Biochemical Changes and Cytotoxicity Associated with Methionine Depletion in Paediatric Central Nervous System Tumour Cell Lines

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Abstract. *Background: The aim of this study was to investigate the importance of the extent and duration of methionine depletion as a cause of cytotoxicity for CNS tumour cell lines, and also to investigate the associated in vitro cellular biochemical responses. Materials and Methods: Cell growth inhibition was assayed by the SRB assay. Intracellular methionine levels were measured by GC/MS following dervatization with MTBSTFA. After methionine depletion, methionine synthase and MGMT activities were also determined. Glutathione levels were assayed by HPLC after derivatization with OPA. Results: Medulloblastoma (Daoy) and glioma (D54) cells were found to be methionine dependent and effects on proliferation, apoptosis and clonogenic survival were dependent on time and degree of methionine depletion. Methionine depletion also caused a demonstrable decrease in L-methionine levels and an increase in glutathione levels for both cell lines, with a decrease in MGMT activity for Daoy cells. Conclusion: Daoy and D54 cells are methionine dependent; the degree and duration of methionine depletion is related to cell death. The associated biochemical changes in MGMT and glutathione may be expected to modulate chemosensitivity and this will be investigated in future studies.*

Abbreviations: CNS, central nervous system; MGMT, O^6-methylguanine-DNA-methyltranferase; SRB, sulphorhodamine B; GC/MS, Gas chromatography/mass spectrometry; MTBSTFA, *N*-methyl-*N*-(*tert*-butyl-dimethylsilyl) trifluoroacetamide; OPA, *ortho*-phathalaldehyde; HPLC, high-performance liquid chromatography.

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A characteristic feature of many solid tumours is their requirement for exogenous methionine and their inability to proliferate in a methionine-depleted environment supplemented with homocysteine, the immediate metabolic precursor of methionine (1, 2). In contrast, normal cells are generally methionine-independent and able to efficiently utilize extracellular homocysteine and sustain growth in the absence of an external source of methionine (3). Methionine depletion is known to have marked effects upon tumour cell cycle kinetics, producing cell cycle arrest at different phases of the cell cycle, depending on the cell line studied (4-6). Methionine depletion also affects the survival and growth of tumour cells, and this phenomenon has been considered as a means for the selective targeting of methionine-dependent cancer cells (4-6).

Methionine synthase (MS; EC 2.1.1.13, 5-methyltetrahydrofolate-homocysteine methyltransferase) has an important role in maintaining methionine levels and cellular functions *via* the methylation pathway. MS catalyses the intracellular conversion of L-homocysteine to L-methionine, with a requirement for 5-methyltetrahydrofolate as a methyl-group donor and methylcobalamin as a cofactor (7). The deficiency and/or inhibition of MS has potentially other effects on cellular processes, including an increase in intracellular and extracellular levels of homocysteine, and also affects the availability of *S*-adenosylmethionine, which is required for methylation and polyamine synthesis.

MGMT (EC 2.1.1.64, also known as *O^6-methylguanine-DNA-methyltransferase* gene) activity, which is a major mechanism of resistance for tumour cells to alkylating agents such as β-chloronitrosourea (BCNU) and temozolomide (TMZ), can be down-regulated by methionine depletion in methionine-dependent tumour cells due to an inhibition of *MGMT* gene transcription (8, 9). Methionine has also been shown to maintain intracellular glutathione (GSH) levels by acting as a sulphur donor for the synthesis of cysteine and by preventing efflux of glutathione from isolated rat hepatocytes within cells (10). Therefore methionine restriction potentially

could inhibit tumour growth by increasing oxidative stress in cancer cells (11). Lowering plasma methionine levels *in vivo* by diet alone is difficult due to a wide variety of methionine sources in foods. Alternatively, the enzymatic degradation of methionine could be used to induce extracellular methionine depletion and therefore methionine deprivation in dependent cancer cells.

