Methionine Restriction Reduces the Chemosensitivity of Central Nervous System Tumour Cell Lines

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Abstract. *Background: The aim of this study was to investigate the effects of methionine depletion with cytotoxic agents that are potentially influenced by depletion of methionine, and are known to have a role in CNS tumour treatments for children. Materials and Methods: Cytotoxicity studies and synergistic interactions were assayed by SRB assay. Glutathione levels were assayed by HPLC after derivatization with OPA. MGMT activity was determined by a restriction endonuclease inhibition assay. Results: Methionine depletion causes a demonstrable increase in glutathione levels for medulloblastoma (Daoy) and glioma (D54) cells, with a decrease in MGMT activity for Daoy cells. For both cell lines, methionine depletion reduces their sensitivity to a range of chemotherapy agents that interface at the level of methionine metabolism, namely temozolomide, cisplatin and methotrexate. Conclusion: The results show that methionine depletion increases the resistance of tumour cells to the chemotherapeutic agents tested. However, in methionine-replete conditions, we have demonstrated synergistic activity for various combinations of chemotherapeutic agents that are hitherto unreported and may have clinical utility for the treatment of children with CNS tumours.*

Methionine dependency, which is a phenomenon confined to malignant cells, is amenable to pharmacological manipulation both *in vitro* and *in vivo*. Indeed, methionine depletion results in cellular biochemical changes in malignant cells such as a reduction in O^6-methylguanine-DNA-methyltranferase (MGMT) activity, which can enhance cellular sensitivity to alkylating agents such as TMZ. Therefore, pre-clinical studies are needed to begin the investigation of the appropriateness of this maneuver in relation to the therapy of childhood CNS tumours, where new treatments are needed both to improve survival and reduce late-effects. For example: in Daoy, SWB77 and D54 xenografts in athymic mice, tumour regression can be achieved with prolonged methionine restriction. However, cessation of this restriction results in tumour regrowth (1). Because of potential toxicity and quality of life problems, prolonging methionine restriction with the diet is not suitable for clinical application. Therefore, the potential role of methionine restriction may be best mediated by methioninase as a part of a combination therapy. Indeed, methionine restriction may act synergistically with other cancer treatments to increase their efficacy and/or reduce their toxic side-effects (1).

Methionine restriction is known to have effects on the activity of MGMT (EC 2.1.1.64), which is a major mechanism of resistance for tumour cells to alkylating agents such as β-chloronitrosourea (BCNU) and temozolomide (TMZ), whereby methionine depletion in methionine-dependent tumour cells results in an inhibition of *MGMT* gene transcription (2, 3).

Modulation of methionine status may also affect certain factors that determine cellular sensitivity to cisplatin (CDDP). For example, Mineura *et al*. (4) demonstrated that CDDP is a potent chemical modulator in the chemotherapy of brain tumours because CDDP inhibits methionine uptake selectively in brain tumour tissue. In addition, methionine maintains intracellular glutathione levels by acting as a sulphur donor for the synthesis of cysteine and by preventing efflux of glutathione from isolated rat hepatocytes within cells (5). Moreover, methotrexate (MTX) may conceivably potentiate the effects of methionine depletion by an indirect affect on residual methionine synthase activity by depletion of the reduced folate cofactor pool (6-8), and all of the above agents have clinical utility for CNS tumours in children.

Abbreviations: CNS, Central nervous system; MGMT, O^6-alkylguanine-DNA alkyltransferase; MS, methionine synthase; SRB, sulphorhodamine B; OPA, *ortho*-phathalaldehyde; HPLC, high-performance liquid chromatography.

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Therefore, the potential role of methionine restriction may be best mediated by methioninase as a part of a combination therapy with conventional cytotoxic agents. Indeed, methionine restriction may act synergistically with other cancer treatments to increase their efficacy and/or reduce their toxic side-effects. For example, the combination of dietary methionine and choline restriction with administration of methioninase are known to treble the efficacy of TMZ against a glioblastoma xenograft model in athymic mice (SWB77), in comparison to TMZ alone (1).

