[CANCER RESEARCH 61, 4017–4023, May 15, 2001]

Synergy between Methionine Stress and Chemotherapy in the Treatment of Brain Tumor Xenografts in Athymic Mice[1]

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ABSTRACT

This study describes a novel approach to the treatment of brain tumors with the combination of recombinant L-methionine-α-deamino-γ-lyase and chemotherapeutic regimens that are currently used against such tumors. The growth of Daoy, SWB77, and D-54 xenografts in athymic mice was arrested after the depletion of mouse plasma methionine (MET) with a combination of a MET- and choline-free diet and recombinant L-methionine-α-deamino-γ-lyase. The treated tumor-bearing mice were rescued from the toxic effects of MET withdrawal with daily i.p. homocystine. This regimen suppressed plasma MET to levels below 5 μM for several days, with no treatment-related deaths. MET depletion for 10–12 days induced mitotic and cell cycle arrest, apoptotic death, and widespread necrosis in tumors but did not prevent tumor regrowth after cessation of the regimen. However, when a single dose of 35 mg/m^2 of *N*,*N*′-bis(2-chloroethyl)-*N*-nitrosourea (BCNU), which was otherwise ineffective as a single therapy in any of the tumors tested, was given at the end of the MET depletion regimen, a more than 80-day growth delay was observed for Daoy and D-54, whereas the growth of SWB77 was delayed by 20 days. MET-depleting regimens also trebled the efficacy of temozolomide (TMZ) against SWB77 when TMZ was given to animals as a single dose of 180 mg/m^2 at the end of a 10-day period of MET depletion. The enhanced responses of both Daoy and SWB77 to DNA alkylating agents such as BCNU and TMZ could be attributed to the down-regulation of *O*^{6}-methylguanine-DNA methyltransferase activity. However, the synergy of MET depletion and BCNU observed with D-54 tumors, which do not express measurable *O*^{6}-methylguanine-DNA methyltransferase protein, is probably mediated by a different mechanism. MET depletion specifically sensitizes tumors to alkylating agents and does not significantly lower the toxicity of either BCNU or TMZ for the host. In this regard, the combination approach of MET depletion and genotoxic chemotherapy demonstrates significant promise for clinical evaluation.

INTRODUCTION

The prognosis for patients with glioblastoma and anaplastic astrocytoma has changed little despite aggressive cytoreductive surgery, radiation, and chemotherapy (1). The propensity of individual neoplastic cells to migrate along white matter pathways (2) renders treatment directed solely at the main tumor mass of palliative benefit only. To date, no treatment has been developed that is capable of destroying all residual neoplastic cells within tissue remaining after surgery or radiation therapy, and these residual cells serve as the reservoir from which brain tumors may progress and recur (2). The failure of chemotherapy to significantly enhance survival by killing or delaying regrowth of tumors from residual neoplastic reservoirs remains challenging. Bifunctional nitrosoureas such as BCNU[3] and *N*-(2-chloroethyl)-*N*′-cyclohexyl-*N*-nitrosourea have had only modest objective responses in patients with anaplastic gliomas (3). The limited responses appear to be due to mechanisms of resistance (4–6). More recently, the methylating drug TMZ has shown promising activity against astrocytomas and glioblastomas, but again, resistance mechanisms in the majority of astroglial tumors have limited its effectiveness (7, 8).

An important and well-characterized mechanism contributing to the resistance of brain tumors to the nitrosoureas and DNA methylating drugs is the up-regulation of AGT during neoplastic progression (9, 10). AGT confers resistance to agents that exert their cytotoxic action via the formation of *O*^{6}-alkylguanine adducts, which either form lethal double-strand cross-links, as is the case with bifunctional nitrosoureas such as BCNU and *N*-(2-chloroethyl)-*N*′-cyclohexyl-*N*-nitrosourea (11), or interfere with mismatch repair mechanisms resulting in an abortive repair and cell death, as is the case with TMZ, procarbazine, and other DNA methylating agents (12–14). The role of AGT in the resistance of brain tumors to DNA alkylating drug therapy has been demonstrated with the use of AGT inactivators, such as BG and *O*^{6}-benzyl-2′-deoxyguanosine (15–17), which enhance the efficacy of a variety of DNA alkylating agents against brain tumor xenografts in mice (18–20). These observations suggested that drugs that have been marginally active against brain tumors in the past could be successfully reintroduced in combination with AGT inhibitors (21). A number of clinical trials are currently in progress to determine the feasibility of such a strategy (21–23). Unfortunately, the AGT inhibitors currently available are not tumor specific, so they sensitize normal tissues as well as the tumor to the cytotoxic effect of the alkylating drug (24, 25). An alternate strategy to achieve specific depletion of tumor AGT has been considered by introducing AGT inhibitors as prodrugs that are activated exclusively by the tumor, but synthesis of tumor-specific AGT inhibitors has not yet been realized.

A method to deplete AGT activity in certain tumors that display strong MET dependence has been developed in culture by MET deprivation and substitution of this essential amino acid with HCYS precursors (26). MET-dependent tumor cells down-regulate their AGT activity in response to MET deprivation and, as a result, show enhanced tumor cell kill with BCNU (26). Because we have demonstrated that various degrees of MET dependence are a common finding in brain tumors and because we have developed a method to achieve consistent and protracted depletion of plasma MET levels (27), the current study examines the effect of MET deprivation on the growth of human brain tumor xenografts in athymic mice. In addition, it evaluates the potential synergism of the MET-depleted state and chemotherapy with BCNU and TMZ as compared with the normal nutritional state. The MET depletion was achieved by dietary restric-

Received 11/8/00; accepted 3/16/00.

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[1] Supported by Grants CA 78561, CA 57725, and CA 86166 from the National Cancer Institute and the Children's Brain Tumor Foundation of the Southwest.