Therefore, the studies presented here aimed to investigate proliferation, survival, MGMT activity and glutathione levels for exponentially growing cells (Daoy and D54) during pharmacologically-relevant periods of exposure to methionine depletion, as a prelude to the investigation of the potential for this pharmacological manipulation to enhance the chemosensitivity of Daoy and D54 cells.

Materials and Methods

Cell lines and culture conditions. Two different CNS tumour cell lines were used in this study. The Daoy cell line, was a gift from Dr Steve Clifford, Northern Institute for Cancer Research, Newcastle, and the D54 cell line was obtained from Dr Darell Bigner at the Duke University Medical Centre, Durham, North Carolina, U.S.A. The Daoy cell line was adapted to grow in Dulbecco's modified Eagle's medium (DMEM), and the D54 cell line was cultured in DMEM-F12 (Biowest, East Sussex, UK). In both cases, the culture medium was supplemented with 10% foetal calf serum (FCS), 2 μM glutamine, and 1 mM sodium pyruvate. Methionine-free medium was also purchased from Biowest and cells were cultured at 37˚C and 5% CO_2.

Methionine dependency and cell growth inhibition studies. The methionine-dependent cell lines (Daoy and D54) were grown in the following medium, for different experiments: methionine-replete medium (abbreviated MET 100, *i.e.* not depleted) or methionine-free medium (abbreviated MET 0, *i.e.* 100% depleted). Each media was supplemented with 10% fetal calf serum or with dialysed fetal calf serum (dFCS, in the case of methionine depleted medium, Biowest). Cells were plated in 24-well plates at 5×10^4 cells/ml in MET 100 and incubated for 24 hours. The medium was changed after 24 hours' incubation to start the experiments. Control cells were grown in MET 100, while test cells were treated in different methionine concentrations (for Daoy cells: 200, 160, 120, 80, 40, 30, 20, 10, 0 μM; and for D54 cells: 115, 90, 70, 50, 20, 15, 10, 5, 0 μM) and at different exposure times (4, 24, 48, and 72 hours). At the end of each exposure to reduced methionine conditions, the media were changed to fresh MET 100 (recovery) and the cells were harvested at 72 hours. At the termination of the experiment, the cells were washed in phosphate-buffered saline (PBS) twice and trypsinised. Cell viability was determined and cells counted using a flow cytometer (CyFlow Space, Partec, Germany).

Methionine depletion and clonogenic survival assay. Cells were plated in 6-well plates in MET 100 for 24 hours (500 cells/well for D54 and 1,500 cells/well for Daoy). The cells were treated as described above. At the end of each exposure to reduced methionine conditions, the media in the control and the treated wells were removed and washed with 1 ml PBS, the cells resuspended in fresh MET 100 and then incubated for another 2 weeks. At the termination of the experiment, the number of colonies on each plate was counted and average percentage survival was calculated as described by Wilson (12).

Cell cycle phase distribution. Cells in exponential growth were trypsinised, counted and plated in 6-well plates at a cell density 1×10^5 cells/ml in MET 100 medium for 24 hours. Following the treatments as described earlier, cells were harvested according to the method of Kalejta *et al*. (13) and analysed using flow cytometry as described previously (13).

Determination of methionine dependency by apoptosis assay. Annexin V-fluoroscein isothiocyanate (Annexin V-FITC) apoptosis detection kit (50 μg/ml Annexin V-FITC conjugate, 100 μg/ml propidium iodide solution, and 10 × binding buffer, Sigma, UK) along with flow cytometry were used to detect the apoptotic cells (14-16).

GC/MS determination of intracellular methionine levels following methionine depletion. The retention time and major ions were determined for L-methionine (Sigma) and isotopically- labelled methionine (Sigma) by GC/MS, and a calibration line was established. In the case of L-methionine, major peaks were present at m/z values of 320 and 292 corresponding to $[M-57]^+$ and $[M-85]^+$ respectively (17, 18); for the internal standard, m/z values of 324 and 296 were obtained, the difference in mass/charge ratio provided by the isotopes.

