

Mitotic Arrest, Apoptosis, and Sensitization to Chemotherapy of Melanomas by Methionine Deprivation Stress

Demetrius M. Kokkinakis,^{1,4} Anthony G. Brickner,^{2,3,4} John M. Kirkwood,^{2,4} XiaoYan Liu,⁴ Jason E. Goldwasser,⁴ Anastasiya Kastrama,⁴ Cindy Sander,² Dora Bocangel,⁵ and Sunil Chada⁵

Departments of ¹Pathology, ²Medicine, and ³Immunology and ⁴Cancer Institute, University of Pittsburgh, Pittsburgh, Pennsylvania and ⁵Introgen Therapeutics, Inc., Houston, Texas

Abstract

Methionine deprivation stress (MDS) eliminates mitotic activity in melanoma cells regardless of stage, grade, or TP53 status, whereas it has a negligible effect on normal skin fibroblasts. In most cases, apoptosis accounts for the elimination of up to 90% of tumor cells from the culture within 72 hours after MDS, leaving a scattered population of multinucleated resistant cells. Loss of mitosis in tumor cells is associated with marked reduction of cyclin-dependent kinase (CDK) 1 transcription and/or loss of its active form (CDK1-P-Thr¹⁶¹), which is coincident with up-regulation of CDKN1A, CDKN1B, and CDKN1C (p21, p27, and p57). Expression of the proapoptotic *LITAF*, *IFNGR*, *EREG*, *TNFSF/TNFRSF10* and *TNFRSF12*, *FAS*, and *RNASEL* is primarily up-regulated/induced in cells destined to undergo apoptosis. Loss of Aurora kinase B and BIRC5, which are required for histone H3 phosphorylation, is associated with the accumulation of surviving multinucleated cells. Nevertheless, noncycling survivors of MDS are sensitized to temozolomide, carmustin, and cisplatin to a much greater extent than normal skin fibroblasts possibly because of the suppression of *MGMT/TOP1/POLB*, *MGMT/RAD52/RAD54*, and *cMET/RADD52*, respectively. Sensitivity to these and additional genotoxic agents and radiation may also be acquired due to loss of *cMET/OGG1*, reduced glutathione reductase levels, and a G₂-phase block that is a crucial step in the damage response associated with enhancement of drug toxicity. Although the genes controlling mitotic arrest and/or apoptosis in response to low extracellular methionine levels are unknown,

it is likely that such control is exerted via the induction/up-regulation of tumor suppressors/growth inhibitor genes, such as *TGFB*, *PTEN*, *GAS1*, *EGR3*, *BTG3*, *MDA7*, and the proteoglycans (*LUM*, *BGN*, and *DCN*), as well as the down-regulation/loss of function of prosurvival genes, such as *NFκB*, *MYC*, and *ERBB2*. Although MDS targets several common genes in tumors, mutational variability among melanomas may decide which metabolic and signal transduction pathways will be activated or shutdown. (Mol Cancer Res 2006;4(8):575–89)

Introduction

Melanoma is a prevalent disease among fair-skinned Caucasians and has been linked to sunlight exposure (1). High constitutive expression of extracellular signal-regulated kinase 1 and 2 and the amplification of cyclin D1 (*CCND1*) in most melanomas has been linked with the activation of the RAS-RAF mitogen-activated protein kinase signaling pathway (2-4). Mutations in RAS and BRAF, which occur in 15% and 66% of melanomas, respectively, could account for enhanced proliferative activity (5-8). In addition, loss of PTEN's regulatory control of AKT and epigenetic silencing of APAF1 confer a high rate of proliferation (9-11). In metastatic melanoma, resistance to treatment could be linked to defective apoptosis due to high expression of the antiapoptotic BCL-2 (12-14). The majority of melanomas (>80%) are curatively treated when surgery is applied early before metastatic disease. However, advanced metastatic melanoma is incurable (15, 16). The methylating agents dacarbazine given i.v. and its oral analogue temozolomide are the most frequently used drugs with a response of only 15% (17-21). Nitrosoureas (22, 23), platinum analogues (24, 25), *Vinca* alkaloids (26), and taxanes (27) have shown limited promise. Attempts to combine chemotherapy with surgery have been fruitless in significantly extending survival (28). The poor response to chemotherapy and the steady increase of the incidence of melanoma worldwide show the need for the development of new therapeutic approaches. One of the best understood reasons for the failure of methylating agents and nitrosoureas to kill tumor cells is the presence of high levels of O⁶-methylguanine-DNA methyltransferase (MGMT), a ubiquitously expressed DNA repair protein that is vital for preventing mutations (29-31). MGMT-induced resistance can be overcome by MGMT inhibition with

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Requests for reprints: Demetrius M. Kokkinakis, Hillman Cancer Center Research Pavillion, Room G12.e, 5117 Centre Avenue, Pittsburgh, PA 15213. Phone: 412-585-0833; Fax: 724-625-3036. E-mail: kokkinakisd@thiogenics.com
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a pseudosubstrate, such as *O*⁶-benzylguanine (32). In the clinic, MGMT is inversely correlated with improved response to carmustine [1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)] and overall survival in patients with gliomas, but the relationship is less clear in patients with melanoma (33, 34). An association between the expression of MGMT and the response to dacarbazine has been suggested by one study (33), but this has not been confirmed in patients treated with temozolomide (22). The extent of MGMT expression in melanoma remains uncertain and the methodologies used to determine such expression have yielded contradictory results. In developing treatments against metastatic melanomas, one must consider the resistance of these tumors beyond the involvement of MGMT.

In this communication, we examine the effect of methionine deprivation stress (MDS) on the abrogation of pro-survival pathways and in the induction of cell cycle arrest and apoptosis in several melanoma cell lines. MDS has been shown to inhibit AKT, RAS, and nuclear factor- κ B (NF- κ B) activities in medulloblastomas, glioblastoma multiforme, and pancreatic adenocarcinomas (35, 36) and to greatly enhance the response of these tumors to chemotherapy (37). Resistance of tumors to alkylating chemotherapeutic agents may be eliminated by MDS due to the down-regulation of MGMT (38) and the induction of tumor suppressor genes, such as *MDA7*, *IFNG*, and/or *transforming growth factor- β* (*TGF β*), which control the expression of p21 independently of p53 status and may exert tumor growth inhibition in melanomas by the up-regulation and nuclear translocation of this cyclin-dependent kinase (CDK) inhibitor (35, 36). In addition to p21, MDS can also enhance the response of tumors to alkylating agents by a synergistic induction of growth arrest in response to DNA damage (GADD) genes, which act downstream of p53 and interact with p21 and CDK1, thus regulating the cell cycle and cell proliferation in a p53-dependent or p53-independent manner (35, 36). Furthermore, MDS has the potential to enhance the

efficacy of chemotherapy by reducing the expression of genes involved in the resistance to antitumor agents and radiation. Most importantly, MDS has a pleiotropic action and kills or sensitizes tumor cells by using a variety of mechanisms, thus overriding variable genetic and epigenetic restrictions that are related to tumor heterogeneity.

Results

Physiologic Changes of Melanoma Cells in Response to MDS

MDS retarded the growth of eight melanoma cell lines in culture. Five of eight cell lines tested (M591, M255, M108, M103, and MEWO) showed accumulation of large cells with multiple micronuclei and decondensed chromatin 96 hours after MDS (Figs. 1 and 2). These cells survived a burst of cellular death that occurred between 48 and 72 hours after replacing methionine with homocysteine in the medium (Table 1). Multinucleated cells persisted for several days in culture; however, they resumed mitotic activity and proliferated when methionine was replenished (data not shown). In three additional cell lines (M105, WM35, and A375), there was no evidence for progression of the cycle and mitotic activity for at least 10 days following methionine deprivation; however, there was no accumulation of multinucleated cells. In these tumors, cellular death was protracted and continued for several days. In contrast to tumor cells, the growth of normal human CCD-187sk and horse fibroblasts E.DERM under MDS was moderately affected with no significant evidence of cell death. To determine the cell cycle distribution during a MDS regimen and derive information regarding possible cell cycle check points, melanoma and normal skin fibroblast cells cultured in either methionine or homocysteine medium for various time intervals were stained with propidium iodide and analyzed by flow cytometry. Cells with a multiple to diploid DNA content were exclusively present at 96 hours after MDS in cultures of