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[3] The abbreviations used are: BCNU, *N*,*N*′-bis(2-chloroethyl)-*N*-nitrosourea; AGT, *O*^{6}-alkylguanine-DNA alkyltransferase; BG, *O*^{6}-benzylguanine; HCYS, homocysteine; HCYSS, homocystine; MET, methionine; METdr, methionine-depleting regimen; rMETase, recombinant methioninase; TMZ, temozolomide; HPLC, high-performance liquid chromatography; T − C, growth delay; *O*^{6}-MeG, *O*^{6}-methylguanine.

tion and the use of recombinant L-methionine-α-deamino-γ-lyase (methioninase.)

MATERIALS AND METHODS

Chemicals. rMETase was prepared at AntiCancer Inc. from an rMETase high expression clone derived from *Pseudomonas putida*. This was used for the production of rMETase in *Escherichia coli* (28, 29). The rMETase was purified with a DEAE-Sepharose (fast flow) column. Endotoxin was removed with an Acticlean Etox (Sterogen, Arcadia, CA) column. The isolated rMETase was 98% pure by HPLC and a single band of M_r 43,000 on SDS-PAGE. The specific activity of rMETase used in this study was ~20 units/mg protein, and the endotoxin level was <0.2 units/mg (28). The K_m for MET and HCYS was 0.7 and 1.7 mM, respectively, whereas the V_{max} for these two substrates was determined as 0.07 μM/min/unit.[4] HCYSS was not a substrate for rMETase.[4] BCNU was purchased from Bristol-Myers Squibb (Princeton, NJ), and TMZ was donated by Schering-Plough Inc. (Madison, NJ).

Animals. Four-week-old BALB/c-*nu/nu* athymic mice were purchased from Harlan Laboratories (San Diego, CA). Mice were maintained under filter air barrier conditions and given sterilized food and water.

Tumor Lines. MET-dependent tumor cell line D-54 and SWB77 (human glioblastomas) and Daoy (human medulloblastoma) xenografts used in this study were grown in our laboratory. Cell lines were propagated in 5% fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD) in Eagle's MEM (Life Technologies, Inc.) and supplemented with lysine, valine, MET, and leucine (100 mM each); nonessential amino acids (1:100 dilution of stock from Life Technologies, Inc.); 1 mM sodium pyruvate; 1 μM α-hydroxy-cobalamin; 10 μM folic acid; and 0.2 mg/ml gentamicin. s.c. tumors grew after injection of 3–4 million cells/animal. Daoy xenografts were propagated in athymic mice and macerated and frozen in 18% DMSO in fetal bovine serum at −70°C until use. Tumor xenografts of D-54, SWB77, and Daoy had AGT activities of <10, 75, and 376 fmol/mg protein, respectively, as determined by biochemical assay. Respective mitotic indices in the xenografts were 5.5, 5.7, and 18.9 mitotic figures per high power field (×400). Some core necrosis was observed in growing Daoy tumors, in agreement with the rapid growth pattern of this tumor, but D-54 and SWB77 tumors showed no necrotic patterns. However, even in Daoy tumors, necrosis was significant in tumor sizes exceeding 500–600 mm^3; thus, it did not interfere with the assessment of drug-induced necrosis in the tumors treated in this study.

Diets. The following pelleted synthetic, mouse formulated diets were prepared by Dyets (Bethlehem, PA). The amino acid defined Lombardi diet without DL-HCYS (diet 518786) is referred to as MET(+)HCYS(−)CHOL(+). The DL-HCYS, choline-deficient diet (diet 518763) is referred to as MET(+)HCYS(−)CHOL(−). Diet 518787 contained 1.7 g/kg DL-HCYS and was MET and choline deficient. It is referred to as MET(−)HCYS(+)CHOL(−). Diet 518788 was devoid of MET, HCYS, and choline and is referred to as MET(−)HCYS(−)CHOL(−). With the exception of the latter diet, all diets were efficient in maintaining growth of athymic mice. The composition of these entirely defined synthetic diets has been published previously (27). MET(+)HCYS(−)CHOL(+) supported growth of the human tumor xenografts in athymic mice and the growth of athymic mice equally as well as a natural basal diet (Teklad Laboratory diet; Harlan, Indianapolis, IN). All experimental animals received a basal Harlan Teklad laboratory mouse diet until they weighed 16–18 g. At that time, animals were inoculated s.c. with tumors and switched to a MET-deficient synthetic diet with or without choline, as described in individual experiments. METdrs were administered 48 h after withdrawal of dietary MET by switching to the MET(−)HCYS(+)CHOL(−) or the MET(−)HCYS(−)CHOL(−) diets. The latter diet was supplemented with 20 mg/kg daily i.p. injections of HCYSS to maintain the functions of normal tissue.

Drug Treatment. rMETase was administered i.p. at a dose of 1500 units/kg every 8 h (unless otherwise noted) for 10–12 days in animals receiving MET-free diets. Sterile enzyme was given in a volume of 30 ml/m^2 surface area in PBS. BCNU was administered i.p. in ethanol in water from a stock solution of 20 mg/ml in anhydrous ethanol. Depending on the dose of BCNU, the injected ethanol carrier varied from 10–15% and was given in a volume of 20 ml/m^2. TMZ was dissolved in DMSO and administered i.p. in a volume of 20 ml/m^2. Drug doses were calculated as mg/m^2 using the formula $m^2 = \text{weight (g)}^{2/3} \times K \times 10^{-4}$, where K is 10.5 for mice (30). In animals of 20 ± 2 g used in this study, the weight (in kg) of the animal is approximately 2.6 × the area surface (in m^2).