In summary, the methionine metabolic status of cancer cells may result in possible chemopotentiation in the case of CDDP and TMZ, and therapy with MTX may compound the effects of extracellular methionine depletions by virtue of reduced methionine synthase (MS) activity. CDDP, TMZ and MTX may have actions to potentiate the effects of methionine depletion on Daoy and D54 cell lines and all these drugs are currently employed in treatment protocols for childhood CNS tumours worldwide. Therefore, the studies presented here aimed to investigate the effects of methionine depletion for pharmacological-relevant period of time on MGMT activity and intracellular glutathione levels, and relationship of these changes to the chemosensitivity of Daoy and D54 cells to TMZ, CDDP and MTX. In addition, investigations of potential synergistic effects of both novel and known combinations of TMZ, CDDP and/or MTX for Daoy and D54 cells in methionine-replete conditions are reported for their potential clinical utility.

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 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 case, 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.

Determination of MGMT activity following methionine depletion. Cell treatment in methionine-free medium and lyzing cells were carried out as follows: 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 medium was supplemented with 10% FCS or with dialysed FCS (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 with 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 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. The cell pellet was removed from trypsin by centrifugation (50,000 xg, 5 minutes) and the pellets stored at –80˚C until analysis. For analysis, 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. After treatment cell pellets were analysed using methods modified from those of Wu *et al*. (9).

Determination of glutathione levels following methionine depletion. Determination of free glutathione (GSH) in cell lyzate was carried out using a method described previously (10) with a slight modification. Cell treatment in methionine-free medium and lysis of cells are given above. Separation of GSH-OPA adducts was achieved on a 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, UK), 0.1 M at pH 7. The flow rate during elution was 0.7 ml/min for a total analysis time of 15 minutes.

Cytotoxicity studies. Temozolomide (Gift from Dr. Geoff Margison at Paterson Institute of Cancer Research, Manchester, UK) was reconstituted in 100% pure DMSO, MTX (Sigma, UK) was reconstituted in sterilized NaOH solution (0.05 M), and the stock solutions (25 mM MTX and 0.1M TMZ) were stored as aliquots at –20˚C until required. Cisplatin (Sigma, UK) was dissolved in sterilized distilled water, and stock solution stored at room temperature. The cytotoxicity assay (sulphorhodamine B, SRB) used was developed previously by the National Cancer Institute. It measures the cellular protein content in viable cells of adherent and suspension cultures in 96-well plates (11, 12).

Drug treatment in methionine-replete medium. Cell lines in exponential growth were trypsinised, counted and plated in a volume of 100 μl per well at density of 5,000 cells/well in 96-well plates in MET 100 medium. The cells were pre-incubated for approximately 24 hours at 37˚C to allow stabilization prior to addition of drugs. After preparation of optimum drug dilutions (0.02-1.5 μM MTX, 0.02-1.5 mM TMZ, and 0.02-1.5 μM of CDDP for the Daoy cell line, and 0.02-1.5 μM MTX, 0.09-6 mM TMZ, and 1.5-100 μM of CDDP for the D54 cell line); 100 μl aliquots of each dilution were added to the 96-well plate. Following 96 hours' incubation, plates were assayed for cellular growth and cell viability by the SRB assay (11, 12).

Drug treatment in methionine-free medium. Cells were plated in 96-well plates in MET 100 medium at density of 5,000 cells/well. In exponential growth, the medium was replaced by MET 0 medium, and cells were treated with MTX, TMZ, or CDDP. Optimum drug concentrations (0.0001-1000 μM MTX, 0.09-6 mM TMZ, and 0.02-1.5 μM of CDDP for the Daoy cell line, and 0.0001-1000 μM MTX, 0.09-6 mM TMZ, and 1.5-100 μM of CDDP for the D54 cell line) were also diluted with MET 0 medium. Following 96 hours' incubation, cells were assayed for cellular growth and viability by the SRB assay (11, 12).