Following treatments as described earlier, cultured cells were washed with PBS twice and trypsinised with 400 μl trypsin. The cell pellet was removed from trypsin by centrifugation (50,000 × g, 5 minutes) and the resulting cell pellet was lysed by adding an equal volume of water and freeze-thawing three times (0 to –80˚C). An aliquot of this cell lysate was used for protein content determination by the Bio-Rad protein assay (Bio-Rad Laboratories Ltd., Hertfordshire, UK). Subsequently, 50 μl of this cell lysate were mixed with 50 μl of (100 μM) [methyl ^{13}C $^{2}H_3$] methionine internal standard stock solution. The samples were dried using a concentrator system (Eppendorf concentrator 5301, Germany), then 60 μl of *N*-methyl-*N*-(tert-butyl-dimethylsilyl) trifluoroacetamide (MTBSTFA, Sigma) and 40 μl of acetonitrile (Perbio Science, Rockford, USA) were added, followed by heating at 90˚C for 30 minutes on a heating block (Recti-Therm Heating Module; Pierce, Rockford, USA). GC/MS analysis was carried out using a Varian CP 3800 gas chromatograph coupled with a Varian 1200 quadrupole MS/MS (Electron ionization source) with Varian FactorFour capillary column (5% phenyl and 95% dimethylpolysiloxane stationary phase, VF-5 ms; 30 m × 0.25 mm ID, 0.25 μm stationary phase thickness). The sample (1 μl) was injected onto the column using a split (split ratio 100:1). The injector and detector temperature were 250˚C, the oven temperature program was 150˚C (initial) held for 2 minutes, then increasing at a rate of 10˚C /minute until a final temperature of 300˚C and held for 3 minutes for a total analysis time of 22 minutes. The detector voltage was 1100 V, and the carrier gas (helium) was a constant flow of 1 ml/min. Quantitative analysis was carried out with the mass spectrometer in selected ion monitoring (SIM) mode. Ions selected were (292) for methionine and (296) for isotopically-labelled methionine.

Determination of MS activity following methionine depletion. Following exposure to methionine-free medium and cell lysis, a non-radioactive assay for MS measuring tetrahydrofolate (H_4folate) concentration was

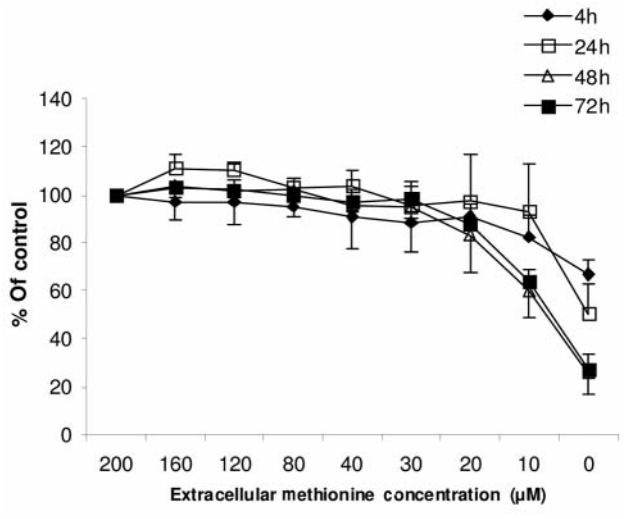

Figure 1. *Effects of methionine depletion on Daoy growth in culture containing different methionine concentrations at different incubation times. The results for each time point represent the mean±SD, n=3.*

