analogues in vivo.

**MATERIALS AND METHODS**

**Cell culture**

HT29 human colon carcinoma cells, MCF7 human mammary carcinoma cell (American Type Culture collection; Rockville, MD, USA) and MCF7-ADR human mammary carcinoma cells (obtained from Dr Zaver Bhujiwalla, The Johns Hopkins University, Baltimore, USA) were cultured in RPMI 1640 medium (Sigma, Dorset, UK) supplemented with 0.4 mM l-glutamine, 20 U ml−1 penicillin, 20 μg ml−1 streptomycin and 10% foetal calf serum (FCS) (Gibco BRL Life Technologies, Renfrewshire, UK). Cells were incubated at 37°C (in 5% CO2 and at 100% humidity) and treated upon reaching approximately 50% confluency.

**Drug treatment**

To determine the time-dependent effect of geldanamycin (Sigma, Dorset, UK) on ERK1/2 phosphorylation, cells were cultured in 90 mm Petri-dishes and treated with 2 μM geldanamycin for 2, 6, and 24 h. Cells were also incubated for 24 h, washed and incubated with drug-free medium for a further 24 h to evaluate recovery of activity. To determine the concentration-dependent effect of geldanamycin on ERK1/2 phosphorylation, cells were treated with geldanamycin (0.01, 0.2, 0.5 and 2 μM) for 24 h. To determine whether geldanamycin suppressed cell viability, cells were seeded in 96 well plates and treated with geldanamycin (0.01, 0.2, 0.5 and 2 μM) for 4, 24, 48 and 72 h. Similarly, the effect of geldanamycin on (methyl-14C)choline uptake by cells growing in 12-well plates was determined at the same concentrations for 1, 2, 4, 24 and 48 h. The effect of geldanamycin on (methyl-14C)phosphocholine production was determined by incubating cells, growing in 90 mm Petri-dishes, for 24 h with 0.01, 0.2, 0.5 and 2 μM geldanamycin. To further understand the role of the ERK pathway on phosphocholine production, validation studies were performed with other inhibitors including 1 μM hemicholinium (Sigma), 100 μM U0126 (Calbiochem Ltd., Nottingham, UK), 10 μM roscovitine (Calbiochem) and 5 μM LY294002 (Sigma). These concentrations of the inhibitors used have been reported previously to be efficacious (Hernandez-Alcoceba et al., 1997; Rudolph et al., 1996; Schulte et al., 1996; An et al., 1997; Meijer et al., 1997; Favata et al., 1998).

**Determination of protein content**

Supernatants of homogenates obtained from treated and untreated cells were kept at 4°C prior to the protein assay. Protein concentrations were determined by a BioRad protein assay kit (Biorad, Hemel Hampstead, UK) which measures the absorbance at 595 nm of an acidic solution of coomassie blue-protein complex (Spector, 1978). Standard concentrations of bovine albumin were used to obtain a calibration curve.

**Western blot analysis**

At the end of drug incubation, ERK protein phosphorylation was induced by treating cells with 100 nM of phorbol 12-myristate 13-acetate (PMA) for 10 min. After three washes with ice cold PBS, cells were lysed in TNES buffer (50 mM Tris-HCl, pH 7.5; 1% nonylphenoxyethanol (NP40); 2 mM ethylene diamine tetraacetic acid; 100 mM NaCl, 1 mM sodium orthovanadate; 1 mM phenyl methyl sulphonyl fluoride (PMSF); 25 mM NaF; and 25 mM β-glycerophosphate) for 1 h. The protein content of the lysates was determined by the BioRad method described above. Aliquots (containing 30 μg of protein) were loaded into each lane of a 10% polyacrylamide gel and eluted. Separated proteins were electrophoresed onto nitrocellulose membranes and incubated with 5% non-fat milk powder in TBS (10 mM Tris-HCl, pH 7.4; 0.9% NaCl) supplemented with 0.05% Tween 20 for 45 min to block non-specific interaction with antibody. The membranes were then incubated with a 1:5000 dilution of either rabbit anti-ERK (total ERK) or anti-phosphoERK polyclonal antibodies (Promega, Southampton, UK). The reaction was carried out overnight, followed by a further 1 h incubation with goat anti-rabbit antibody conjugated to horseradish peroxidase (Sigma) at 1:20 000 dilution. After three
washes, bands were visualised using the ECL method (Nycomed Amersham plc, Buckinghamshire, UK).