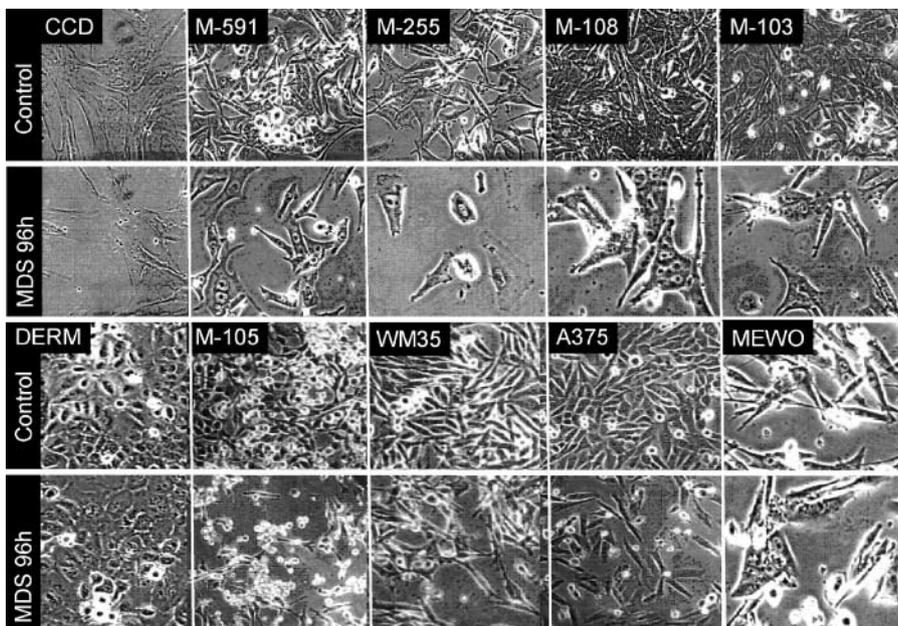


FIGURE 1. Effect of MDS on melanoma cell lines M591 (mutant TP53, infiltrated lymph node), M255 (WT TP53, infiltrated lymph node), M105 (mutant TP53), M103 (mutant TP53, infiltrated lymph node), M108 (WT TP53), A375 (WT TP53), skin melanomas WM35 (WT TP53) and MEWO (mutant TP53), human normal skin fibroblast CCD-187sk, and horse normal dermis fibroblast E.DERM. Cells were cultured in methionine-efficient medium containing 10% dialyzed serum until they were confluent. At that time, methionine was withdrawn and replaced with homocysteine thiolactone. At day 4, there was accumulation of multinucleated cells in M591, M255, M108, M103, and MEWO. Excessive cell death in the above lines was evident at day 2 but continued to occur in M105, WM35, and A375 for several days. Contrast, $\times 200$.

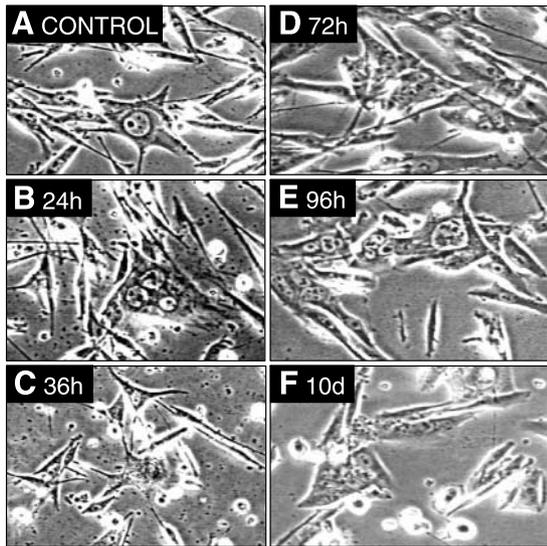


FIGURE 2. Cell death and accumulation of multinucleated cells in M103 following withdrawal of methionine. In methionine (0 hour), cells were elongated and proliferated as determined by the abundance of mitotic figures (A). At 24 hours after methionine deprivation, cells that were normally elongated (B) began to die yielding debris, which is evident at 36 hours (C). At 72 hours, most of the cells were dead or dying (D) while multinucleated cells survived the stress. At 96 hours, the only cells remaining in the culture were those containing multiple nuclei (E); these cells persisted for at least 10 days (F).

M103 and MEWO (Fig. 3A). Cell death in MEWO was due to nuclear fragmentation (apoptosis), which reached a zenith at 48 hours from MDS as shown by flow cytometry (Fig. 3B). The cell cycle distribution was not affected in E.DERM and CCD-187sk cultures by MDS, whereas a G₁-S arrest observed in WM35 cultures may explain the absence of accumulation of multinucleated cells in this line (Fig. 3C). Overall, tumor cells could be classified into two categories: those that undergo massive apoptosis between 48 and 72 hours in response to MDS and finally survive as multinucleated nondividing structures (M591, M255, M108, M103, and MEWO) and those that undergo a more protracted apoptotic response and do not yield multinucleated structures (M105, WM35, and A375).

Genetic Changes of Melanoma Cells to MDS

MDS had a marked effect on gene expression as shown by cDNA oligoarray analysis in MEWO and M103. In these tumor lines, the expression of >1,000 genes was significantly ($P < 0.05$) induced or up-regulated, whereas expression of 1,200 genes was suppressed or down-regulated after a 96-hour MDS. Approximately the same number of genes was affected within the first 48 hours after treatment. Changes in gene expression at 48 and 96 hours followed the same direction (up or down) in 57% and opposite direction in only 6% of all cases. The rest (37%) of the changes occurred only at one time point. The above indicates that the majority of the changes occurred in all cells whether they were destined to undergo apoptosis (48 hours) or survive (96 hours). Changes at 48 hours but not at 96 hours may be related to proapoptotic events. Changes at 96 hours but not at 48 hours are likely to be

associated with the survival of persisting cells in culture. Genes were classified according to the above criteria and were also subclassified into two categories depending on their relation to their association with metabolism or signal transduction.

Genes Regulating Metabolism

Selected genes regulating enzyme levels that control known metabolic reactions (including phosphorylations) with major (>4 fold) changes in their expression under MDS are as follows:

- metabolism-related genes that were induced/up-regulated only in surviving-multinucleated cells:
 - tryptophan 2,3-dioxygenase (TDO2)*,
 - adenylate cyclase 3 (ADCY3)*,
 - glucose-6-phosphate dehydrogenase (G6PD)*,
 - glutathione S-transferase M1 (GSTM1)*,
 - glutathione S-transferase M2 (GSTM2)*,
 - glutathione S-transferase A4 (GSTA4)*, and
 - heparan sulfate 3-O-sulfotransferase 3A1 (HS3ST3A1)*;
- metabolism-related genes that were silenced/down-regulated only in surviving-multinucleated cells (96 hours):
 - glutamine-fructose-6-phosphate transaminase 1 (GFPT1)*,
 - acyl-CoA hydrolase (BACH)*,
 - protein kinase C (PRKCH)*,
 - casein/kinase 2 α 1 (CSNK2A1)*,
 - soluble thymidine kinase 1 (TK1)*,
 - fucosyltransferase 7 (FUT7)*, and
 - phosphoinositide-3-kinase (PIK3CB)*;
- metabolism-related genes that were induced/up-regulated in cells undergoing apoptosis:
 - kynureninase (KYNU)*,
 - arachidonate 5-lipoxygenase (ALOX5)*,
 - carbonic anhydrase II (CA2; 100 \times)*,
 - phosphatidic acid phosphatase type 2C (PPAP2C)*,

Table 1. Cell Populations in Cultures of Normal Fibroblasts and Melanomas under Control and MDS Conditions as a Function of Time

Cell line	Days under MDS						
	1	2	3	4	5	6	10
CCD-187sk	103*	105	94	92	93	95	91
E.DERM	99	94	92	94	91	90	95
M591	95	87	32	23	21	21	18
M255	91	82	22	11	9	9	7
M108	85	41	19	9	11	11	11
M103	91	45	15	12	8	8	9
M105	102	91	84	83	47	43	13
WM35	99	93	75	45	31	28	11
A375	111	92	66	28	23	19	8
MEWO	93	78	28	13	12	13	11

NOTE: Cells were plated in 27 μ mol/L methionine-efficient medium in 10% dialyzed serum. Cell numbers were monitored daily, and when cultures reached confluence, methionine was replaced with 27 μ mol/L homocysteine thiolactone. Medium was replaced every 36 hours. Controls were supplied with methionine and 10% dialyzed serum every 36 hours throughout the experiment. Cells were counted at five different fields every 24 hours and the median number was expressed as percent of cells in control cultures. Time period of massive apoptosis is shown in bold for each culture.

*Median of 15 determinations (3 plates, 5 fields each) showing change in cell numbers as percent of control (methionine-efficient medium).