Tumor Implantation and Treatment. Approximately 3×10^6 D-54, SWB77, or Daoy cells in 200 μl of 5% serum media were injected s.c. in the left flank of 6-week-old athymic mice weighing between 18 and 20 g. Visible tumors appeared in most of the animals within 3–5 weeks after implantation. The tumors were subsequently measured in two perpendicular dimensions, and their volumes were estimated using the formula $(\alpha^2 \times \beta)/2$, where α is the shorter of the two dimensions, and β is the longer of the two dimensions. Treatment was administered to animals with tumors ranging between 120 and 160 mm^3 (approximately 4–5 weeks after implantation, depending on the tumor). Tumor mass was measured every other day or as otherwise indicated until tumor volumes exceeded 5× the volume of the tumor at treatment. The data were analyzed using Wilcoxon's rank-sum test, comparing the time from treatment to 5× treatment volume in individual animals in each of the groups. T − C was the difference between the median time to 5× treatment volume in the treatment group minus the median time to 5× treatment volume in the control group. The number of tumor regressions was also determined. The time tumors started to regress as a result of MET-depleting conditions depended on the tumor. When the METdr was not combined with the chemotherapeutic agent or chemotherapy was applied alone, regression was scored as a >10% maintained reduction of original tumor size over three consecutive daily measurements. When these two treatments were combined, regression was scored for the reduction of tumor size by at least 20% during the entire treatment period (10 days of MET deprivation and 5 days after BCNU or TMZ treatment) as compared with tumor size at the beginning of treatment. A complete response was defined as the disappearance of measurable tumor mass (<1 mm) at some point within a 12-week period after initiation of treatment. A maintained complete response was defined as no tumor regrowth within 12 weeks of completing the treatment regimen. Toxicity was evaluated by animal weight reduction after treatment with the METdr and the drug. Tumor regressions were compared among groups with the two-tailed Fisher's exact test. Experimental groups were treated with (*a*) METdrs alone, (*b*) METdrs followed by a single injection of BCNU, or (*c*) METdrs followed by a single injection of TMZ. Various dietary combinations were tested with each of the treatments. Control animal groups included (*a*) animals treated with BCNU alone, (*b*) animals treated with TMZ alone, and (*c*) animals treated with the carrier (15% ethanol or DMSO) alone. Control animals received either a MET(+)HCYS(−)CHOL(+) or a MET(+)HCYS(−)CHOL(−) diet throughout.

Determination of MET and HCYS. MET and HCYS content in plasma was determined by HPLC with electrochemical detection using a Coularray detector equipped with a four-channel 5010 graphite electrode (ESA, Chelmsford, MA) set at 520, 650, 750, and 850 mV operating potentials according to the following method: 150 μl of plasma were mixed with 75 μl of penicillamine (internal standard) and 25 μl of 60 mg/ml Tris 2-carboxy-ethyl-phosphine. Tubes were capped, vortexed for 60 s, and allowed to stand for 10 min at room temperature. The samples were cooled on ice, and 500 μl of ice-cold 0.3 N perchloric acid were added. The tubes were capped tightly, vortexed for 30 s, and centrifuged for 5 min at 10,000 rpm. A 100-μl aliquot of the supernatant was transferred into an ESA 542 autosampler operating at 5°C, and 20 μl were injected onto an 80 × 4.6-mm ESA HR-80 C-18 three μm HPLC column (ESA) equilibrated at 30°C. The sample was eluted isocratically with a mixture of 10% acetonitrile in 0.15 M phosphate buffer (pH 2.9) containing 1.0 mM SDS. HCYS, penicillamine, and MET were eluted at 5.1, 6.7, and 8.6 min, respectively, and identified and quantitated by the ratios of the detector's response at the operating potentials.

AGT Assay. Tumors were divided in 0.2–0.3-cm-thick sections with a razor. These sections were sampled, avoiding apparent necrotic and vascular areas. Half of the samples were processed for histology (H&E staining), whereas the other half, adjacent to those selected for histology, were used to measure AGT levels. Samples were homogenized in 20 mM Tris, 0.1 mM EDTA, and 2 mM DTT at 0°C, and protein was determined by the Bradford assay (31). The AGT assay was performed by using a slightly modified version of a method described previously (26). [^{3}H]DNA dissolved in 20 mM Tris, 0.1 mM EDTA, and 2 mM DTT (pH 7.8) and containing 60 fmol of O^6-MeG (total dpm, 24×10^3) was incubated with 100–500 mg of protein for 1 h at 37°C in

[4] D. M. Kokkinakis, unpublished observations.

a final volume of 500 μl. The reaction was quenched with 0.1 ml of 1 N HCl, and samples were incubated for an additional 45 min at 70°C. Samples were cooled on ice for 1 h, 500 μl of cold ethanol were added, and the mix was centrifuged at 14,000 × *g* for 5 min. The supernatant was removed and neutralized with sodium bicarbonate and dried by lyophilization. Lyophilized samples were dissolved in 0.12 ml of 0.01 M HCl, spun at 14,000 × *g*, and analyzed by HPLC using a Supelcosil-C18DB analytical column (Supelco, Inc.). Samples were eluted at a flow rate of 1.5 ml/min with 2% acetonitrile in 0.1 M phosphate buffer (pH 3.5; 0–5 min) followed by a gradient scintillation counting. 7-Methylguanine (7-MeG) and O^6-MeG were eluted at 4.5 and 13 min, respectively. The ratio of radioactivity of O^6-MeG:7-MeG from four samples of varying protein concentration was derived and plotted against the amount of protein. The intercept of the central linear response of the curve (between ratios 0.9 and 0.3) with the *X* axis marks the amount of protein needed to remove 60 fmol of O^6-MeG from DNA. The assay is highly specific for AGT activity and can detect AGT levels as low as 5 fmol/mg protein with an error of ~10%.