**Cell viability assay**

Relative cell viability (that reflects survival and proliferation) was measured by a colorimetric assay for determining the number of viable cells. At the end of drug treatment, the plates were washed twice and incubated at $37^\circ C$ for 90 min with medium containing 20 μl of CellTiter 96®AQueous One Solution Reagent (Promega). This solution is comprised of a tetrazolium compound and an electron-coupling reagent, phenazine ethosulphate. Reduction of the solution by growing cells leads to the formation of a water-soluble formazan derivative, which absorbs light at 490 nm (US Patent no. 5,185,430). Plates were vibrated for 2 min prior to reading their optical density in a scanning spectrophotometer (96-well plate reader; Anthos Labtech Instruments, Salzburg, Austria) at a wavelength of 492 and 620 nm (reference).

**Choline uptake assay**

Following drug incubation, cells were washed twice and incubated for 1 h with (methyl-14C)choline chloride (Amersham Life Sciences Ltd., Rainham, UK) at 0.01 μCi well$^{-1}$. After two washings with phosphate buffered saline (PBS), cells were detached by trypsinisation (0.5 ml, 5 min) and the action of trypsin neutralised with 0.5 ml of FCS enriched growth medium. Cells were then centrifuged (180 g, 5 min) and the cell pellets were re-suspended in 0.2 ml PBS and transferred into glass vials. Cells were solubilised by incubation with 0.8 ml of Soluene (Packard, Pangbourne, UK) at $50^\circ C$ for 3 h. The radioactivity retained in cells was determined by scintillation counting after addition of 9 ml of Hionic fluor (Packard) to each glass vial. This assay measures the net accumulation of radioactivity following a 1 h pulse with radiolabelled choline.

**Phosphocholine production (whole-cell choline kinase assay)**

Following drug incubation for 24 h (geldanamycin, roscovitine and LY294002) or 30 min (U0126), cells were washed twice with PBS to remove drug and incubated with medium containing (methyl-14C)choline chloride (Amersham) at 2 μCi per 7 cm$^2$ flask. In the case of hemicholinium, radioactivity was added to drug containing medium at 30 min and incubated for 1 h. Cells were washed twice with PBS, then detached by trypsinisation (5 ml, 5 min) and the action of trypsin neutralised with 5 ml of FCS enriched growth medium. Cells were then centrifuged (3000 g, 10 min), resuspended in 2 ml of PBS and 3 ml of ice cold methanol, and then stored at $-70^\circ C$ for a minimum of 10 min in order to inactivate choline kinase. To release the intracellular (radioactively-labelled) metabolites, the cell suspensions were homogenised with a Polytron-Ultraturrax homogenizer (Jank and Kunkel KG, Breisgan, Germany) and centrifuged at 500 g for 10 min. Phosphocholine levels were analysed by high performance liquid chromatography (HPLC) with radiochemical detection using a μBondapak C18 column (7.8 x 300 mm, 10 μm; Waters, Watford, UK) and a mobile phase comprising of 1.5 mm K$_2$HPO$_4$ and 5 mm tetramethylammonium hydrogen sulphate, pH 7.0. The flow rate of mobile phase and scintillant (Monoflow 2; National Diagnostics, Atlanta, GA, USA) were 2 and 6 ml min$^{-1}$, respectively. Aliquots (50 μl) of (methyl-14C)betaine (prepared by oxidation of (methyl-14C)choline) were used as internal standard per ml of supernatant. Dilutions of (methyl-14C) phosphocholine (Amersham) spiked with (methyl-14C)betaine were used for calibration. Three to five samples were analysed for each dose level and at each time point. A set of samples was also eluted without the internal standard to ensure that no (methyl-14C)betaine peak was present in the samples. Phosphocholine levels (pmol μg$^{-1}$ protein) were derived by converting the radioactivity associated with the phosphocholine produced to concentration units using the specific activity of phosphocholine. Protein concentrations of aliquots of the homogenate (50 μl) were determined as described above to ensure normalisation of phosphocholine production to protein content.