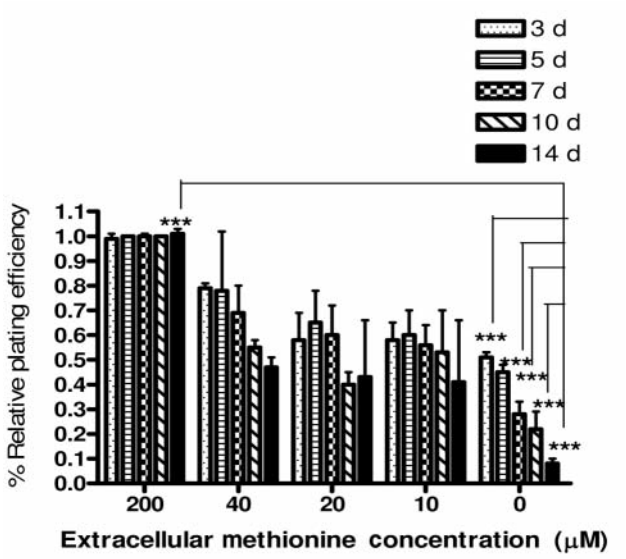

Figure 2. *Colony formations in Daoy cells cultured in different methionine concentrations at different time intervals. Values are means±SD (n=3). All comparisons were made using two-tailed Student's t-test (*p<0.05, **p<0.01, ***p<0.001).*

used. The H_4folate may be converted to methenyltetrahydrofolate (CH^+=H_4folate) by heating in formic acid. (The procedure was identical to those described previously (19, 20)).

Determination of glutathione levels following methionine depletion. Determination of free glutathione (GSH) in cell lysate was carried out using a method described previously (21) with a slight modification. Cells were treated and lysed a described above. Separation of GSH-OPA adducts was achieved on as Synergi 4u C-18 Hydro-RP column (150×4.6 mm ID; Phenomenex, Macclesfield, Cheshire, UK). Glutathione was detected using fluorimetry (Varian Model 363) at 420 nm after excitation at 340 nm. Derivatives were eluted isocratically with 20% acetonitrile (VWR, UK) and 80% Tris buffer (Sigma), 0.1 M at pH 7. The flow rate during elution was 0.7 ml/min for a total analysis time of 15 minutes.

Determination of MGMT activity following methionine depletion. After treatment, cell pellets were analysed using methods modified from those of Wu *et al*. (22).

Statistics. All determinations were performed at least in triplicate and means and standard deviations determined. All comparisons were made using two-tailed Student's *t*-test .

Results

Effects of level and duration of methionine depletion on proliferation and survival, cell cycle and apoptosis levels. As the percentage of methionine in the tissue culture medium was reduced to MET 5 (95% methionine depletion) and with longer incubation times of up to 72 hours, a marked reduction in the growth rate of the Daoy and D54 cell lines was observed (Figure 1), and cell growth was inhibited to less than 50% of that found in methionine-replete conditions. For Daoy cells, this effect was seen at methionine concentrations of less than 10 μM, and for D54 cells the threshold was 5.75 μM. (Note: For Daoy cells, MET 100=200 μM, MET 5=10 μM, and for D54 cells MET 100=115 μM, MET 5=5.75 μM.)

Evidence of cell cycle distribution, for both Daoy and D54 cells, showed unchanged cell cycle characteristics under conditions of even total methionine depletion up to 72 hours (Data not shown). In addition, the results show that the apoptotic cell population in Daoy cells increased significantly (3-fold, $p<0.01$) from control levels of 15% ±6 to 52% ±6.8 after exposure to methionine-free medium for 72 hours, and the apoptotic cell populations for D54 cells also increased significantly (2-fold, $p<0.05$) from control levels of 25% ±9 to 50% ±12 under the same conditions (Data not shown).

Clonogenic cell survival was significantly influenced by the degree and duration of methionine-depletion. For Daoy cells, the effect on reducing colony formation increased with both the degree and duration of methionine depletion, with the maximal effect on colony formation found compared to that of the control cells after 14 days at 0 μM methionine (9-fold reduction, $p<0.001$), although there was some reduction in the colony formation observed with extracellular methionine concentrations of 40 to 20 μM (Figure 2). However, this effect was less pronounced for D54 cells, where significant effects on colony formation. In contrast,

this effect was less pronounced for D54 cell lines, where the effects on colony formation where a maximum of a 6-fold reduction ($p<0.01$) was found after 14 days in conditions of complete methionine depletion (Data not shown).