Histology. Fresh livers were harvested from animals in the different dietary groups immediately after sacrifice and immersion fixed in 10% neutral buffered formalin. After overnight fixation, the tissue was dehydrated in graded alcohol solutions to xylene and embedded in paraffin. Sections (6 μm) were cut from the paraffin blocks on a standard rotary microtome, stained with H&E, and evaluated by light microscopy by an observer blinded to the dietary status of the animal.

RESULTS

Effect of Dietary MET and Choline on Animal and Tumor Growth. Growth of animals was not altered when the basal diet was replaced with complete amino acid synthetic diets. In general, animals weighing between 18 and 22 g gained weight at a rate of 0.70 g/day (range, 0.61–1.00 g/day) when fed a synthetic diet sufficient in MET and choline. Growth of 0.60 g/day (range, 0.44–0.77 g/day) was observed when choline was restricted. In comparison, animals given a basal (natural) diet gained 0.55 g/day (range, 0.38–0.78 g/day). Replacement of dietary MET with HCYS in the absence of choline resulted in slower growth of 0.31 g/day (range, 0.22–0.36 g/day). Restriction of MET, choline, and HCYS resulted in severe initial weight loss of 0.9 g/day (range, 0.7–1.1 g/day) and death in 10–14 days. However, when animals fed a MET-, choline-, and HCYS-restricted diet were injected i.p. with 20 mg/kg HCYSS on a daily basis, weight loss was minimized to 0.2–0.3 g/day for the first 10 days. Animals given this type of METdr were alive and healthy for at least 40 days.

None of the tumors tested in these experiments (Daoy, D-54, and SWB77) grew consistently as xenografts in athymic mice fed a MET- and choline-deficient diet, even when HCYS was supplied either in the diet or i.p. In animals fed synthetic diets containing MET and choline, the tumors grew at rates comparable with those observed in animals fed a basal diet. Daoy, D-54, and SWB77 quintupled in 10, 8, and 13 days, respectively, when the host received the MET- and choline-sufficient synthetic diet. Withdrawal of choline resulted in a slight decrease of the rate of animal growth but a marked delay in the establishment of the Daoy and SWB77 tumor xenografts. However, once tumor xenografts were established, they quintupled at rates that were not statistically different from those of xenografts growing in animals fed the complete synthetic diet. These results demonstrate that a synthetic diet sufficient in MET but lacking choline can support the growth of tumor xenografts in athymic mice; therefore, this diet was used after tumor implantation.

Depletion of Plasma MET. Reduction of plasma MET below 5 μM arrests human xenograft growth in athymic mice (27). Such low levels have been achieved with a combination of dietary and pharmacological means. As shown in Table 1, plasma MET was 67 μM in athymic mice bearing 200–300 mm^3 Daoy xenografts and fed a synthetic diet that was sufficient in choline, MET, and folates. A 7-day deprivation of dietary choline resulted in the decrease of plasma MET to a third of its base value. Similar MET levels were obtained by restricting MET and choline while supplementing with dietary HCYS. To lower plasma MET below 5 μM, it was necessary to restrict dietary MET, HCYS, and choline and administer 1500 units of rMETase i.p. every 8 h. HCYSS, 20 mg/kg once a day i.p., was used as a rescue agent. As shown in Table 1, depletion of MET was achieved as early as 2 days after combining a MET-, choline-, and HCYS-deficient diet with rMETase and HCYSS, providing that animals had been adapted to choline deprivation for at least 7 days. With the exception of the MET(−)HCYS(+)CHOL(−) diet, which resulted in a marked increase in the total HCYS in plasma, all other diets and treatments had only a modest effect on HCYS levels. Supplementing HCYSS i.p. during administration of the MET(−)HCYS(−)CHOL(−) diet did not raise the level of plasma HCYS over that of animals fed the MET(+)HCYS(−)CHOL(+) diet.

Table 1 *Dietary and pharmacological modulation of MET and total HCYS in plasma of athymic mice bearing human medulloblastoma (Daoy) xenografts*

Diet/treatment	MET (μM)	HCYS (μM)
Basal[a]	60.3 ± 3.4	3.1 ± 0.8[b]
MET(+)HCYS(−)CHOL(+)	67.2 ± 3.2	5.7 ± 0.1
MET(+)HCYS(−)CHOL(−)	18.5 ± 1.1	7.3 ± 0.8
MET(−)HCYS(+)CHOL(−)	24.3 ± 4.3	89.6 ± 13.3
MET(−)HCYS(−)CHOL(−)/HCYSS[c]	9.0 ± 0.9	5.8 ± 0.8
MET(+)HCYS(−)CHOL(−)/rMETase[d]	15.2 ± 2.9	3.0 ± 0.8
MET(−)HCYS(+)CHOL(−)/rMETase	15.6 ± 1.3	13.3 ± 3.8
MET(−)HCYS(+)CHOL(−)/rMETase+HCYSS	4.3 ± 1.1	7.9 ± 2.1

[a] Diet fed for at least 7 days before determination.
[b] Mean of six determinations (animals) ± SD.
[c] HCYSS (20 mg/kg) once per day i.p., (measurement taken 8 h after dosing).
[d] rMETase, 1500 units/kg i.p. every 8 h (determination 4 h after fourth treatment).

Effect of MET Depletion on AGT in Tumor and Tissues. As has been demonstrated previously, MET deprivation results in a decrease of AGT activity in MET-dependent tumor cells grown in culture due to the inhibition of AGT gene transcription (26). Such inhibition has not been observed in MET-independent cells grown in HCYS-supplemented media without exogenous MET. Selective reduction of expression of AGT in tumors, but not in normal tissue, can be theoretically achieved in animals by depriving tumors of MET by lowering systemic levels of this amino acid while maintaining an adequate HCYS supply to allow MET synthesis in normal tissues. Thus, AGT activity in Daoy xenografts was reduced from 375 to 31 fmol/mg protein after 10 days of treatment with rMETase (every 8 h) and HCYSS (every 24 h) while restricting dietary MET, choline, and HCYS (Fig. 1; Table 2). Down-regulation of AGT was also observed in SWB77 tumor xenografts deprived of MET in the same manner. In contrast to tumor tissue, the AGT activity of normal tissue of the mouse bearing the tumor xenografts remained unchanged during the METdr (Table 2). The down-regulation of AGT in tumors is closely related to the ability of the treatment to lower plasma MET.