Table I. *The effects of methionine depletion on intracellular glutathione levels, 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<0.01, ***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 levels	MGMT activity in Daoy cells (fmol/μg DNA)	Intracellular GSH (nmol/mg protein)	
		Daoy	D54
Control	8.1±0.4***	67±29*	90±45*
Complete depletion	2.8±0.5***	132±49*	167±87*

Drug treatments applied after methionine depletion. Cells were plated in 96-well plates in MET 100 medium at a density of 5,000 cells/well. In exponential growth, the medium was replaced by methionine-free medium (MET 0) and cells were maintained for either 2 or 5 days' incubation prior to drug treatments. Following the incubation period, cells were treated with different concentrations (see above) of each drug for 72 hours at 37˚C, and then chemosensitivity was determined by the SRB assay (11, 12).

Synergistic interactions between MTX, TMZ and/or CDDP. The synergy assay was performed using National Cancer Institute protocol with some minor changes. Culture conditions were optimized for each cell line. The two cell lines (Daoy and D54) were exposed to MTX, TMZ, and CDDP as separate agents or combination simultaneously; different sequential schedules were also used.

Simultaneous combination. At day 1, the cell lines were plated in 96-well plates as described earlier in a volume of 100 μl/well. On day 2, MTX and TMZ were added separately and simultaneously in a volume of 100 μl, for each drug and drug combination, to three wells (same procedure was applied in the combination of MTX and CDDP, or TMZ and CDDP). The IC_{50} for each drug allowed a new concentration range to be derived in which this IC_{50} was mid range, resulting in a series of final concentrations from $IC_{50} \times 0$ to $IC_{50} \times 8$. The same culture time of 96 hours for all cell lines was used, and following exposure, chemosensitivity was determined by the SRB assay (11, 12).

Dose-response interactions (antagonism, additivity, and synergism) between drugs were expressed as a nonexclusive case CI (combination index) for every FA (fraction affected), using the method of Chou and Talaly (13), processed by a computer program (CalcuSyn) developed by Biosoft, Cambridge, U.K. For the separate drugs, the respective growth inhibition parameter has to be introduced and expressed as FA (*e.g.* an FA of 0.25 is a growth inhibition of 25%). The CI was calculated from the dose of the separate drugs and their combination at a fixed ratio (13).

Sequential schedules combination. The cells were not only exposed to the combination simultaneously, but several sequential schedules were also used (at pharmacologically relevant exposure times): (a) 2 hours' pre-incubation with TMZ, after which the cells were exposed to MTX for 24 hours or 48 hours; (b) 2 hours' incubation with TMZ, followed by incubation with CDDP for 6 hours; (c) 6 hours' pre-incubation with CDDP, after which the cells were exposed to MTX for 24 hours or 48 hours; (d) 6 hours' incubation with CDDP, followed by incubation for 2 hours with TMZ; (e) 24 hours' incubation with MTX, followed by incubation with both TMZ for 2 hours and CDDP for 6 hours; and (f) 48 hours' incubation with MTX, followed by incubation with both TMZ for 2 hours or CDDP for 6 hours. Chemosensitivity was determined by SRB assay (11, 12).

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 (*$p<0.05$, **$p<0.01$, ***$p<0.001$) is significantly different from control.

Results

Effects of level and duration of methionine depletion on cellular biochemical parameters. The influence of methionine-depletion on MGMT activity and GSH levels in Daoy and D54 cell lines was investigated. 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 (Table I). An increase (2-fold, $p<0.05$) in GSH levels was observed for both Daoy and D54 cell lines under methionine depletion conditions (Table I).

Chemosensitivity of Daoy and D54 cell lines to TMZ, CDDP and MTX under conditions of methionine depletion. The influence of methionine depletion on the response of Daoy and D54 cell lines to TMZ, CDDP and MTX is presented in Table II. The TMZ IC_{50} for Daoy cells increased 4-fold under conditions of concomitant MET 0, and 3-fold under conditions of 2- or 5-day pre-incubation in MET 0 followed by a single TMZ treatment for 72 hours ($p<0.05$). In the case of the D54 cell line, TMZ IC_{50} values were similar for control and MET 0 conditions, when these exposures began simultaneously. However, TMZ IC_{50} values increased 2-fold under the conditions of 2- or 5-day pre-incubation in MET 0 followed by a single TMZ treatment for 72 hours ($p<0.01$) (Table II).