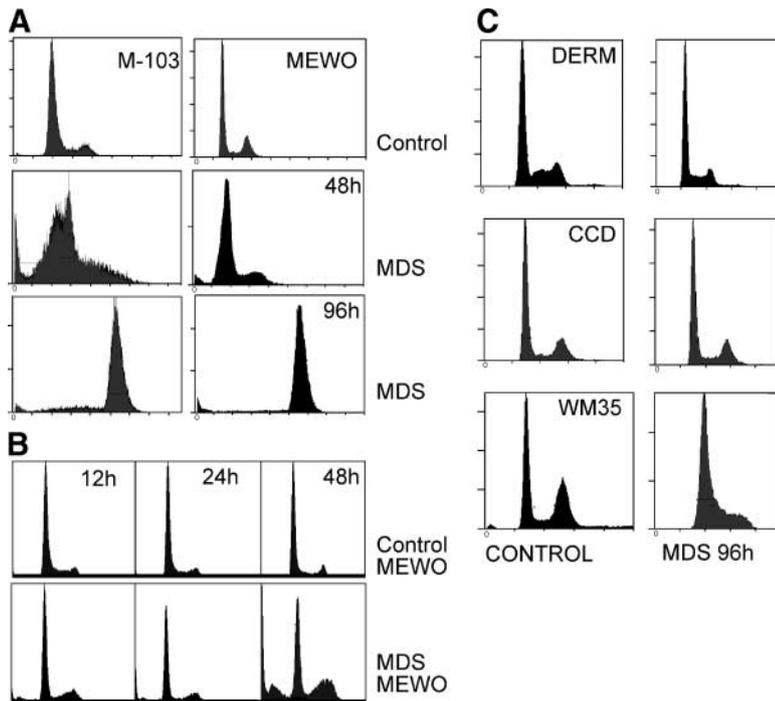


FIGURE 3. Evidence of cell cycle arrest, apoptosis, and accumulation of multinucleated cells. At 48 hours after MDS, live (attached) M103 and MEWO cells appear to move into S phase. At 96 hours, all surviving cells are present as multinucleated structures (A). Apoptosis as determined by nuclear fragmentation is insignificant in melanoma cells growing controls. An increasing (with time) apoptotic incidence is observed in MEWO cultures under MDS. At 48 hours, a G₂-M arrest is evident (B). In contrast to M103 and MEWO, E.DERM and CCD-187sk show nearly unchanged cell cycle characteristics under MDS up to 96 hours, whereas WM35 appears to arrest in G₁-S at 96 hours (C).

transglutaminase 1 (TGM1),
UDP glycosyltransferase 1 family (UGT1A),
cytochrome P450 family 26 subfamily A polypeptide 1 (CYP26A1),
fucosyltransferase 3 (FUT3),
ribonucleotide reductase M2 polypeptide (RRM2),
xanthine dehydrogenase (XDH),
cytochrome P450 family 2 subfamily C polypeptide 9 (CYP2C9),
transglutaminase 2 (TGM2),
inositol polyphosphate-4-phosphatase type II 105 kDa (INPP4B),
3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMGCS1),
 and *sterol-C5-desaturase (SC5DL);*

- (d) metabolism-related genes that were silenced/down-regulated in cells undergoing apoptosis:

CDP-diacylglycerol synthase 2 (CDS2),
polymerase I polypeptide B (POLR1B),
IMP dehydrogenase 1 (IMPDH1),
N-methylpurine-DNA glycosylase (MPG),
glutamyl-prolyl-tRNA synthetase (EPRS), and
protein tyrosine phosphatase receptor type M (PTPRM);

- (e) metabolism-related genes that were induced/up-regulated in all cells:

farnesyl-diphosphate farnesyltransferase 1 (FDFT1),
lysosomal sphingomyelin phosphodiesterase 1 acid (SMPD1),
guanine deaminase (GDA),
plasma glutathione peroxidase 3 (GPX3),
acyl-CoA synthetase 1 (ACSL1),
ornithine aminotransferase (OAT),
glutathione S-transferase π (GSTP1),

adenylate cyclase 7 (ADCY7),
mitochondrial superoxide dismutase 2 (SOD2),
nicotinamide N-methyltransferase (NNMT), and
alcohol dehydrogenase 5 (ADH5); and

- (f) metabolism-related genes that were silenced/down-regulated in dying and in surviving-multinucleated cells (42 and 96 hours):

malate dehydrogenase 1 (MDH1),
isovaleryl CoA dehydrogenase (IVD),
asparagine synthetase (ASNS),
catalase (CAT),
hydroxyacylglutathione hydrolase-like (HAGHL),
adenylate kinase 2 (AK2),
mitogen-activated protein kinase kinase 2 (MAP2K2),
glutaminase (GLS),
aspartate β-hydroxylase (ASPH),
hydroxysteroid dehydrogenase 2 (HSD17B2),
acyl-CoA oxidase 1 (ACOX1),
protein tyrosine phosphatase nonreceptor type 14 (PTPN14),
topoisomerase I (TOP1),
peroxiredoxin 6 (PRDX6),
cytidine deaminase (CDA),
methylthioadenosine phosphorylase (MTAP),
pyruvate dehydrogenase kinase 1 (PDK1),
protein tyrosine phosphatase receptor type A (PTPRA),
adenosine kinase (ADK),
dimethylarginine dimethylaminohydrolase 1 (DDAH1),
ornithine decarboxylase 1 (ODC1),
protein kinase Cδ (PRKCD),
adenylate kinase 3 (AK3),
phosphoinositide-3-kinase class 2α polypeptide (PIK3C2A),

*biliverdin reductase A (BLVRA),
farnesyltransferase CAAX box β (FNTB), and
phosphorylase glycogen brain (PYGB).*

Genes Regulating Signal Transduction Pathways

In addition to the genes controlling metabolic pathways, genes that control signal transduction pathways and were similarly induced/up-regulated or turned off/down-regulated in response to MDS by a factor greater than 4 at both 48 and 96 hours are as follows:

(a) induced genes:

*decorin (DCN),
biglycan (BGN),
insulin-like growth factor-binding protein 2
(IGFBP2),
lumican (LUM),
tensin (TNS),
tissue inhibitor of metalloproteinase 3 (TIMP3),
TGF- β induced (TGFB1),
bradykinin receptor B2 (BDKRB2),
catenin α 1 (CTNNA1),
CDC42 effector protein (CDC42EP5),
cyclin D2 (CCND2),
early growth response 1 (EGR1),
frizzled homologue 7 (FZD7),
growth arrest-specific 1 (GAS1),
insulin-like growth factor-binding protein 7 (IGFBP7),
interleukin-1 receptor type 1 (IL1R1),
lipopolysaccharide-induced TNF factor (LITAF),
signal transducer and activator of transcription 3
(STAT3),
stanniocalcin 1 (STC1),
thrombospondin 2 (THBS2), and
tumor necrosis factor- α -induced protein 6 (TNFAIP6);*

(b) up-regulated ($>10\times$):

*early growth response 3 (EGR3),
neuroblastoma,
suppression of tumorigenicity 1 (NBL1),
phosphatase and tensin homologue (PTEN),
prolactin-induced protein (PIP),
RAB31,
member RAS oncogene family (RAB31),
calmodulin 3 (phosphorylase kinase δ) (CALM3),
hypoxia-inducible factor 3,
 α subunit (HIF3A),
RAS association (RalGDS/AF-6)
domain family 2 (RASSF2),
Bloom syndrome (BLM),
BTG family,
member 3 (BTG3),
epiregulin (EREG),
thrombospondin 1 (THBS1),
CDK inhibitor 1A (p21, Cip1) (CDKN1A),
inositol-1,4,5-trisphosphate 3-kinase C (ITPKC),
metallothionein 1E (functional) (MT1E),
insulin-like growth factor-binding protein 5 (IGFBP5),
polymerase I and transcript release factor (PTRF),
Fas (TNF receptor superfamily, member 6) (FAS), and*

RAB2,

member RAS oncogene family (RAB2);

(c) up-regulated (5- to 10-fold):

*amyloid β (A4) precursor protein (protease nexin II,
Alzheimer's disease) (APP),
gap junction protein, α 1,
43 kDa (connexin 43) (GJA1),
tumor necrosis factor- α -induced protein 3 (TNFAIP3),
interleukin-4 receptor (IL4R),
adrenomedullin (ADM),
RAB5C, member RAS oncogene family (RAB5C),
retinoic acid receptor β (RAR β),
SHC (Src homology 2 domain containing) trans-
forming protein 1 (SHC1),
TGF- β receptor III (betaglycan, 300 kDa) (TGFB3),
tumor necrosis factor receptor superfamily,
member 10D (TNFRSF10D),
tumor necrosis factor- α -induced protein 9
(TNFAIP9), DNA replication factor (CDT1),
interleukin-11 receptor α (IL11RA),
interleukin-6 signal transducer (IL6ST),
IFN- γ -inducible protein 16 (IFI16),
BTG family,
member 2 (BTG2),
ELK3,
ETS-domain protein (SRF accessory protein 2) (ELK3),
excision repair cross-complementing rodent repair
deficiency (ERCC1),
insulin-like growth factor-binding protein 3 (IGFBP3),
RAS homologue gene family,
member B (RHOB),
TGF- β 2 (TGFB2),
platelet-derived growth factor receptor-like
(PDGFR),
eukaryotic translation initiation factor 3,
subunit 10 θ , 150/170 kDa (EIF3S10),
eukaryotic translation initiation factor 1A,
Y-linked (EIF1AY),
START domain containing 4,
sterol regulated (STARD4),
suppression of tumorigenicity 13
(colon carcinoma, Hsp70 interacting protein)
(ST13),
and thioredoxin (TXN);*

(d) turned off genes:

*integrin α 1 (PELO),
frizzled homologue 8 (FZD8),
cadherin 1,
type 1,
E-cadherin (epithelial) (CDH1),
desmoplakin (DSP),
androgen-induced proliferation inhibitor (APRIN),
nitrilase 1 (NIT1),
IFN-stimulated gene 20 kDa (ISG20),
caspase 2,
apoptosis-related cysteine protease (CASP2),
N-myc downstream regulated gene 1 (NDRG1),
CDK inhibitor 2C (p18, inhibits CDK4) (CDKN2C),
interleukin-1 receptor-like 1 (IL1RL1),*

Table 2. Fold Change in Gene Expression in MEWO Melanoma Cells at 48 Hours (before Extensive Apoptosis) and 96 Hours (after Completion of the Apoptotic Response) following the Transition from a Methionine-Efficient State to the MDS State as Determined by Reverse Transcription-PCR Analysis

	48 h	96 h
Effector genes		
Loss of proliferative activity		
<i>TP53</i>	4*	12
<i>DCN</i>	11	110
<i>BGN</i>	2	11
<i>LUM</i>	92	3,200
<i>TGFBR</i>	-7	12
<i>PTEN</i>	22	58
<i>TNS</i>	2	18
<i>GAS1</i>	13	110
<i>EGR3</i>	14	58
<i>MDA7</i>	270	490
<i>NBL1</i>	9	24
<i>RASSF2</i>	6	23
<i>NBL1</i>	9	22
Apoptosis related		
<i>QUIESCIN</i>	7	3
<i>LITAF</i>	212	87
<i>BTG3</i>	38	16
<i>IFNGR</i>	13	2
<i>EREG</i>	110	22
Mediator genes		
Cell cycle/mitosis inhibitors		
<i>YWHAH</i>	2*	2
<i>YWHAQ</i>	2	2
<i>CDKN1A (p21)</i>	23	29
<i>CDKN1B (p27Kip1)</i>	1	3
<i>CDKN1C (p57Kip2)</i>	2	53
<i>CDKN2B (p15)</i>	-3	-110
<i>CDKN2C (p18)</i>	-7	-16
<i>CDKN2D (p19)</i>	1	-6
<i>CDK1</i>	1	-28
<i>Cyclin B</i>	2	-3
<i>Aurora kinase B</i>	-2	-13
Apoptosis related		
<i>TNFSF10</i>	22	-2
<i>TNFRSF10</i>	6	2
<i>TNFRSF12</i>	3	-14
<i>FAS</i>	20	5
<i>BCAT1</i>	120	410
<i>BRCA1</i>	7	2
<i>RNASEL</i>	98	32
Resistance-related genes		
<i>MGMT</i>	-5	-18
<i>TOP1</i>	-3	-14
<i>TOP1MT</i>	-22	-34
<i>RAD52</i>	-5	-13
<i>RAD54</i>	1	-6
<i>HGF</i>	1	1
<i>cMET</i>	-20	-121
<i>OGG1</i>	1	-4

NOTE: Effector genes were selected based on their high intensity of expression under MDS as determined by cDNA oligoarray analysis. Mediator genes were selected based on their high intensity of expression and their likelihood to be involved in the physiologic changes observed in response to MDS. Genes involved in the resistance to chemotherapy/radiation were selected according to similar criteria.

*Amplification was carried for a maximum of 30 cycles. Three serial dilutions of control and MDS samples having equal levels of β -actin were amplified and runs were analyzed with light cycle software under conditions of a low error rate (<0.2). Slopes determined from plots of cDNA concentration versus cycles required to achieve 30% of the maximum SYBR green fluorescence (Santa Cruz Biotechnology) were used to relative to β -actin expression of each gene in MDS and control samples. Fold increase (positive) or fold decrease (negative) in comparison with methionine controls were determined from ratios of MDS to control relative RNA concentrations under equal β -actin levels.

amphiregulin (schwannoma-derived growth factor) (AREG),
CDK2-associated protein 2 (CDK2AP2),
heat shock 70-kDa protein 6 (HSPA6),
polymerase (RNA) III (DNA-directed) polypeptide A (POLR3A),
frizzled homologue 8 (FZD8),
inhibin β B (INHBB),
protein kinase (cyclic AMP-dependent, catalytic) inhibitor β (PKIB),
neuropilin 1 (NRP1),
CDK inhibitor 3 (CDKN3), and
eukaryotic translation elongation factor 1 α 2 (EEF1A2); and

(e) down-regulated genes (5- to 10-fold):
IFN-induced protein with tetratricopeptide repeats 1 (IFIT1),
ETS homologous factor (EHF),
TGF- α (TGFA),
transcription factor CP2-like 2 (TFCP2L2),
phosphatidylinositol glycan class C (PIGC),
protein inhibitor of activated STAT 2 (PIAS2),
eukaryotic translation initiation factor 3, subunit 4 δ , 44 kDa (EIF3S4),
RAS and EF hand domain containing (RASEF),
growth factor receptor-bound protein 10 (GRB10),
trafficking protein particle complex 5 (TRAPPC5),
cullin 2 (CUL2),
tenascin XB (TNXB),
CD24 antigen (small cell lung carcinoma cluster 4 antigen) (CD24),
RAN-binding protein 3 (RANBP3), and
retinol dehydrogenase 10 (all-trans) (RDH10).

Classification

In an effort to elucidate the signaling pathways that may lead to death or survival, we have clustered significant changes in gene expression ($P < 0.05$) according to their known function in regulating cell cycle, apoptosis, mitosis, receptor control, DNA synthesis, DNA repair, resistance to genotoxic stimuli, metabolism (methionine, steroid biosynthesis, and pyrimidine biosynthesis), and growth control at both 48 and 96 hours (Supplementary Table S1). This type of clustering facilitates the interpretation of physiologic changes based on the expression of genes of known function. It also guides the selection of genes for further verification of expression by reverse transcription-PCR and Western blot analysis and in determining the genes involved as effectors of cell physiology under MDS conditions.

Verification by Reverse Transcription-PCR

Gene expression was determined by reverse transcription-PCR for 50 gene products shown in Table 2. These genes were classified as controlling proliferation activity, apoptosis, cell cycle progression, mitosis, and resistance to DNA damage. Determinations were done on cells exposed to MDS for 48 or 96 hours representing populations that were either destined to die or survive, respectively. Major changes

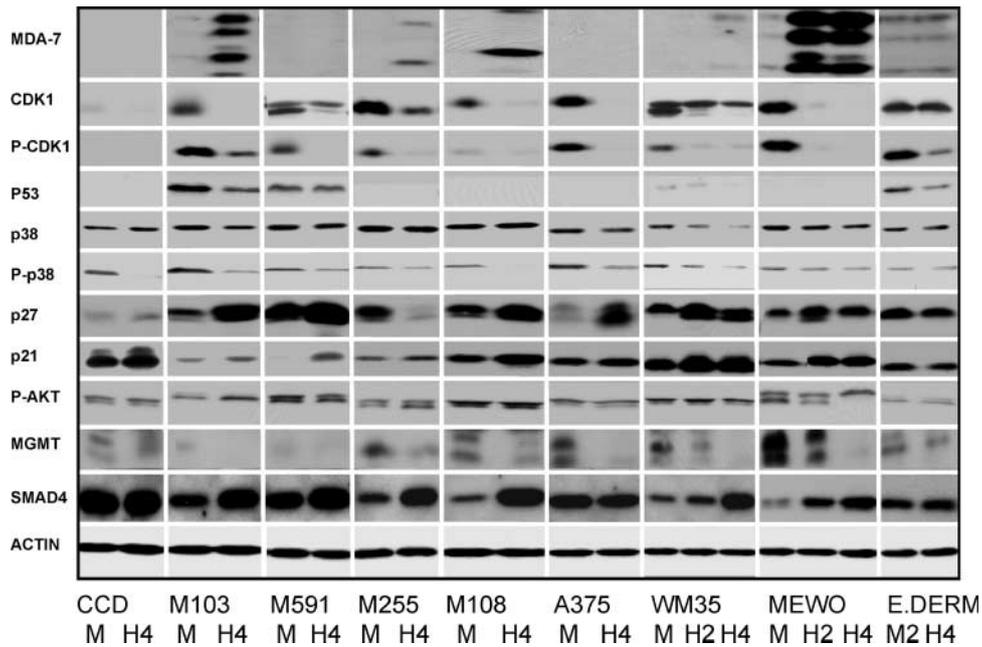


FIGURE 4. Western blot analysis showing changes in the expression of proteins that are likely to be associated with induction of cell cycle check points under MDS. Controls (*M*) or MDS at days 2 (*H2*) or 4 (*H4*) were harvested free of dead cells and proteins were extracted in SDS buffer and loaded to gels at equal protein concentrations. MDS induces synthesis of the tumor suppressor gene *MDA7* in M103, M255, M108, MEWO, and, to some extent, WM35. M591 and M103 expressed measurable levels of the mutant TP53 protein, which was down-regulated under MDS conditions. Enhanced expression of p21^{CIP1} under MDS was evident in all melanoma cell lines regardless of the inducibility of *MDA7* or TP53 status. Higher expression of p27 in response to MDS in most lines (except M255) indicates inhibition of G₁-S transition. CDK1 expression was lost under MDS in M103, M108, A375, and MEWO and reduced in M591, M255, and WM35. Weak presence of P-Thr¹⁶¹ CDK1 (activated) in melanoma cell lines under MDS is correlated with loss of proliferation compared with controls. Most impressive was the down-regulation of MGMT protein levels at 48 hours after MDS in melanoma but not in normal cell lines. Loss of MGMT at 96 hours was nearly complete.