Daoy Xenografts. Depletion of plasma MET with the combination of dietary and pharmacological means results in the complete retardation of tumor growth of Daoy medulloblastoma xenografts (Fig. 2). Five of six Daoy xenografts regressed 4–5 days after the initiation of treatment (Fig. 2; Table 3). The tumors continued to regress as long as the animals were treated with rMETase and HCYSS. However, tumor growth resumed when treatment was discontinued and animals were given a MET-sufficient diet. Rates of tumor regrowth were similar to those seen for the growth of tumors in untreated animals, but lag periods depended on the extent of regression these tumors experienced during the METdr. In a subsequent experiment, treatment of animals with a low dose of BCNU 8 h after discontinuation of the treatment with a METdr and reestablishment of dietary MET resulted

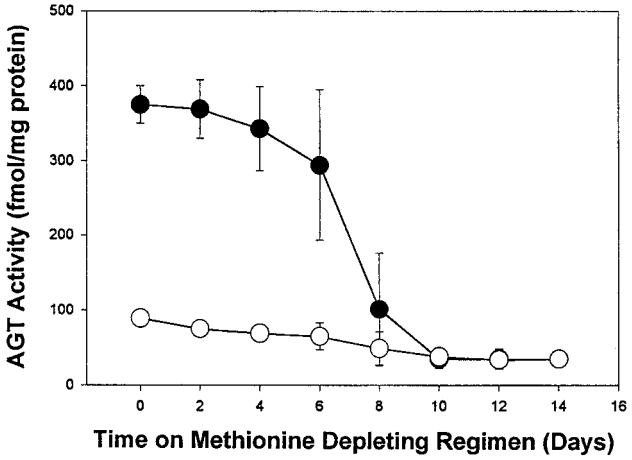

Fig. 1. Time course of down-regulation of AGT activity in viable tumor segments during treatment of athymic mice bearing Daoy (●) or SWB77 (○) tumor xenografts with a METdr. Animals were placed on a MET(+)HCYS(−)CHOL(−) diet after implantation of the tumor, and when tumors reached 200 mm^3, the diet was switched to MET(−)HCYS(−)CHOL(−), and animals were treated with 1500 units/kg rMETase i.p. every 8 h and 20 g/kg HCYSS i.p. every 24 h for the time intervals indicated. Data are the mean from five animals ± SD.

Table 2 *Effect of 10-day-long MET depletion on AGT activity in human medulloblastoma (Daoy) xenografts and athymic mouse normal tissues*

Diet/treatment	AGT activity in[a]		
	Brain	Liver	Tumor
MET(+)HCYS(−)CHOL(+)[b]	18 ± 3	108 ± 14	368 ± 32
MET(+)HCYS(−)CHOL(−)[b]	18 ± 4	132 ± 18	372 ± 48
MET(−)HCYS(+)CHOL(−)[b]	21 ± 6	185 ± 22	333 ± 35
MET(+)HCYS(−)CHOL(−) + rMETase[c]	20 ± 6	108 ± 12	327 ± 55
MET(−)HCYS(+)CHOL(−) + rMETase[c]	29 ± 9	111 ± 18	147 ± 98
MET(−)HCYS(−)CHOL(−) + rMETase[c] + HCYSS[d]	23 ± 9	95 ± 12	31 ± 9

[a] fmol/mg protein after 10 days of treatment, mean of three determinations.
[b] Animals were fed the respective diet for at least 10 days before being killed.
[c] rMETase, 1500 units/kg, thrice a day, every 8 h for 10 days.
[d] HCYSS, 20 mg/kg, once a day, for 10 days.

in maintained complete response of five of six tumor xenografts. BCNU alone at the dose used in combination with METdr (35 mg/m^2) had no effect on the rate of growth of the Daoy xenograft in athymic mice, regardless of diet (Table 3). The maximum tolerated dose of BCNU of 65 mg/m^2, *i.e.*, maximum dose without deaths, induced a growth delay of only 6 days. In contrast, a METdr administered for 12 days resulted in a marked increase in the time required for the xenograft to quintuple as compared with controls or BCNU-treated animals (T − C = 20 days; $P < 0.005$). Furthermore, the METdr sensitized the xenograft to BCNU, apparently by eliminating AGT-related resistance. Despite the relatively large tumor volume at the time of treatment with BCNU, tumors that were deprived of MET for 12 days responded to the low dose of BCNU (35 mg/m^2) by further regression and by delayed regrowth for at least a period of 90 days after the initiation of MET-depleting treatment. The overall toxicity on the animals treated with the MET depletion and with a combination of MET depletion and BCNU was reflected by a modest loss of weight.

D-54 Xenografts. The effect of depletion of plasma MET on the resistance of the AGT-negative glioblastoma D-54 xenografts to BCNU is shown in Table 4. D-54 xenografts are moderately resistant to BCNU, which has an effect only at its maximum tolerated dose of 65 mg/m^2 by inducing a median growth delay of 19 days (range, 16–25 days). The dose of 35 mg/m^2, on the other hand, had no significant effect in inducing tumor regressions or tumor growth delays, despite the lack of AGT in this tumor. A METdr to animals bearing D-54 xenografts resulted in a 26-day tumor growth delay and was marginally more effective than treatment with 65 mg/m^2 BCNU ($P = 0.045$). However, when the MET depletion was combined with a single treatment of 35 mg/m^2 BCNU, tumors that were regressing in response to MET deprivation showed a sustained complete response for at least 90 days. Unlike Daoy, in which sensitization was due to the depletion of AGT activity by MET deprivation, the D-54 xenografts were sensitized to BCNU by a mechanism that appears to be AGT independent.