**Statistical analysis**

Statistical analysis of the data was performed using StatView version 1.04, 1991 (Abacus Concepts Inc., Berkeley, CA, USA). The statistical significance of differences in choline uptake, choline kinase activity and cell viability was determined using the Mann–Whitney U-test (two-tailed). $P$ values $\leq 0.05$ were considered to be significant.

**RESULTS**

**Suppression of ERK1/2 phosphorylation**

We monitored the effect of geldanamycin on PMA-induced ERK1/2 phosphorylation in HT29 colorectal cancer cells by Western blotting (Figure 1A-B). PhosphoERK1/2 were detected as 44/42 kDa proteins. There was a concentration-dependent reduction in PMA-induced ERK-1/2 phosphorylation after 24 h treatment with geldanamycin (Figure 1A). Maximum inhibition of PMA-induced ERK1/2 phosphorylation was observed with 2 μM geldanamycin. With regards to kinetics, inhibition of PMA-induced ERK1/2 phosphorylation by 2 μM geldanamycin was apparent at 6 h and highest at 24 h (Figure 1B). PMA-induced phosphoERK1/2 were detected when cells were incubated with geldanamycin for 24 h, washed, and incubated for a further 24 h in drug free medium suggesting partial recovery of PMA-induced phosphoERK1/2 levels within 24 h. The assay was insensitive to detection of non-PMA-induced phosphoERK1/2 levels beyond 6 h post-treatment. The differences in band intensities between Figure 1A and B were due to longer film exposure for the latter (to allow detection of non-PMA-induced phosphoERK1/2). Overall, the changes in phosphoERK1/2 levels were not accompanied by changes in total ERK1/2 protein (Figure 1A,B), indicating that the effect of geldanamycin were at the level of ERK1/2 phosphorylation rather than depletion of the protein. When cells were incubated with 10–100 μM of the MEK inhibitor, U0126 for 30 min, there was complete inhibition of ERK phosphorylation (data not shown).

**Suppression of cell growth**

Geldanamycin produced a concentration- and time-dependent decrease in viability of HT29 cells (Figure 2). The percentage decrease in viability (compared to control) at a dose level of 2 μM was 25, 43 and 49% at 24, 48 and 72 h, respectively.

**Inhibition of choline uptake**

The net accumulation of radiolabelled choline (choline uptake) is a function of both choline transport into cells and trapping through phosphorylation (by choline kinase). Figure 3 shows that the treatment of HT29 cells with geldanamycin produced a concentration- and time-dependent inhibition of (methyl-14C)choline uptake. Inhibition of (methyl-14C) choline uptake by geldanamycin was apparent at 2 h and maximal at 48 h post-treatment. After 4 h, doses as low as 0.01 μM produced a significant decrease in (methyl-14C) choline uptake (35% at 4 h; 33% at 48 h). The percentage decrease in (methyl-14C) choline uptake compared to control at a dose level of 2 μM was 61 and 70% at 24 and 48 h, respectively. As a positive control, 90 min incubation of HT29 cells

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with 1 mM of the choline transport and choline kinase inhibitor hemicholinium inhibited (methyl-\(^{14}\)C)choline uptake by 89 ± 2%.

Combined with the cell viability measurements, these data indicate that the decline in choline accumulation is due in part to fewer cells rather than less accumulation per cell.

**Suppression of phosphocholine production**

Choline kinase activity was evaluated by incubating cells with (methyl-\(^{14}\)C)choline and measuring intracellular phosphocholine

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**Figure 1** Western blots showing PMA-induced and uninduced phosphoERK1/2 and total ERK protein levels. (A) Treatment with different concentrations of geldanamycin (GA) for 24 h. (B) Treatment with 2 \(\mu\)M GA for different lengths of time. At 24 h cells were washed and placed in fresh media for a further 24 h (48 h).