Effects of degree and duration of methionine depletion on cellular biochemical parameters. The depletion of extracellular methionine in Daoy cells resulted in a significant decrease in intracellular methionine levels within 48 hours of culture in 40 μM methionine (MET 20), with an approximate 2-fold decrease resulting from culture in 0 μM methionine (MET 0) concentrations for 72 hours (Table I). In the case of D54 cells, the decrease in methionine levels was found to be less pronounced than for Daoy cells, where the control levels of methionine were largely preserved (Table I). In Daoy cells, with increasing degree and time of methionine-depletion there was an increase (4-fold) in methionine synthase activity at 48 hours, but this was not sustained for example, a significant decrease observed (75%) in 4 hours followed by a 2-fold and 4-fold increase at 24 and 48 hours respectively and then a fall to control values at 72 hours. In contrast, for D54 cells a significant increase in methionine synthase activity 72 hours after culture in conditions of complete methionine depletion was found for all time points measured between 4 and 72 hours, with 1.5-fold increase maximal at 48 hours (Table I).

The influence of methionine-depletion on MGMT activity and glutathione levels in Daoy and D54 cell lines was investigated (Table I). D54 cells did not express MGMT activity and a significant (4-fold, $p<0.001$) reduction in MGMT activity in Daoy cells was observed in the face of complete methionine depletion for 72 hours. An increase (2-fold, $p<0.05$) in GSH levels was observed for both cell lines under conditions of complete methionine depletion.

Discussion

Methionine-dependence is a known characteristic of certain cancer cells, and Daoy and D54 cells were found to be methionine-dependent and unable to survive and proliferate when methionine was replaced in the medium with homocysteine (data not shown), and this agrees with previous studies for other cancer types, as reviewed by Cellarier *et al.* (23).

This present study has shown that the degree and duration of methionine depletion influences the growth, survival and cellular biochemistry of Daoy and D54 cells *in vitro*, when investigated for a pharmacologically-relevant duration of methionine depletion. The growth of Daoy and D54 cells is inhibited with extracellular methionine concentrations below 10 μM and 5.75 μM respectively, with these effects evident after 4 hours and 24 hours of methionine depletion, respectively. Moreover, our studies clearly demonstrate that the growth inhibitory, pro-apoptotic and cytotoxic effects of methionine depletion depend both upon the degree and duration of depletion over the time course that the D54 and Daoy cells would have otherwise been in exponential growth. A similar effect with respect to the degree and duration of methionine depletion has been found for pre-clinical xenograft models *in vivo*. For example, growth delay was observed below 5 μM plasma methionine for Daoy, D54, SWB77 (24) and SWB40 xenografts in athymic mice after exposure to dietary deprivation of methionine combined with methioninase and homocystine (25).

Although in this present study Daoy and D54 cells were found to undergo apoptosis under conditions of methionine depletion, there is no evidence for disruption of the cell cycle and mitotic activity following methionine depletion for 72 hours, when compared to the cell cycle in control cells in methionine-replete medium. This is in agreement with the findings of Kokkinakis *et al.* (26) who reported that the cell cycle distribution was not affected in E.DERM and CCD-187sk cells cultured in conditions of methionine deprivation for up to 96 hours (27). In contrast, other studies have described G2 cell cycle blockade in Daoy cell lines at day two in methionine-deficient medium (9), and similar finding have been described in U-138, H-1632 and D-341 cell lines (9).

During the time course of the variations in methionine depletion investigated for this current study, intracellular methionine levels were significantly depleted to 40% and 60% of control values for Daoy and D54 cells, respectively, with the maximum effect observed after 72 hours in the absence of extracellular methionine. A similar observation for the reduction of tumour methionine levels has been found *in vivo* for mice bearing Daoy xenografts, where a 90% reduction in tumoural methionine levels was achieved within 4 days in the face of plasma concentrations of less than 5 μM after treatment with methioninase and homocystine supplementation in the setting of deprivation of dietary methionine (4).