SWB77 Xenografts. The SWB77 glioblastoma xenograft is resistant to BCNU. BCNU at a dose of 65 mg/m^2 yielded a T − C of only 2 days, which is not significant (32). The combination of a METdr + BCNU (35 mg/m^2) induced a 20-day tumor growth delay as compared with untreated animals and an 18-day delay as compared with those treated with 35 mg/m^2 BCNU (Table 5). This may be related to a 2-fold reduction of AGT activity caused by the METdr (Fig. 1). MET depletion alone caused a delay of growth of 11 days, a period that was markedly shorter than that observed for Daoy or D-54 ($P < 0.001$).

Unlike BCNU, TMZ had some activity against SWB77 (32). TMZ at doses of 180 mg/m^2 caused tumor regressions in 8 of 10 animals and induced a tumor delay of 12 days. As shown in Table 5, the combination of MET deprivation followed by TMZ resulted in a tumor delay of 39 days *versus* a delay of 11 days for the METdr alone. A significant reduction of T − C was observed in animals fed a choline-sufficient diet after the depleting/TMZ combination treatment instead of the usually used choline-deficient diet. However, even in this case, TMZ was markedly more effective when combined with a METdr. These results indicate synergy between TMZ and MET deprivation especially because there is no substantial increase in toxicity for the combination of the two as compared with the use of either one of these treatments (Table 5).

Toxicity of METdrs. Of the organs examined, only the liver was histopathologically affected in mice subjected to METdrs. Histological evaluation of the livers from all animals undergoing MET-

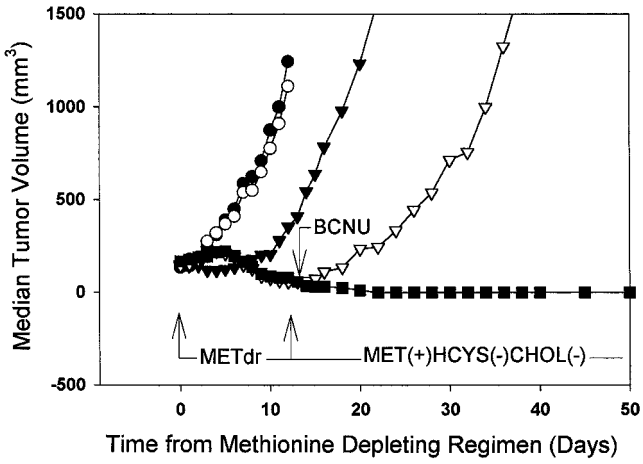

Fig. 2. Effect of a 12-day METdr and subsequent BCNU treatment on the growth of human (Daoy) medulloblastoma xenografts implanted s.c. in athymic mice. The METdr [animals were fed a MET(−)HCYS(−)CHOL(−) diet and treated with 1500 units/kg RMETase i.p. every 8 h and 20 mg/kg HCYSS i.p. every 24 h] was initiated when tumors reached a volume of 120–160 mm^3. Animals were given a MET(+)HCYS(−)CHOL(−) diet before METdr and returned to the same diet after 12 days of treatment. ○, untreated controls fed a MET(+)HCYS(−)CHOL(−) diet; ●, animals given BCNU (35 mg/m^2) with a MET(+)HCYS(−)CHOL(−) diet throughout; ▼, animals given BCNU (65 mg/m^2) with a MET(+)HCYS(−)CHOL(−) diet throughout; ▽, animals on a 12-day METdr followed by a MET(+)HCYS(−)CHOL(−) diet; ■, animals on a METdr followed by a MET(+)HCYS(−)CHOL(−) diet + a single treatment with BCNU (35 mg/m^2) administered 8 h after termination of METdr.

Table 3 *Effect of MET depletion on the efficacy of BCNU against human medulloblastoma (Daoy) tumor xenografts in athymic mice*

Treatment	Median time (range) to 5× treatment volume (days)	Tumor regressions	Mortality	Mean weight loss (range) %	T − C[a] (days)	*P*
Untreated[b]	10 (8–12)	0/6	0	0		
BCNU (35 mg/m^2)[c]	9 (8–11)	0/6	0	0	−1	NS[d]
BCNU (65 mg/m^2)	16 (11–24)	3/5	1	10 (9–12)	6	<0.045
METdr (12 days)	30 (18–42)	5/6	0	9 (6–12)	20	<0.005
METdr (12 days) plus BCNU (25 mg/m^2)[e]	>90 (35–>90)	6/6	0	12 (9–12)	>80	<0.001

[a] Tumor delay (T − C) as compared with the untreated animals.
[b] Animals were given a synthetic MET(+)HCYS(−)CHOL(−) diet.
[c] Drugs were administered i.p. at a volume of 20 ml/m^2.
[d] *P*s as compared to untreated animals, NS, not significant.
[e] BCNU was administered 8 h after animals were transferred from a MET(−)HCYS(−)CHOL(−) diet to a MET(+)HCYS(−)CHOL(−) diet and from the last treatment with rMETase.

depleting treatments revealed preservation of the native hepatic architecture, with variable degrees of hepatocellular lipid accumulation (steatosis) in all animals. The degree of steatosis was influenced considerably by the dietary status of the animal. A 12-day administration of a MET(−)HCY(−)CHOL(−) diet with concomitant treatment with i.p. HCYSS and rMETase was associated with mild hepatocellular lipid accumulation most conspicuously in the midzonal areas of the liver (Fig. 3*a*). A METdr based on the MET(−)HCY(+)CHOL(−) diet induced more severe steatosis, with similar mid-zonal distribution (Fig. 3*b*). Histological sections of livers obtained from animals after reintroduction of dietary MET after termination of a 12-day METdr revealed the persistence of macrovesicular steatosis similar to that seen in the animals maintained previously on MET-deficient diets, with superimposed, more generalized microvesicular steatosis (Fig. 3, *c* and *d*).