compared with untreated were observed for *DCN*, *LUM*, *PTEN*, *GAS1*, *EGR3*, *MDA7*, *LITAF*, *BTG3*, *EREG*, *p21*, *BCAT1*, *RNASEL*, *TOP1MT*, and *cMET* in both cell populations, indicating a robust direct effect of MDS on the expression of these genes. Changes observed in cells destined

to undergo apoptosis alone included *IFNGR*, *TNFSF10*, and *FAS*. This indicates that apoptosis may be mediated by these genes. Changes in cells undergoing mitotic arrest, but not dying, included *BGN*, *TGFBR*, *TNS*, *NBL1*, *RASSF2*, *p57*, *p15*, *p18*, *AURKB*, *TNFRSF12*, *MGMT*, and *RAD52*. These

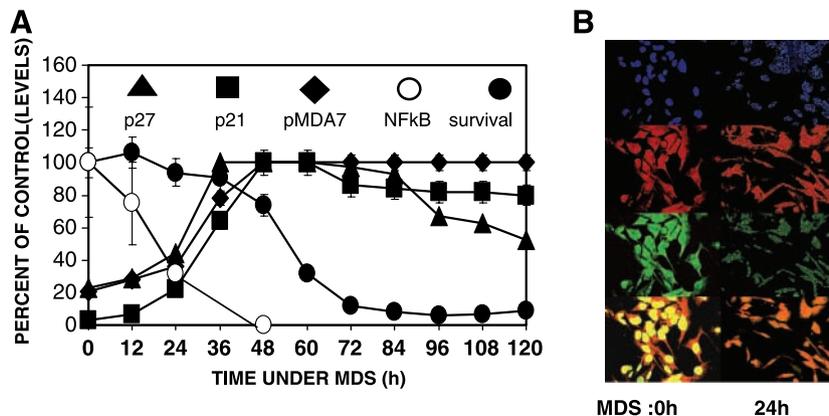


FIGURE 5. **A.** Kinetic analysis of the response of MEWO cells to MDS. MEWO cells under MDS were harvested at the time intervals indicated and counted. Percent survival was established as the number of live cells present in the culture compared with the number at day 0, which represents the untreated control (*circles*). *MDA7*, p21, and p27 protein expression was determined with Western blot analysis. Levels of *MDA7* (*diamonds*), p21 (*squares*), and p27 (*triangles*) were expressed as percent of their maximum value. Simultaneous presence of NF- κ B p50 (*red*) and p65 (*green*) subunits in the nucleus was determined by immunohistochemistry (*open circles*). **B.** Activation of NF- κ B was determined by colocalization (*yellow*) of NF- κ B p50 (*red*) and p65 (*green*) subunits in the nucleus of MEWO cells. On the other hand, intense activation of NF- κ B was observed in MEWO cells grown in methionine (*left*). Staining of nuclei with 4',6-diamidino-2-phenylindole (*top*) show differences in nuclear morphology under MDS. Points, mean of five determinations (cell survival) or three determinations (immunohistochemistry/Western blot); bars, SD.

Table 3. Enhancement of the Efficacy of Cisplatin, Temozolomide, and BCNU against Melanoma Tumor Cell Lines with MDS

Cell line	<i>O</i> ⁶ -benzylguanine/MDS	+Cisplatin*	+Temozolomide*	+BCNU*
Treatment 1 (<i>O</i> ⁶ -benzylguanine 24 h, MDS 96 h, chemotherapy, methionine 2 wk)				
MEWO	1	0.18 ± 0.3 [†]	0.105 ± 0.073	0.07 ± 0.04
M103	1	0.05 ± 0.02	0.012 ± 0.004	Eradicated
WM35	1	0.08 ± 0.02	0.025 ± 0.012	Eradicated
E.DERM	1	0.64 ± 0.13	0.87 ± 0.12	0.55 ± 0.08
Treatment 2 (<i>O</i> ⁶ -benzylguanine 24 h, MDS 96 h, methionine 24 h, chemotherapy, methionine 2 wk)				
MEWO	1	0.27 ± 0.6	0.15 ± 0.05	0.13 ± 0.06
M103	1	0.12 ± 0.04	0.032 ± 0.01	0.03 ± 0.02
WM35	1	0.13 ± 0.04	0.033 ± 0.01	0.04 ± 0.01
E.DERM	1	0.72 ± 0.14	0.89 ± 0.09	0.67 ± 0.12

NOTE: MEWO, M103, WM35, and E.DERM were treated with *O*⁶-benzylguanine (30 μmol/L) for 24 hours followed by MDS alone for an additional 96 hours. When multinucleated cells were fully arrested, they were treated with cisplatin (30 μmol/L), temozolomide (200 μmol/L), or BCNU (30 μmol/L) and placed in methionine medium, and their ability to grow was tested using a clonogenic assay (Materials and Methods). Colonies of ≥50 cells were counted and expressed as fraction of control (no cytotoxic drug). Mean ± SD of three determinations.

*Concentrations of the drugs used did not have a significant effect on the survival and proliferation of cells growing in methionine-efficient medium as determined by clonogenic assays.

[†]Fraction of surviving cells compared with their respective controls. These fractions were determined by counting the number of colonies in agar after 2 weeks of growth and comparing such numbers to controls (no cytotoxic agent).

results clearly suggest that MDS survivors are arrested and vulnerable to DNA-damaging agents.

Verification by Western Blot

Effector genes were examined by Western blot analysis. As shown in Fig. 4, MDS induced synthesis of the tumor suppressor gene *MDA7* in M103, M255, M108, and MEWO and to a lesser extent in WM35. Significant p53 down-regulation was observed in wild-type (WT) TP53 WM35 and E.DERM in response to MDS, whereas the expression of TP53 was not evident in CCD-187sk, M255, M108, and A375, which are WT TP53. M591 and M103 expressed measurable levels of the mutant TP53 protein, which either remained the same or was down-regulated (M103) under MDS conditions. There was no evidence of *MDA7* influence on p53 expression in either p53 mutant or WT cells, indicating that these two suppressor genes act independently. Evidence for increased TGF-β activity in conformity with microarray data (enhanced *TGFBR*, *PAL*, *PLAU*, *PLAUR*, and *integrin*) was supported by enhanced expression of SMAD4 in all tumor cell lines examined under MDS regardless of p53 or *MDA7* status. The effect of MDS on p21 was also evident in all tumor cell lines regardless of *MDA7* or TP53 status. Constitutive expression of p21 was significantly higher in the WT TP53 lines M108 and WM35, in marked contrast to its expression in the mutant TP53-expressing cell lines M103 and M591, where it was hardly detectable. Enhanced expression of p21 protein under MDS was correlated with the abrogation of expression of CDK1, which was most pronounced in M108, WM35, MEWO, and M591. Loss of CDK1 in M103 and A375 was correlated with an increase of p27 rather than p21. p27 expression correlated with the induction of SMAD4 in all tumor cell lines, except in M255 and A375. A large fraction of CDK1 was found in its activated form P-Thr¹⁶¹ CDK1 in M103, M591, M255, A375, and MEWO in methionine-efficient cultures but was lost under MDS. MDS and the induction of *MDA7* had minor effects on total p38. An unexpected decline of P-p38 indicates that this protein was not a major contributor in cell death even in lines in which MDS induced *MDA7*. Unlike previous reports indicating loss of P-AKT in response to

MDS (35), such treatment seems to have a minor effect in melanomas. In TP53 mutant melanomas and control cells, there was only a minor effect of MDS on levels of P-AKT. MEWO was an exception to this observation by showing a decline in AKT phosphorylation. In spite of untouched AKT activity, MDS inhibited NF-κB translocation into the nucleus and such inhibition preceded up-regulation of *MDA7*, p21, and p27 (Fig. 5).