Although intracellular methionine levels in Daoy and D54 cell lines were significantly lowered under conditions of increasing methionine depletion - this was not mirrored by a consistent increase in MS activity. This is in agreement with Hoffman *et al.* (1) who demonstrated that MS activity for cancer cells is sometimes found to be high, as reflected in their increased metabolic requirements. For Daoy cells, a biphasic pattern of change in MS activity was found, with an initial increase in activity over 48 hours being followed by a reduction in comparison to control levels thereafter. For D54 cells, methionine depletion resulted in a more consistent pattern of increased MS activity. This is in keeping with the findings of Kamely *et al.* (28) and Tisdale (29), who demonstrated that MS activity increased in BHK cells (28) and melanoma cells (29) when they were shifted from a methionine-replete medium to a methionine-depleted

Table I. *Effects of methionine depletion on intracellular methionine and glutathione levels, and methionine synthase and MGMT activity in control conditions as compared to the levels after 72 hours' exposure to 0 μM methionine in Daoy and D54 cells. Values are means±SD, n=3. All comparisons were made using two-tailed Student's t-test (*p<0.05, ***p<0.001). (For Daoy cells 0% methionine-free=200 μM, for D54 cells 0% methionine-free=115 μM, and 100% methionine-free=0 μM).*

Extracellular methionine level	MGMT activity in Daoy cells (fmol/μg DNA)	Intracellular GSH (nmol/mg protein)		Methionine synthase activity (nmol/min/mg protein)		Intracellular methionine level (nmol/mg protein)	
		Daoy	D54	Daoy	D54	Daoy	D54
Control	8.1±0.4***	67±29*	90±45*	4.7±2.5*	2.6±1.0***	7.7±0.9***	3.7±0.4***
Complete depletion	2.8±0.5***	132±49*	167±87*	3.6±1.0*	4.7±0.9***	2.8±1.1***	2.8±0.5***

medium supplemented with homocysteine. Thus, the effects of methionine depletion were less pronounced for D54 when compared with Daoy cells, with the latter cells demonstrating lower intracellular methionine levels, a failure to maintain an adaptive increase in MS activity and reduced clonogenic survival at all degrees and durations of methionine depletion tested in these experimental conditions.

The influence of methionine-depletion on measurement of MGMT activity and glutathione levels, *in vitro*, of Daoy and D54 cell lines was determined for this pharmacologically relevant period of methionine depletion. A 4-fold reduction in the MGMT activity of Daoy cells was observed in the absence of methionine within 72 hours. This finding is in keeping with previous studies for Daoy cell line and Daoy xenografts (8). We have shown that the D54 cell line does not exhibit detectable MGMT activity under different degree and durations for methionine-depletion of up to 72 hours, in agreement with results obtained by Kokkinakis *et al*. (9) and Bocangel *et al*. (30) who demonstrated that D54 cell line and xenografts in athymic mice have a very low MGMT activity.

The results presented in this study also show that methionine depletion gives rise to an increase in intracellular glutathione levels for Daoy and D54 cells, with the maximum effect found with extracellular methionine concentrations below 10 μM for 72 hours. The effect seen was a 2-fold increase in intracellular glutathione levels, and was observed within 48-72 hours in the absence of extracellular methionine for both cell types. However, there was a decrease in intracellular methionine levels in both cell lines, and methionine is involved in glutathione biosynthesis as a precursor for cysteine. Thus, the cause of this phenomenon is unknown. Different observations for glutathione status have been found for different tissues in the body in the face of methionine restriction. For example, an increase in plasma glutathione was observed after deprivation of dietary methionine in athymic mice bearing human brain tumour xenografts (4).