DISCUSSION

MET stress has been shown to block the cell cycle at G_2 in tumor cells *in vitro* with resultant cell death (26, 33, 34). MET- and HCYS-depleted diets prevent metastasis in tumor-bearing animals (35). A G_2 cell cycle blockade in tumors and extension of the life span of the animals bearing human tumor xenografts has also been demonstrated (36–38). Impressive tumor regressions can be induced by reduction of the plasma MET level to a steady state of <5 μM in athymic mice bearing human tumor xenografts (27). These levels can be achieved with the use of rMETase during restriction of dietary MET, HCYS, and choline, combined with the rescue of normal tissue with i.p. HCYSS. An important related observation is that even when tumor regression was achieved with prolonged MET depletion, discontinuation of the MET-depleted state resulted in tumor regrowth. Because the application of METdrs is cumbersome, these observations suggest

Table 4 *Effect of MET depletion on the efficacy of BCNU against D-54 human glioblastoma xenografts in athymic mice*

Treatment	Median time (range) to 5× treatment volume (days)	Regressions	Mortality	Mean weight loss (range) %	T − C[a] (days)	*P*
Untreated[b]	9 (7–11)	0	0/6			
BCNU (35 mg/m^2)[c]	8 (7–12)	0	0/6	0	−1	NS[d]
BCNU (65 mg/m^2)	26 (21–33)	3/6	0/6	12 (9–13)	19	<0.001
METdr (12 days)	33 (19–41)	6/6	0/6	14 (11–17)	26	<0.001
METdr (12 days) + BCNU (35 mg/m^2)[e]	>90 (45–>90)	6/6	0/6	17 (11–21)	>80	<0.001

[a] Tumor delay (T − C) as compared to the untreated animals.
[b] Animals were given a synthetic MET(+)HCYS(−)CHOL(−) diet.
[c] Drugs were administered i.p. at a volume of 20 ml/m^2.
[d] *P*s as compared to untreated animals, NS; not significant.
[e] BCNU was administered 8 h after animals were transferred from a MET(−)HCYS(−)CHOL(−) diet to a MET(+)HCYS(−)CHOL(−) diet and from the last treatment with rMETase.

Table 5 *Effect of MET depletion on the efficacy of BCNU and TMZ against SWB77 human glioblastoma xenografts in athymic mice*

Treatment	Animal no.	Median time (range) to 5× treatment volume (days)	Regressions	Mean weight loss[a] (range) %	T − C (days)	*P*
MET(+)HCYS(−)CHOL(−)[b]						
Untreated	10	13 (9–21)[c]				
BCNU (35 mg/m^2)	10	15 (9–22)	0/10	0	2	NS[d]
BCNU (65 mg/m^2)	10	17 (11–25)	3/10	10 (9–12)	4	NS
TMZ (180 mg/m^2)	10	25 (18–35)	8/10	10 (5–16)	12	<0.001[e]
TMZ (300 mg/m^2)	10	32 (19–62)	9/10	11 (7–18)	19	<0.001[e]
MET(−)HCYS(−)CHOL(−). . .MET(+)HCYS(−)CHOL(−)[f]						
METdr	10	24 (18–51)	9/10	19 (16–22)	11	<0.005[e]
METdr + BCNU (35 mg/m^2)	10	33 (19–51)	10/10	21 (17–22)	20	<0.005[d,e]
METdr + TMZ (180 mg/m^2)	10	52 (42–74)	10/10	23 (17–24)	39	<0.005[d,e]
MET(−)HCYS(−)CHOL(−). . .MET(+)HCYS(−)CHOL(+)[f]						
METdr	10	22 (17–22)	9/10	13 (10–15)	9	<0.005[e]
METdr + BCNU (35 mg/m^2)	10	30 (18–40)	10/10	18 (12–21)	17	<0.005[d,e]
METdr + TMZ (180 mg/m^2)	10	41 (32–63)	10/10	21 (16–24)	28	<0.005[d,e]

[a] Weight loss as a percentage of animal weight during treatment. For combined treatments, this number represents total loss.
[b] Diet administered throughout study.
[c] Median (range).
[d] *P*s as compared to animals treated with METdr alone; NS, not significant.
[e] *P*s as compared to untreated animals.
[f] Sequence of diets representing the diet before METdr and after METdr.