CDDP IC_{50} in Daoy cells increased 2-fold under conditions of MET 0 ($p<0.05$) and 15-fold under conditions of 2- or 5-day pre-incubation in MET 0 followed by a single CDDP treatment for 72 hours. A marked reduction in chemosensitivity

Table II. *Cytotoxicity values (Mean±S.D, n=3) of TMZ, CDDP and MTX in Daoy and D54 cell lines. All comparisons were made using two-tailed Student's t test (*p<0.05, **p<0.01, ***p<0.001).*

Extent of methionine depletion	TMZ IC_{50} (mM)		CDDP IC_{50} (μM)		MTX IC_{50} (μM)	
	Daoy	D54	Daoy	D54	Daoy	D54
Concomitant in MET 100	0.46±0.15*	0.77±0.01**	0.08±0.05*	8±1***	0.07±0.01***	0.1±0.01***
Concomitant in MET 0	1.95±0.68*	0.8±0.22**	0.20±0.03*	21±4***	>1000***	>1000***
2 Days' pre-incubation in MET 0	1.45±0.27*	1.75±0.15**	1.56±0.72*	17±7***	>1000***	>1000***
5 Days' pre-incubation in MET 0	1.36±0.2*	1.55±0.05**	1.33±0.72*	22±8***	>1000***	>1000***

was also observed in the case of D54 cell lines treated with CDDP, with increased IC_{50} values almost 3-fold under conditions of MET 0 and 5-day pre-incubation in MET 0 followed by a single CDDP treatment for 72 hours ($p<0.001$) (Table II). In the case of MTX, both cell lines (Daoy and D54) were significantly resistant to MTX in MET 0, and pre-methionine depletion for 2 days and 5 days resulted in MTX IC_{50} values greater than 1000 μM ($p<0.001$) (Table II).

Studies of cytotoxic combinations. When the cell lines were exposed to the sequential schedule combinations of MTX:TMZ, MTX:CDDP or TMZ:CDDP in methionine-replete conditions, very different patterns of sensitivity were observed for the combinations and the cell lines studied (Table III). For Daoy cells, and for exposure duration of 2 hours of TMZ followed by 6 hours CDDP or 24 hours MTX, synergistic effects were observed. Moreover, exposure to 2 hours' TMZ followed by 48 hours' MTX was additive for Daoy cells and demonstrated synergistic effects for D54 cells. At an exposure duration of 6 hours for CDDP, additive effects were observed following subsequent incubation for 24 hours with MTX and synergism observed following incubation with MTX for 48 hours in Daoy cells. Pre-incubation of CDDP for 6 hours followed by 2 hours' TMZ was also synergistic for Daoy cells. For an initial MTX exposure of 24 hours, synergism was observed following subsequent treatment with TMZ for 2 hours, while antagonism was observed following incubation for 6 hours with CDDP in Daoy cells. Pre-incubation of MTX for 48 hours followed by 2 hours' TMZ or 6 hours' CDDP resulted in antagonistic effects. In the case of D54 cells, synergistic effects were observed in all cases except pre-incubation of MTX for 24 hours and 48 hours followed by CDDP incubation for 6 hours, which was antagonistic.

Table III. *Shows the combination index values and a summary of the effect for scheduling of combination of MTX, TMZ and CDDP for Daoy and D54 cells. A value of CI=0.1-0.3 indicates strong synergism; 0.3-0.7 synergism; 0.7-0.85 moderate synergism; 0.85-0.9 slight synergism; 0.9-1.1 nearly additive; 1.1-1.2 slight antagonism; 1.2-1.45 moderate antagonism; 1.45-3.3 antagonism; 3.3-10 strong antagonism. All experiments were performed at least in triplicate.*