**Figure 2** Inhibition of HT29 cell viability by geldanamycin (GA). Cells were incubated with drug from 4 to 72 h. At the end of the incubation, cells were washed and cell viability determined by a colorimetric assay as described in Materials and Methods section. There was a significant difference between untreated cells and cells treated for 4 h (\(P=0.016, 0.009\) and 0.009 for 0.01, 0.2, 0.5 and 2 \(\mu\)M, respectively), 24 h (\(P=0.049, 0.049\) and 0.015, for 0.2, 0.5 and 2 \(\mu\)M, respectively), 48 h (\(P=0.003, 0.004\) and 0.0001 for 0.2, 0.5 and 2 \(\mu\)M, respectively), and 72 h (\(P=0.0001, 0.0003\) and 0.000001 for 0.2, 0.5 and 2 \(\mu\)M, respectively). Error bars=1 s.d. (n=8 – 10).

**Figure 3** Effect of geldanamycin on the net accumulation of radiolabelled choline following a 1 h pulse in HT29 cells. Cells were treated with geldanamycin at the concentrations indicated and incubated for 1 to 48 h. Following drug treatment, cells were washed and (methyl-\(^{14}\)C)choline uptake assessed as described in the Materials and Methods section. There was a significant difference between untreated cells and cells treated for 1 h (\(P=0.038\) for 2 \(\mu\)M), 2 h (\(P=0.007\) for 2 \(\mu\)M), 4 h (\(P=0.042, 0.008, 0.001\) and 0.002 for 0.01, 0.2, 0.5 and 2 \(\mu\)M, respectively) and for 24 h (\(P=0.032, 0.013\) and 0.003 for 0.2, 0.5 and 2 \(\mu\)M, respectively) and 48 h (\(P=0.0004\) and 0.00005 for 0.5 and 2 \(\mu\)M, respectively). Error bars=1 s.d. (n=3 – 5).
levels by a novel HPLC assay. (methyl-\(^{14}\)C)betaine was used as an internal standard after having shown that (methyl-\(^{14}\)C)betaine was absent in these cells under identical conditions. Typical chromatograms are shown in Figure 4A.B. Data were expressed as phosphocholine levels normalised to total cellular protein content (Table 1A).

Treatment of HT29 cells with geldanamycin produced a concentration-dependent decrease in phosphocholine production (Table 1A). At the highest concentration used (2 \(\mu\)M), the degree of inhibition was comparable to that seen with 1 mM hemicholinium. We hypothesised that choline kinase may lie immediately downstream of ERK. The MEK inhibitor, U0126, the cyclin dependent kinase 2 inhibitor, roscovitine and the PI3K inhibitor, LY294002 were used to probe the validity of this hypothesis. Brief (30 min) incubation of cells with 100 \(\mu\)M U0126 suppressed phosphocholine formation to the same extent as hemicholinium. In contrast, 24 h incubation with 10 \(\mu\)M roscovitine and 5 \(\mu\)M LY294002 had no effect on choline kinase activity. Phosphocholine production was also inhibited in MCF7 cells but not in the resistant MCF7-ADR cells (Table 1B).

**DISCUSSION**

The focus of new drug development is now on abrogating the malignant phenotype and several agents are in preclinical and clinical development that target oncogenic signal transduction pathways (Garrett and Workman, 1999; Gibbs, 2000). Since traditional endpoints, such as tumour shrinkage and growth delay, may be insufficient or inappropriate for the objective assessment of such therapies, there is an urgent need for new surrogate endpoints to monitor pharmacodynamics during early clinical trials (Gelman et al, 1999; Gibbs, 2000).

In this study, we have investigated the ability to monitor the pharamodynamic effect of geldanamycin, an inhibitor of HP90 and Raf-1, using carbon-14-labelled choline. All cells utilise extracellular choline (via bloodstream from food) as a precursor for the biosynthesis of the membrane phospholipid phosphatidylcholine (Zeisel, 1981). Phosphocholine is produced in cells via the action of choline kinase utilising ATP as the phosphate donor. This constitutes the major source of phosphocholine (see reviews by Pelech and Vance, 1989; Exton, 1994). The phosphocholine produced is trapped in cells as phosphocholine due to its charge, or is converted to phosphatidylcholine via cytidine diphosphate choline (see reviews by Pelech and Vance, 1989; Exton, 1994). Choline can be produced endogenously via the action of phospholipase D on phosphatidylcholine or from the hydrolysis of glycerophosphocholine and phosphocholine can be produced via the action of phospholipase C on phosphatidylcholine (Pelech and Vance, 1989). Due to the slow turnover of phosphatidylcholine, however, no cellular metabolites of (methyl-\(^{14}\)C)choline, other than (methyl-\(^{14}\)C)phosphocholine derived via the exogenous pathway, are detected in tumour cells after the ‘pulse time’ of 1 h used in this work (see also HPLC analysis). This makes radio-labelled choline an attractive tracer for monitoring this pathway. We have demonstrated that geldanamycin inhibited (methyl-\(^{14}\)C)-choline uptake at concentrations that inhibit Raf-1 dependent ERK1/2 phosphorylation and cell viability. The inhibition of