Enhanced Efficacy of Toxicity by MDS

The results obtained by protein and gene expression analysis suggest that the surviving cells should be susceptible to several chemotherapeutic agents. Here, we examined the enhanced efficacy of temozolomide, BCNU, and cisplatin on the cell lines MEWO and M103 for which we have extensive information regarding gene expression and also in MW35, which is WT TP53. Temozolomide was tested because of the observed down-regulation of MGMT and TOP1 (39, 40) under MDS. BCNU was expected to also be effective because of the down-regulation of MGMT and of proteins involved in the repair of double-strand breaks by homologous recombination under MDS and cisplatin was tested for the latter reason. The doses of 200 and 30 μmol/L for temozolomide and BCNU/cisplatin, respectively, were selected because they had no significant effect on clonogenic growth of MEWO, M103, MW35, or E.DERM (data not shown). *O*⁶-benzylguanine was used to deplete MGMT activity 24 hours before the administration of MDS because this treatment has been shown previously to enhance the chemotherapeutic efficacy of temozolomide due to a more complete suppression of MGMT than that achieved by MDS alone (36). Under this treatment schedule, MGMT was reduced to undetectable levels and remained undetectable under MDS conditions due to rapid down-regulation of MGMT expression. As shown in Table 3, E.DERM cultures treated with *O*⁶-benzylguanine for 24 hours followed by 96-hour MDS were further sensitized to chemotherapeutic agents. However, such sensitization (2-fold) was considered to be marginal compared with that observed in tumor lines subjected to the same

treatment. Thus, BCNU eradicated cultures of M103 and WM35 and enhanced the efficacy of cisplatin and temozolomide by >10 times in MEWO. Although MEWO was more resistant to this treatment, it was markedly more responsive than E.DERM. Temozolomide and cisplatin were less effective than BCNU in tumor cells; however, both of these agents achieved much greater rates of cell death in tumor cells than in the E.DERM. Interestingly, the efficacy of treatment was greater when the chemotherapeutic agent was given at the end of MDS instead of 24 hours after MDS treatment. This difference cannot be attributed to up-regulation of MGMT during methionine repletion because MGMT remained undetectable for 24 hours after disrupting MDS (data not shown). Additional experiments are required to assess the optimal timing for administering chemotherapy in combination with MDS.

Discussion

MDS in melanoma tumor cells results in an overall antiproliferation and proapoptotic phenotype. Gene expression and epigenetic changes agree with the massive cell death observed within 48 to 72 hours from MDS and subsequently with the survival of a nondividing tumor cells that are nevertheless more susceptible to chemotherapy than untreated tumor cells. MDS induced changes in gene expression and epigenetic events in melanomas that are somewhat similar to those observed in other tumors (35, 36). However, significant differences are observed not only between melanomas and other classes of tumors but also within melanomas. It is hypothesized that although MDS may target several common genes regardless of tumor type or grade, mutational variability among tumors may decide which metabolic and signal transduction pathways will be activated or shutdown. A case in point is the induction of MDA7 by MDS, which occurs in a subset of melanomas and central nervous system tumors but not in pancreatic tumors (35, 36). Genetic variability may be also seen in the physiologic response of tumor to MDS and may differ as seen in pancreatic tumors that undergo mitotic death while attempt to divide in contrast to melanomas that die by classic apoptosis. Nevertheless, the loss or gain of genetic/epigenetic

function follows always the same direction by shutting down mitosis and prosurvival pathways, activating cell death signaling, and down-regulating resistance of tumor cells to toxic assault.

The question arises of how all these processes are affected and coordinated by MDS. Coordination could be attributed to either an external sensor that regulates cellular response based on the absence of exogenous methionine or by the stress imposed as a result of changes in the metabolic reactions controlling methionine levels within the cell. A third possibility, that the observed effects result from the toxic action of homocysteine thiolactone via protein thiolation, is unlikely because of the absence of a toxic effect on nontumor fibroblasts and some tumor cells (methionine independent; ref. 35). Furthermore, homocysteine thiolactone has no effect on growth of methionine-dependent tumor cells when methionine is supplied at the same time. Our data indicate that MDS imposes changes in sulfur amino acid metabolism that could alert tumor cell to conserve methionine by not proliferating. Methionine conservation was shown by the ability of tumor cells to maintain a relatively high ratio of methionine to homocysteine even when deprived from exogenous methionine (Table 4). Despite of the loss of methylthioadenosine phosphorylase (EC 2.4.2.28) expression under MDS (Supplementary Table S1), which suggests limitations in the methionine salvage pathway, methionine could have been conserved by the suppression of cystathioninase (EC 4.4.1.1), which limits conversion of methionine to cysteine, and by up-regulation of 5-methyltetrahydrofolate-homocysteine methyltransferase (EC 2.1.1.13), which drives reutilization of homocysteine. At the same time, protein synthesis may be suppressed by down-regulation of L-methionyl-tRNA synthetase (EC 6.1.1.10). Suppression of DNA (cytosine-5-)-methyltransferase 1 (EC 2.1.1.37) in survivors indicates difficulty of these cells to complete DNA transmethylation, thus sending a direct signal to stop cell cycling and mitosis. The above indicates that the mitotic arrest in MDS survivors may be associated with limited transmethylation activity. The antiproliferative effect of MDS is most evident on pyrimidine metabolism. Overall, pyrimidine processing is severely repressed by MDS, with most of the

Table 4. Levels of Extracellular Methionine and Total Homocysteine in Cultures of Cells after Exposure to Either Methionine (27 $\mu\text{mol/L}$) or Homocysteine Thiolactone (27 $\mu\text{mol/L}$)

Cell line	Medium	Cellular*	Time (h)	Extracellular MET ($\mu\text{mol/L}$)	HCYS ($\mu\text{mol/L}$)
E.DERM (MDS-nonresponding)	MET(+)/HSYS.TL(-)	47.3 \pm 4.1	24	26.4 \pm 1.9 [†]	ND
		45.1 \pm 4.6	48	22.8 \pm 3.4	1.0 \pm 0.3 [‡]
	MET(-)/HCYS.TL(+)	43.6 \pm 2.2	24	ND [§]	23.5 \pm 1.1
M103 (MDS-responding)	MET(+)/HSYS.TL(-)	41.2 \pm 1.5	48	ND	22.6 \pm 1.6
		43.8 \pm 3.3	24	22.1 \pm 1.4	3.2 \pm 1.7
		44.7 \pm 2.5	48	19.2 \pm 1.1	4.5 \pm 2.3
MEWO (MDS-responding)	MET(-)/HSYS.TL(+)	33.6 \pm 2.8	24	0.5 \pm 0.1	25.9 \pm 2.2
		31.5 \pm 3.4	48	0.6 \pm 0.1	22.8 \pm 1.8
	MET(+)/HCYS.TL(-)	42.3 \pm 1.9	24	23.7 \pm 2.1	3.9 \pm 1.1
		44.5 \pm 4.9	48	20.9 \pm 1.9	3.5 \pm 1.3
	MET(-)/HCYS.TL(+)	33.4 \pm 5.0	24	0.9 \pm 0.3	21.0 \pm 2.4
		33.3 \pm 2.1	48	0.9 \pm 0.4	18.6 \pm 2.8

*Ratio of methionine to homocysteine in cell extracts. Mean \pm SD of three determinations. Changes in the ratio of methionine to homocysteine was mainly due to increased intracellular homocysteine levels. However, less pronounced but significant reduction of methionine levels contributed to the changes in MDS responding cells.

[†]Mean \pm SD methionine concentration ($\mu\text{mol/L}$) of three determinations.

[‡]Mean \pm SD total homocysteine concentration of three determinations.

[§]Nondetectable.

reactions leading to the formation of TDP and TTP being repressed. This situation is compounded by up-regulation of pathways involved in uracil catabolism, primarily by levels of expression of dihydropyrimidine dehydrogenase in survivors, which drops to a fifth of the control level. On the other hand, thymidylate synthetase is down-regulated in cells destined to undergo apoptosis, whereas deoxythymidylate kinase and uridine phosphorylase 1 are down-regulated and shutdown, respectively, in both those undergoing apoptosis and in survivors.

Effector Genes

One of the most convincing correlations between physiologic responses and genetic modulation by MDS is observed with the melanoma cell lines MEWO and M103. Major changes in gene expression in these lines include the induction of the tumor suppressor/antiproliferative genes *TGFB*, *DCN*, *BGN*, *LUM*, *TNS*, *PTEN*, *GAS1*, *LITAF*, *IFNGR*, *EGR3*, *NBL1*, *KIP2* and *QUIESCIN Q6*, *E-cadherin*, *NBL1*, *RASSF2*, *BTG3*, *EREG*, *BIRC5* (*survivin*), and *MDA7*. The small leucine-rich proteoglycans DCN, BGN, and LUM are strongly induced by MDS and may be involved in apoptosis by binding and neutralizing growth factors (41, 42). DCN inhibits growth of different cancer cells (43, 44) by neutralizing the epidermal growth factor receptor-dependent kinases, by up-regulating p21^{CIP1/WAF1} through interaction with the epidermal growth factor receptor (45), or by a mechanism involving the SMAD4 transduction pathway (46). LUM is involved in controlling melanoma growth and invasion (41) by restricting proliferation and capacity to invade extracellular matrix. Loss of PTEN function in melanomas is believed to be a major factor in tumorigenic progression and its reintroduction retards tumor development (9, 47). Mechanistically, loss of PTEN is believed to lead to the activation of AKT, which consequently down-regulates the apoptotic pathway in melanoma cells. Loss of BIRC5 (*survivin*) by MDS causes abnormalities in cell division and failure of cytokinesis that results in the accumulation of multinucleated cells and apoptosis (48-50).