Sequential schedule combination	Daoy (CI)	D54(CI)
2-h TMZ followed by 6-h CDDP	0.78	0.56
2-h TMZ followed by 24-h MTX	0.69	0.25
2-h TMZ followed by 48-h MTX	1.02	0.31
6-h CDDP followed by 2-h TMZ	0.5	0.53
6-h CDDP followed by 24-h MTX	1.10	0.66
6-h CDDP followed by 48-h MTX	0.9	0.53
24-h MTX followed by 2-h TMZ	0.76	0.43
48-h MTX followed by 2-h TMZ	1.23	0.94
24-h MTX followed by 6-h CDDP	1.3	1.59
48-h MTX followed by 6-h CDDP	1.62	7.32

Discussion

The aims of these studies were to investigate the influence of methionine depletion on the sensitivity of Daoy and D54 cells exposed to TMZ, CDDP and MTX, and relate any changes in sensitivity found to the effect of methionine depletion on MGMT activity and glutathione levels. In addition, the potential synergies of TMZ, CDDP and/or MTX for Daoy and D54 cells were investigated in methionine-replete 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 (2). We have shown that the D54 cell line does not exhibit detectable MGMT activity under different degrees and durations of methionine depletion of up to 72 hours, in agreement with results obtained by Kokkinakis *et al*. (3) and Bocangel *et al*. (14), 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

GSH levels for Daoy and D54 cells, with the maximum effect found with extracellular methionine concentrations below 10 μM for 72 hours. The effect seen is a 2-fold increase in intracellular GSH levels, and was observed within 48-72 hours in the absence of extracellular methionine for both cell types. Thus, the cause of this phenomenon is unknown. Different observations for GSH status have been found for different tissues in the body in the face of methionine restriction. For example, an increase in plasma GSH was observed after deprivation of dietary methionine in athymic mice bearing human brain tumour xenografts (15).

For the D54 and Daoy cells studied here, methionine depletion resulted in an increased resistance to CDDP, and this is in keeping with a human colon cancer TC71-MA which was poorly sensitive to CDDP and displayed high levels of GSH and expressed multidrug resistance proteins (16). Although the decrease in sensitivity to CDDP for D54 and Daoy cells might at least in part be explained by the increased intracellular GSH levels that occurred in the face of extracellular methionine depletion, the concomitant reduction in MGMT activity for Daoy cells did not translate to increased sensitivity to TMZ. Indeed, the reverse was found, and both D54 and Daoy cells also became markedly resistant to MTX under these conditions of prior methionine depletion.

Therefore, other factors may be related to the decrease in chemosensitivity found for D54 and Daoy cells in the face of methionine depletion. For example, resistance to CDDP has been found to relate to overexpression of multi-drug resistance proteins such as MRP2 (17). Mechanisms for resistance to TMZ such as inefficient *p21* induction (14) and mismatch repair deficiency (18), and changes in expression for the reduced folate carrier, folylpolyglutamyl synthetase, and the various target enzymes for MTX such as dihydrofolate reductase are known to occur, but were not the subject of study for this present investigation (8). Our findings for a reduction of chemosensitivity contrast with the findings of Pavillard *et al*. (19) although in these latter studies, increased chemosensitivity was found when cells were released from the synchronised cell cycle arrest induced by methionine depletion (19). Because methionine depletion increases resistance to MTX, CDDP and TMZ, drug combinations were tested under methionine-replete conditions. However, in methionine-replete conditions, we have identified drug combinations that may have clinical utility. For example, our results have confirmed the previously reported synergy for the combinations of CDDP and TMZ in either sequence of administration, and we have also found some hitherto unreported combinations that may be of clinical interest. For example, the combinations of CDDP followed by MTX, and TMZ followed by MTX are synergistic for D54 and Daoy cells. However, antagonistic effects were found for MTX followed by CDDP.

In summary, the phenomenon of methionine dependency may yet be the subject of a clinical study for children with CNS tumours, but our results advocate caution in terms of the potential for adjuvant chemosensitization. In our studies, methionine depletion confers a reduction in chemosensitivity to a variety of different agents, and the mechanisms responsible for this phenomenon should be the subject of further study.

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