| Drug treatment                  | Phosphocholine level (pmol \(\mu\)g \(^{-1}\) protein) |
|--------------------------------|------------------------------------------------------|
| Untreated Control              | 0.016±0.004                                          |
| Geldanamycin (0.5 \(\mu\)M, 24 h) | 0.014±0.001                                          |
| Geldanamycin (2 \(\mu\)M, 24 h)  | 0.005±0.001                                          |
| Roscovitine (10 \(\mu\)M, 24 h)  | 0.014±0.010                                          |
| U0126 (100 \(\mu\)M, 30 min)    | 0.004±0.002                                          |
| Hemicholinium (1 mM, 90 min)    | 0.004±0.004                                          |
| LY294002 (5 \(\mu\)M, 24 h)     | 0.014±0.000                                          |

Phosphocholine levels in untreated and drug treated cells MCF7 and MCF7-ADR cells

| Drug treatment               | MCF7 | MCF7-ADR |
|------------------------------|------|----------|
| Untreated Control            | 0.250±0.041 | 0.054±0.001 |
| Geldanamycin (0.5 \(\mu\)M, 24 h) | 0.085±0.018 | 0.043±0.005 |
| Geldanamycin (2 \(\mu\)M, 24 h)  | 0.131±0.070 | 0.073±0.032 |
| U0126 (100 \(\mu\)M, 30 min)   | 0.027±0.005 | 0.012±0.016 |
| Hemicholinium (1 mM, 90 min)  | 0.025±0.011 | 0.000±0.000 |

(A) Unit of phosphocholine: the amount in pmol of phosphocholine formed in 1 h normalised to total cellular protein content. There was a significant difference between untreated and treated cells with 100 \(\mu\)M U0126 (\(P=0.001\)), 2 \(\mu\)M Geldanamycin (\(P=0.045\)) and 1 mM Hemicholinium (\(P=0.02\)). Error bars=1 s.e.m. (n=3 to 5).

(B) Unit of phosphocholine: the amount in pmol of phosphocholine formed in 1 h normalised to total cellular protein content. There was a significant difference between untreated and treated cells with 100 \(\mu\)M U0126 (\(P<0.005\)), 1 mM hemicholinium (\(P<0.005\)) and 2 mM hemicholinium (\(P<0.005\)) in both cell lines; there was a significant difference between untreated and geldanamycin (0.5 and 2 \(\mu\)M) treated MCF7 cells (\(P<0.005\)) but not MCF7-ADR cells (\(P>0.005\)). Error bars=1 s.e.m. (n=3 to 5).

![Figure 4](image-url) Representative chromatograms obtained from (A) untreated cells and (B) cells treated with U0126 (100 \(\mu\)M for 30 min). The chromatograms show a decrease in (methyl-\(^{14}\)C)phosphocholine levels following treatment with U0126. (methyl-\(^{14}\)C)Betaine was used as internal standard.
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We have chosen to develop an endpoint for drugs, which act on the Raf-1-MEK-ERK signal transduction pathway. This is attractive for several reasons. One could potentially use (methyl-11C)choline-PET to select patients likely to respond to geldanamycin analogues. It remains to be seen whether (methyl-11C)choline could be used as a pharmacodynamic marker to monitor the effects of other inhibitors of the ERK cascade, including inhibitors of receptor kinases and Ras prenylation, (Gibbs, 2000). In the event of resistance arising during treatment with ERK pathway inhibitors, choline uptake studies should be able to detect this resistance phenotype, whereas a specific marker for say the receptor tyrosine kinase assay will not. Apart from these biological properties, (methyl-11C)choline is an attractive PET tracer since it is converted into phosphocholine, which is ‘trapped’ in cells.