A significant finding in this study is the robust induction of MDA7 by MDS in four of seven melanoma lines tested. MDA7 (*IL24*) is induced in several central nervous system neoplasms by MDS (35) but is curiously not inducible in pancreatic tumors (36). Enforced expression of MDA7 by transduction with adenovirus vector (Ad.MDA7) is associated with growth inhibition of early-stage and late-stage melanoma cell lines but not normal human epithelial melanocytes (51-53). MDA7 is believed to act via the up-regulation of GADDs and activation of p38 mitogen-activated protein kinase. Although there is no evidence for the up-regulation of p38 mitogen-activated protein kinase by MDS, the up-regulation of GADD45 and GADD34 has been verified by oligoarray analysis in MEWO and M103 as well as in central nervous system tumors in which MDA7 is induced by MDS (35). Expression of MDA7 may lead to apoptosis by several pathways, two of which (i.e., up-regulation of IFN- γ and loss of MYC) agree with gene expression data shown here. Marked up-regulation of BRCA1 (5 \times) and IFNGR (10 \times) in cells destined to die under MDS results in the up-regulation of OAS1 (2 \times) and the proapoptotic gene RNASEL (100 \times). The IFN- γ /BRCA1 signal transduction scheme is

therefore consistent with apoptotic death in MEWO and M103 and may be triggered by the expression of MDA7. Loss of MYC in cells undergoing apoptosis is accompanied by down-regulation of expression of PTMA (3.4 \times), ODC1 (12 \times), and DFFB (11 \times) and up-regulation of BCAT1 (100 \times). The latter enhances levels of α -ketoisocaproic acid, which is associated with apoptosis and loss of cytokinesis (54). The apparent inhibition of the methionine salvage pathway by the down-regulation of *MTAP* expression under MDS implies elevation of polyamines (55, 56). Elevated polyamines may result in either cell death or transformation depending on cell type (57-59). The methionine salvage pathway, although restricted by the down-regulation of *MTAP* expression, may however, remain operational in melanoma cells undergoing apoptosis in response to MDS. The utilization of adenosylmethionine to convert putrescine to spermidine may result in the depletion of methionine with concomitant overproduction of polyamines. This could be associated with the robust apoptosis in MEWO observed between 48 and 72 hours under MDS. In addition to the above, MDA7 may be involved in the up-regulation of *TGFB*/*TIEG*, and *TNF- α* , in a manner that could influence cell cycle progression, apoptosis, and mitosis. However, many of the above processes are up-regulated by MDS in tumors in which MDA7 is not expressed; therefore, it is premature to assign the induction of these genes to MDA7 alone.

More common than MDA7 is the induction/up-regulation of the TGF- β pathway not only in melanomas but also in the majority of tumors examined under MDS. In melanomas, TGF- β -mediated signal transduction by MDS is suggested by the increase of expression of SMAD4, PAI, PLAU, PLAUR, and integrin, which inhibit transformation of plasminogen to plasmin. Vitronectin, which causes dissociation of PAI from integrin, thus limiting its availability, was also down-regulated. Additional support for enhanced TGF- β involvement comes from the presence of markedly high mRNA levels of TGB1, BMP2-8, *TIEG*, SMAD1, SMAD5, and SMAD8 in cells exposed to MDS for 96 hours. The significance of TGF- β in MDS is best described in conjunction with c-MYC down-regulation. TGF- β must down-regulate c-MYC expression to stimulate cell cycle arrest and prevent cell proliferation. Both c-MYC and TGF- β signaling influence gene transcription, and their common targets include the genes encoding the CDK inhibitors p15, p21, and p27. These genes are stimulated by TGF- β (60-64) and suppressed by c-MYC (62, 65-70). TGF- β also stimulates redistribution of p27 within cyclin-CDK complexes (70). In proliferating cells, p27 is found predominantly in complexes with cyclin D-CDK4 and cyclin D-CDK6, which remain active despite their association with p27. Stimulation with TGF- β causes displacement of p27 from these complexes by p15 and binding of p27 to CDK2, resulting in cell cycle arrest (70). On the other hand, the loss of transcriptional activation of the CDK inhibitor p15 by MDS may be due to the suppression of p15 promoter by factors other than MYC. The multifaceted response to MDS can be described as the result of the integrated action of several signal transduction pathways that are regulated by the expression of known master genes, such as *MDA7*, *TGFB*, and *TNFA*, which share common downstream effectors genes with an overall action to stop cell cycle progression and cell

proliferation and induce conditions under which tumor cells either die or are sensitized to external proapoptotic stimuli.

Downstream Events

Rising TGF β levels under MDS conditions enhanced p27 and p21 protein expression. Parallel down-regulation of expression of CDC25A and cyclin D1 could lead to inactivation of CDK2, CDK4, and CDK6 complexes resulting in inhibition of G₁-S transition in MDS survivors. Overall, a less strict inhibition of G₁-S transition is predicted in cells that are determined to undergo apoptosis, in which growth factors and their receptors as well as cyclin D1, cyclin E, and CDK2 are up-regulated as contrasted with their levels in survivors. Although it is reasonable to assign activation of G₁-S checkpoints to the up-regulation of TP53, the question of this protein being actually activated in a known TP53 mutant tumor remains open to speculation. Uncertainty regarding the direct role of TP53 in the up-regulation of its dependent genes raises the question for involvement of other suppressor genes, such as TGF β and MDA7, in such an effect.

Variation in gene expression between 48- and 96-hour cultures was most profound in relation to genes involved in apoptotic pathways. At 48 hours, when cultures contained mostly cells destined to undergo apoptosis, expression of APO2L, APO3, DR3/4/5, caspase-3/6/7/8/10, TRADD, RIPK1, BID, FLIP, IKK, IKK, IAP, and Fodrin was enhanced in relation to controls. In contrast, cells that survived the apoptotic flush expressed reduced levels of APO2L/3L, DR3, APO3, TRADD, TRAF2, and IKK. It is postulated that expression of DR3 promotes apoptosis via the adaptor proteins TRADD/FADD and the activation of caspase-8. Therefore, TRADD and its associated regulation of IKK, IKK, and BID may be the key for survival versus death under MDS. It must be noted, however, that activation of NF- κ B does not occur under MDS possibly because of the severe down-regulation of TRAF2 and increase of the I-TRAF levels, both contributing to enhanced cytoplasmic binding of NF- κ B with IKK. Apoptosis could be further enhanced by several independent pathways that are affected in most melanoma cells under MDS. These pathways include up-regulation of PTEN, the down-regulation of MYC in combination with BCAT1 activation, and the overexpression of BRCA1/IFNGR. In WT TP53 melanoma cells and in certain TP53 mutants, MDS yields up-regulation of the proapoptotic genes *BAX* (12 \times), *BID* (13 \times), *APAF1* (3 \times), *DR5* (6 \times), *p21^{CIP1}* (20 \times), and *DAPK1* (11 \times) but does not trigger the induction of MDM2 and MDM4, ensuring a prolonged TP53-mediated effect that could be associated with the persistence of p21 and other TP53-regulated genes into a prolonged G₂-M cycle arrest.

The central change involved in the inhibition of G₂-M transition under MDS regardless of the final destination of the targeted tumor cell is the down-regulation of CDK1 and cyclin B. Increased levels of tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein θ (14-3-3 θ) could also account for the decline in CDK1 protein levels. Inhibition of CDK1 by dephosphorylation of Thr¹⁶¹ and phosphorylation of Tyr¹⁵ is most likely related to enhanced expression of p21 (20 \times) and GADD45A (5 \times). Decrease in the phosphatase CDC25B levels (5 \times) and increase in the levels of 14-3-3 θ (2 \times) along with an increase of WEE (3 \times) during the first 48 hours

from MDS agree with the absence of Thr¹⁶¹-CDK1 in several melanoma lines under MDS. The above observations indicate that apoptosis may occur under conditions of severe cell cycle arrest in G₂-M as suggested previously for pancreatic tumor cells. However, unlike pancreatic tumor cells, most melanoma destined to die do not enter a mitotic state as can be assessed by the absence of changes in the expression of mitosis-related genes (BUB1 and CDC20) compared with controls. In survivors, loss of CDK1 function could be related to the absence of expression of genes that are vital for mitosis, such as *AKAP95* and *AURBK*. Because these two genes are down-regulated in multinucleated cells but not in those destined to undergo apoptosis, it is suggested that their function in histone H3 is required for chromatin condensation and segregation of chromosomes.

Loss of Resistance to Radiation/Chemotherapy

Enhancement of the efficacy to radiation by MDS is supported by the observed down-regulation of the hepatocyte growth factor receptor cMET, a strong inhibitor of γ -irradiation-induced apoptosis, by 2 orders of magnitude in survivors. Homologous repair of DNA strand breaks is also expected to be inhibited in survivors due to down-regulation of RAD54B, ATRX, and RAD52. Finally, the near elimination of 8-oxoguanine (OGG1) is indicative of the increased sensitivity of MDS to radiation and/or oxidative stress. It is noteworthy that possible sensitization of melanomas to radiation by MDS could be independent of p53 status. Because p53 mutations in melanomas are frequent (71-73), MDS could overcome this serious problem in the treatment of this disease. MDS is also known to enhance the efficacy of alkylating agents by inhibiting the expression of MGMT, a protein that repairs mutagenic/cytotoxic alkylations at the sixth position of guanine. Down-regulation of MGMT mRNA in melanomas is quite effective and nearly complete within 96 hours from MDS. Loss of TOP1 expression with synchronous loss of MGMT could be synergistic in enhancing the efficacy of alkylating agents, especially that of temozolomide (39, 40). A minimal effect of MDS on DNA-glycosylase levels, along with the observed loss of POLB, is also supportive of an enhanced response of melanomas to alkylating agents under MDS (74).