In summary, methionine depletion causes growth inhibition, increases apoptosis and decreases cell survival of Daoy and D54 cells in a manner proportional to the degree and time of depletion to extracellular methionine concentrations below 10 μM and 5.75 μM respectively. In addition, the degree of intracellular methionine depletion, and consequent effects on clonogenic survival, was greater for Daoy cells and this may be due to the fact that methionine synthase activity is not maintained and falls as methionine depletion continues after 48 hours. Methionine depletion causes apoptosis and cell death for both cell lines, but without effects upon the cell cycle. Furthermore, methionine depletion down-regulates *MGMT* activity for Daoy cells, and increases intracellular glutathione levels for both Daoy and D54 cells. The potential effects of theses methionine-related cellular biochemical changes for Daoy and D54 cells for chemotherapeutic synergy will be the subject of further investigation.

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References

1 Hoffman R and Erbe R: High *in vivo* rates of methionine biosynthesis in transformed human and malignant rat cells auxotrophic for methionine. Proc Natl Acad Sci USA *73*: 1523-1527, 1976.

2 Mecham J, Rowitch D, Wallace CD, Stern PH, Hoffman RM: The metabolic defect of methionine dependence occurs frequently in human tumour cell lines. Biochem Biophys Res Commun *117*: 429-434, 1983

3 Halpern B, Clark B, Hardy DN, Hardy RM, Smith RA: The effect of replacement of methionine by homocysteine on survival of malignant and normal adult mammalian cells in culture. Proc Natl Acad Sci USA *71*: 1133-1136, 1974.

4 Kokkinakis D, Schold SC Jr and Nobori T: Effect of long-term depletion of plasma methionine on the growth and survival of human brain tumour xenografts in athymic mice. Nutr Cancer *29*: 195-204, 1997.

5 Pavillard V, Drbal A, Swaine DJ., Phillips RM, Double JA, Nicolaou A: Analysis of cell-cycle kinetics and sulfur amino acid metabolism in methionine-dependent tumour cell lines; the effect of homocysteine supplementation. Biochem Pharmacol *67*: 1587-99, 2004.

6 Pavillard V, Nicolaou A, Double JA and Phillips RM: Methionine dependence of tumours: A biochemical strategy for optimizing paclitaxel chemosensitivity *in vitro*. Biochem Pharmacol *71*: 772-778, 2006.

7 Fiskerstrand T, Christensen B, Tysnes, OB, Ueland PM and Refsum H: Development and reversion of methionine dependence in a human glioma cell line: relation to homocysteine remethylation and cobalamin status. Cancer Res *54*: 4899-4906, 1994.

8 Kokkinakis D, von Wronski M, Vuong TH, Brent TP and Schold SC: Regulation of O^6-methylguanine-DNA methyltransferase by methionine in human tumour cells. Br J Cancer *75*: 779-788, 1997.

9 Kokkinakis D, Bocangel D, Schold SC, Moschel RC and Pegg AE: Thresholds of O^6-alkylguanine-DNA alkyltransferase which confer significant resistance of human glial tumour xenografts to treatment with 1,3-*bis*(2-chloroethyl)-1-nitrosourea or temozolomide. Clin Cancer Res *7*: 421-428, 2001.

10 Aw T, Ookhtens M and Kaplowitz N: Inhibition of glutathione efflux from isolated rat hepatocytes by methionine. J Biol Chem *259*: 9355-9358, 1984.

11 Epner D: Can methionine restriction increase the effectiveness of chemotherapy in treatment of advanced cancer? J Am Coll Nutr *5*: 443S-449S, 2001.

12 Wilson A: Cytotoxicity and Viability Assays in Animal Cell Culture: A Practical Approach. Eshrey RI (ed.). Oxford, Oxford IRL Press 1986.

13 Kalejta RF, Shenk T and Beavis AJ: Use of a membrane-localized green fluorescent protein allows simultaneous identification of transfected cells and cell cycle analysis by flow cytometry. Cytometry *29*: 286-291, 1997.