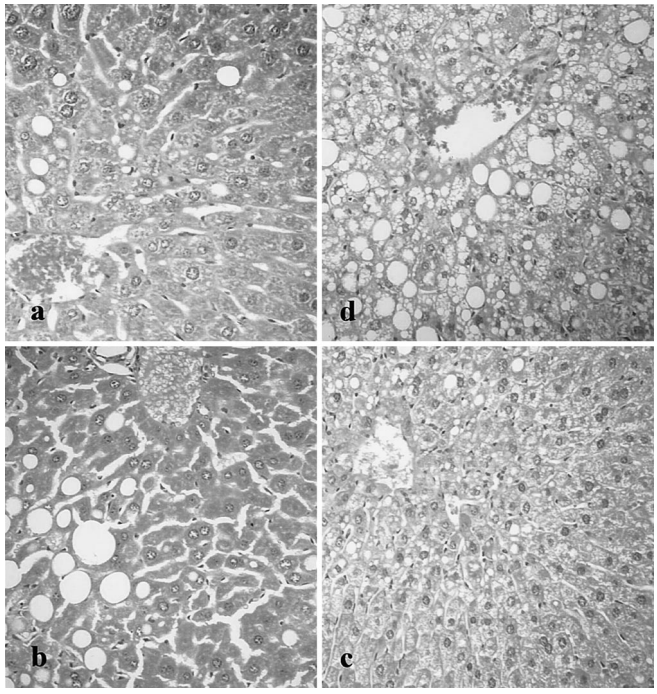

Fig. 3. Toxicity of METdrs on the liver of athymic mice. *a*, liver from an animal maintained on a MET(−)HCYS−)CHOL(−) diet and i.p. HCYSS (20 mg/kg) daily while on treatment with rMETase for 12 days, demonstrating very mild macrovesicular fat accumulation within hepatocytes (stained with H&E; original magnification, ×150). *b*, liver from an animal maintained on a MET(−)HCYS(+)CHOL(−) diet while on treatment with rMETase for 12 days, demonstrating the presence of large lipid droplets within hepatocytes, most conspicuously within the mid-zonal region (stained with H&E; original magnfication, ×150). *c*, liver from an animal initially maintained on a MET(−)HCYS(−)CHOL(−) diet + i.p. HCYSS (20 mg/kg) while on treatment with rMETase for 12 days followed by reintroduction of dietary MET for 10 days reveals a mixed macro- and microvesicular steatosis pattern (stained with H&E; original magnification, ×100). *d*, liver from an animal initially maintained on MET(−)HCYS(+)CHOL(−) diet while treated with rMETase for 12 days followed by reintroduction of dietary MET for 10 days. A widespread accumulation of small fat droplets (microvesicular steatosis) is present, in addition to the macrovesicular fat noted in MET-deficient animals (stained with H&E; original magnification, ×100).

that the real potential role of METdrs is as part of a combined modality therapy approach (39–44). This is further supported by observations made during limited treatment of cancer patients with r-METase (45, 46).

Despite the failure of MET deprivation alone to sustain prolonged inhibition in tumor growth, such an approach has considerable potential when used in combination with chemotherapy due to the remarkable synergy between MET depletion and DNA-damaging chemotherapeutic agents such as BCNU and TMZ. Both Daoy and D-54 xenografts did not regrow for at least 80 days after combined treatment with MET-deprivation and BCNU, despite the resistance of both tumors to BCNU (32). Synergy between MET deprivation and BCNU (35 mg/m^2) was also observed in the highly resistant glioblastoma SWB77 xenografts. A more pronounced synergy was observed with TMZ, which, in combination with MET depletion, delayed SWB77 growth by 39 days, which was significantly longer ($P < 0.005$) than the delays induced by TMZ (12 days) or MET deprivation alone (11 days).

The mechanism by which MET stress sensitizes tumors to alkylating agents is not understood. Resistance to BCNU in Daoy is primarily due to the presence of AGT (18, 20). The strong correlation between AGT depletion with BG administration or AGT down-regulation by MET depletion therapy on one hand, and the acquisition of BCNU sensitivity on the other, provides strong support that elimination of AGT levels in Daoy is a major pathway for the reversal of the resistance to BCNU when BCNU is combined with METdrs. The role of AGT down-regulation in sensitizing SWB77 is less clear. Growth of SWB77 xenografts is not delayed by BCNU unless AGT is first depleted with an AGT inhibitor, such as BG (32). However, even with BG pretreatment, the BCNU-induced growth delay for SWB77 is only 11 days (32) as compared with 20 days shown for BCNU combined with a METdr. A similarly greater synergy of METdrs + TMZ than BG + TMZ (32) indicates that the greater tumor growth delay observed with TMZ in combination with MET depletion (39 days) than with TMZ alone (12 days) is probably not the result of AGT down-regulation alone. The possible modulation of mechanisms of DNA repair, other than AGT, by MET deprivation is further corroborated by the unexpected response of D-54 to MET stress + BCNU treatment. MET deprivation enhances the efficacy of BCNU against the AGT-deficient D-54 by far more than the expected marginal effect observed previously by direct AGT inhibition (43, 44). Additional studies are needed to explore the full effect of MET stress on the resistance mechanisms of gliomas to alkylation damage.

The synergy between MET depletion and alkylating agents becomes even more interesting when we take into account its tumor specificity. Unlike DNA repair system inhibitor drugs, which sensitize normal tissue to genotoxic agents, MET depletion does not appear to affect the resistance of normal tissue to either BCNU or TMZ. MET depletion does cause certain changes in the liver of the mouse hosting the tumor, but these changes are diet related and reversible. Thus the reduction of the animal weight during MET deprivation regimens is due to nutritional imbalances rather than to the toxicity of the regimen. The down-regulation of the tumor xenograft AGT, but not of that of the liver or brain, during MET deprivation is in full agreement with a specific sensitization of the tumor xenograft to BCNU and TMZ in the athymic mouse model.

The experiments presented here show a strong synergy between marked depression of plasma MET and the genotoxic drug BCNU against Daoy and D-54 brain tumor xenografts. Near eradication of these two tumors suggests that MET deprivation not only eliminates AGT-related resistance, which was expected, but probably incapacitates other mechanisms and pathways that render BCNU ineffective against some low AGT-containing tumors. Despite the synergy between MET deprivation and TMZ that was also observed with glioblastoma tumor xenograft SWB77, this tumor still resisted this combined modality therapy approach. Such persistent resistance suggests that MET deprivation may not be best combined with these two agents against all MET-dependent tumors. Additional genotoxic compounds must be evaluated in combination with MET-depleting conditions to take advantage of the apparent modification of tumor cell resistance by the stress induced from a short supply of exogenous MET.

ACKNOWLEDGMENTS

We thank Qing Xian Zhou for excellent technical contributions and Dr. Dennis K. Burns for assistance in histology.

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