The assay of (methyl-11C)choline by PET may be of immediate potential relevance in the clinical evaluation of the geldanamycin analogue, 17AAG. Unlike current invasive pharmacodynamic assays which measure Raf-1 depletion and HSP70 induction (O’Dwyer et al, 1999; Clarke et al, 2000), the measurement by (methyl-11C)choline does not require the biopsy sampling of tumours which can be problematic. Furthermore, necrosis, scar tissue formation and macrophage infiltration can confound assessment of response to cancer therapeutics by conventional anatomical imaging. Thus, a marker that is capable of detecting changes in proliferation and metabolism may be superior to conventional anatomical imaging. A potentially powerful application of this technology will be the ability to assess drug effects at concentrations consistent with maintained cell viability. The effects of U0126 on phosphocholine production (30 min treatment) and geldanamycin on (methyl-11C)choline uptake at early time points (a larger decrease in (methyl-11C)choline uptake compared to relative cell viability at 2 and 4 h), appear to support a potential role for radiolabelled choline in assessing inhibition of signal transduction at concentrations consistent with maintained cell viability. This hypothesis needs to be further tested. Geldanamycin treatment, however, inhibits growth of (or kills) HT29 cells and does not provide a very good model to fully assess cytostasis. In vivo studies to establish the pharmacokinetics and metabolism of (methyl-11C)choline will further support the usefulness of radiolabelled choline as a non-invasive pharmacodynamic marker.

In summary, we have shown that radiolabelled choline can measure the response of cells to geldanamycin treatment. Our results suggest that choline could be used as a non-invasive PET or MRS imaging probe to monitor the effects of geldanamycin analogues, which target the tyrosine kinase receptor-Ras-Raf-1-MEK cascade.

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We have chosen to develop an endpoint for drugs, which act on the Raf-1-MEK-ERK signal transduction pathway. This is attractive for several reasons. One could potentially use (methyl-11C)choline-PET to select patients likely to respond to geldanamycin analogues. It remains to be seen whether (methyl-11C)choline could be used as a pharmacodynamic marker to monitor the effects of other inhibitors of the ERK cascade, including inhibitors of receptor kinases and Ras prenylation, (Gibbs, 2000). In the event of resistance arising during treatment with ERK pathway inhibitors, choline uptake studies should be able to detect this resistance phenotype, whereas a specific marker for say the receptor tyrosine kinase assay will not. Apart from these biological properties, (methyl-11C)choline is an attractive PET tracer since it is converted into phosphocholine, which is ‘trapped’ in cells.

The assay of (methyl-11C)choline by PET may be of immediate potential relevance in the clinical evaluation of the geldanamycin analogue, 17AAG. Unlike current invasive pharmacodynamic assays which measure Raf-1 depletion and HSP70 induction (O’Dwyer et al, 1999; Clarke et al, 2000), the measurement by (methyl-11C)choline does not require the biopsy sampling of tumours which can be problematic. Furthermore, necrosis, scar tissue formation and macrophage infiltration can confound assessment of response to cancer therapeutics by conventional anatomical imaging. Thus, a marker that is capable of detecting changes in proliferation and metabolism may be superior to conventional anatomical imaging. A potentially powerful application of this technology will be the ability to assess drug effects at concentrations consistent with maintained cell viability. The effects of U0126 on phosphocholine production (30 min treatment) and geldanamycin on (methyl-11C)choline uptake at early time points (a larger decrease in (methyl-11C)choline uptake compared to relative cell viability at 2 and 4 h), appear to support a potential role for radiolabelled choline in assessing inhibition of signal transduction at concentrations consistent with maintained cell viability. This hypothesis needs to be further tested. Geldanamycin treatment, however, inhibits growth of (or kills) HT29 cells and does not provide a very good model to fully assess cytostasis. In vivo studies to establish the pharmacokinetics and metabolism of (methyl-11C)choline will further support the usefulness of radiolabelled choline as a non-invasive pharmacodynamic marker.

In summary, we have shown that radiolabelled choline can measure the response of cells to geldanamycin treatment. Our results suggest that choline could be used as a non-invasive PET or MRS imaging probe to monitor the effects of geldanamycin analogues, which target the tyrosine kinase receptor-Ras-Raf-1-MEK cascade.

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