14 Martin S, Reutelingsperger C, McGahon AJ, Rader JA, van Schie RC and LaFace DM: Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J Exp Med *182*: 1545-1556, 1995.

15 Pigault C, Follenius-Wund A, Schmutz M, Freyssinet J and Brisson A: Formation of two-dimensional arrays of annexin V on phosphatidylserine-containing liposomes. J Mol Biol *236*: 199-208, 1994.

16 Trotter P, Orchard M and Walker JH: Ca^{2+} concentration during binding determines the manner in which annexin V binds to membranes. Biochem J *308*: 591-598, 1995.

17 Mashima R, Nakanishi-Ueda T and Yamamoto Y: Simultaneous determination of methionine sulfoxide and methionine in blood plasma using gas chromatography–mass spectrometry. Anal Biochem *313*: 28-33, 2003.

18 Stabler S, Marcell P, Podell ER and Allen RH: Quantitation of total homocysteine, total cysteine, and methionine in normal serum and urine using capillary gas chromatography–mass spectrometry. Anal Biochem *162*: 185-196, 1987.

19 Drummond J, Jarrett J, Gonzalez JC, Huang S and Matthews RG: Characterization of nonradioactive assays for cobalamin-dependent and cobalamin-independent methionine synthase enzymes. Anal Biochem *228*: 323-329, 1995.

20 Jarrett J, Goulding C, Fluhr K, Huang S and Matthews RG: Purification and assay of cobalamin-dependent methionine synthase from *Escherichia coli*. Methods Enzymol *281*: 196-213, 1997.

21 Cereser C, Guichard J, Drai J, Bannier E, Garcia I and Boget S: Quantitation of reduced and total glutathione at the femtomole level by high-performance liquid chromatography with flourescence detection: application to red blood cells and culture fibroblasts. J Chromatogr B Analyt Technol Biomed Life Sci *752*: 123-132, 2001.

22 Wu RS, Hurst-Calderone S and Kohn KW: Measurement of O^6-alkyl-DNA alkyltransferase activity in human cells and tumour tissues by restriction endonuclease inhibition. Cancer Res *47*: 6229-6232, 1987.

23 Cellarier E, Durando X, Vasson MP, Farges MC, Demiden A and Maurizi JC: Methionine dependency and cancer treatment. Cancer Treat Rev *29*: 489-499, 2003.

24 Kokkinakis D, Hoffman R, Frenkel EP, Wick JB, Han Q and Xu M: Synergy between methionine stress and chemotherapy in the treatment of brain tumour xenografts in athymic mice. Cancer Res *61*: 4017-4023, 2001.

25 Kokkinakis D, Wick J and Zhou QX: Metabolic response of normal and malignant tissue to acute and chronic methionine stress in athymic mice bearing human glial tumour xenografts. Clin Res Toxicol *15*: 1472-1479, 2002.

26 Kokkinakis D, Brickner A, Kirkwood JM, Liu X, Goldwasser JE and Kastrama A: Mitotic arrest, apoptosis, and sensitization to chemotherapy of melanomas by methionine deprivation stress Mol Cancer Res *4*: 575-589, 2006.

27 Kokkinakis D: Methionine-stress: A pleiotropic approach in enhancing the efficacy of chemotherapy. Cancer Lett *233*: 195-207, 2006.

28 Kamely D, Littlefield J and Erbe R: Regulation of 5-methyltetrahydrofolate:homocysteine methyltransferase activity by methionine, vitamin B12, and folate in cultured baby hamster kidney cells. Proc Natl Acad Sci USA *70*: 2585-2589, 1973.

29 Tisdale M: Methionine metabolism in Walker carcinoma *in vitro*. Eur J Cancer *16*: 407-414, 1980.

30 Bocangel D, Finkelstein S, Schold SC, Bhakat K, Mitra S and Kokkinakis DM: Multifaceted resistance of glioma to temozolomide. Clin Cancer Res *8*: 2725-2734, 2002.

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