Among the reasons for the resistance of melanomas to chemotherapy is the overexpression of multidrug resistance proteins MRPs (75-77) and GSTs (78, 79). In cells that are resistant to apoptosis, there is up-regulation of GSTP, GSTM1, GSTM2, GSTM3, and GSTM4, indicating that these cells would be resistant to BCNU under MDS conditions. However, associations between cellular resistance to anticancer agents and expression of GSTs are not always conclusive, and the role of GSTs in the protection of cells against anticancer drugs remains equivocal. The function of GSTs may be impaired under MDS due to elevation of glutathione peroxidase and down-regulation of glutathione reductase, which result in functional reduction of reduced glutathione.

Toxicity experiments done in this study indicate that melanomas, like brain and pancreatic tumors, are sensitized to agents that exert their toxicity via the formation of O⁶-alkylguanine adducts or the formation of double-strand DNA lesions. Thus, sensitization to temozolomide may be

derived from the down-regulation of MGMT, TOP1, and POLB. Combining the use of *O*⁶-benzylguanine, which eliminates MGMT protein levels, and MDS, which prevents synthesis of the MGMT protein and ensures the accumulation of *O*⁶-methylguanine adducts that pair with either T or C during DNA synthesis in temozolomide-treated survivors, has a synergistic effect in killing melanoma cells. Loss of POLB will result in the induction of cell cycle arrest in G₁-S that might be associated with apoptosis as shown previously in cells exiting G₂-M arrest and undergoing mitosis to yield a G₁ population with unrepaired DNA (80). A G₁ cell cycle block and the retention of *O*⁶-methylguanine are expected to enhance MMR and cell death by DNA fragmentation. Down-regulation of homologous recombination in melanomas in response to MDS is expected to further facilitate cell death. Suppression of MGMT by MDS and concomitant loss of homologous recombination is expected to also enhance the efficacy of carmustin. Evidently, the increase of GSTs by MDS does not impede the ability of BCNU to induce DNA damage. An explanation for enhanced sensitivity to cisplatin cannot be given without additional experiments. However, this agent is expected to cause DNA fragmentation. In addition to the above agents, radiation could also be effective against MDS melanomas based on the down-regulation of cMET, OGG1, and RADs.

Materials and Methods

Cell Lines and Culture

The metastatic human melanomas M591 (mutant TP53), M255 (WT TP53), M105 (mutant TP53), M103 (mutant TP53), and M108 (WT TP53) were obtained from infiltrated lymph nodes of melanoma patients. A375 (WT TP53), the skin melanomas WM35 (WT TP53) and MEWO (mutant TP53), and the human normal skin fibroblast CCD-187sk and horse normal dermis fibroblast E.DERM lines were obtained from American Tumor Culture Collection (Manassas, VA).

Cultures and MDS Conditions

Cultures were tested for *Mycoplasma* and cloned using RPMI 1640/DMEM and 10% fetal bovine serum. Consequently, all cell lines were adapted to DMEM (BioWhittaker, Walkersville, MD) with 10% fetal bovine serum (Mediatech, Herndon, VA) in 6.5% CO₂ at 37°C until confluent. Cultures were finally adapted to DMEM, supplemented with an additional 584 mg/L L-glutamine to a final concentration of 8 mmol/L, 10 mL/L penicillin/streptomycin, and 100 mL/L dialyzed fetal bovine serum (Life Technologies, Grand Island, NY; full medium A: MET medium). MDS was induced by transferring cell cultures to methionine free DMEM-B (Mediatech) supplemented with 27 mg/L homocysteine thiolactone hydrochloride (Sigma, St. Louis, MO), 62.5 mg/L L-cysteine dihydrochloride (Sigma), L-glutamine to a final concentration of 8 mmol/L, α-cobalamine (1 μmol/L), penicillin/streptomycin, and dialyzed fetal bovine serum (full medium B: HCYS medium). The concentration of folate in all media, excluding those in serum, was 20 μmol/L as reported by the manufacturer (BioWhittaker). Measurements of methionine and homocysteine concentrations in both cellular components and the medium were done to ensure that levels of methionine or

homocysteine did not decline below 20 μmol/L (Table 4). To ensure this, medium was replaced in all cultures every 36 hours.

Flow Cytometry

Cell cultures were harvested every 24 hours, washed to remove dead cells and debris, trypsinized, washed with PBS twice, and centrifuged (800 rpm) at 4°C. The cell count was adjusted to 2 × 10⁵ to 3 × 10⁵/mL cells in PBS per sample. In experiments in which determination of nuclear fragmentation indicative of apoptosis was desirable, cell cultures were not washed before trypsinization. From this cell suspension, 1 mL was taken in triplicate, centrifuged at 800 rpm for 10 minutes at 4°C, reduced to 100 μL, and fixed in 500 μL ice-cold 70% ethanol overnight at 4°C. Fixed cells were centrifuged at 500 rpm at 4°C for 10 minutes and suspended in 500 μL PBS containing 50 μL of 50 μg/mL propidium iodide (Sigma) and 10 μL of 5 μg/mL RNase for 45 to 60 minutes at 4°C. Flow cytometric analysis was done within 24 hours of propidium iodide staining on a Beckman-Coulter (Fullerton, CA) Epics XL. EXPO32 software was used for optimum analysis of cell cycle (G₁, G₂, and S phases).

Affymetrix Chip Hybridization

RNA was isolated from MEWO and M103 cultures at 48 hours following MDS when cells were fully arrested and were primarily in G₁ as determined by flow cytometry or at 96 hours when cell cultures were composed of multinucleated resting cells containing four times the DNA of their respective G₁ counterparts. mRNA expression analysis of >55,000 genes and expressed sequence tags was done on MEWO and M103 tumor cells grown in MET or HCYS medium for 48 and 96 hours with the use of U133 PLUS 2 (54,676 genes) chips from Affymetrix (Santa Clara, CA) in duplicate as described previously (35). Baseline analyses were done using Oligoarray suite 5.0 to identify statistically significant gene expression alterations between samples derived from cells grown in MET and those maintained in HCYS medium. Because samples were analyzed in triplicates, these results were further screened for consistent *P* by the Student's *t* test (*P* < 0.05) to eliminate random sampling errors.

Quantitative PCR

SYBR green light cycler PCR was used to estimate relative mRNA expression with high confidence as described previously (35). Expression for 36 genes was validated using the following forward/reverse sequences:

TP53 (159 bp) gttccgagagctgaatgagg/tctgagtcaggccctctgt,
 DCN (155 bp) ttggctaagtgggattga/agacaacctggatgactatgctc,
 BGN (182 bp) gcttcgactccaagctgt/tggagtgcagatagaccacct,
 LUM (145 bp) cctgggtgagctgctgt/gtaggataatggccccagga,
 TGFBR1(155bp)ccacagagtgggaacaaaag/catgaattccccaatg-
 gaaca,
 PTEN (178 bp) gcagaaagactgaaggcgta/gctgtggtgggttatggtct,
 TNS (175 bp) aaattcaaggcatggagcac/ttagcatacaggctccatct,
 GAS1(1exon;155bp)cctactgcggcaaatgcttc/catgttctccttgacc-
 gactc,
 EGR3 (147 bp) gacaatctgtacccccagga/gtaagagagttccgggtgg,

MDA7 (186 bp) tctgcagaacgtctcggatg/tcattttctgactgggttc,
 NBL1 (188 bp) gccaagaacatcaccagat/cactccagcgtcacaatctc,
 RASSF2 (173 bp) aacggcatttctacaacca/tggccttcagcttctgtttc,
 QSCN6 (167 bp) gaagacgtcaaacgctggag/ccagccactggaaatactgc,
 LITAF (195 bp) atccaattaccgtgcagacg/agaaggggatgagcagcag,
 BTG3 (170 bp) catcgaaggagcaggctac/tctccataccgacagcaca,
 IFNGR (153 bp) tectcagtgctcacaccaact/gcagcgaatcattctctg,
 EGER (179 bp) aggatggagatgctctgtgc/attgacactgagccacag,
 YWHAH (147 bp) acatggcctccgctatga/tggttttctgctcaatgctg,
 YWHAQ (171 bp) aaagtggagtcgagctgag/cgttttctgctcaatgctg,
 CDKN1A (162 bp) acaccagatgaacgaggag/cgcagaacccaaagtctga,
 CDKN1C (170 bp) ggcctctgatctccgatttc/gggctctttgggctctaaat,
 CDKN2B (173 bp) atgcgcgaggagaacaag/ctgccatcatcatgacat,
 CDKN2C (139 bp) acgtcaatgcacaaaatgga/tcatgaatgacagcgaaac,
 CDKN2D (154 bp) ctgcaggtcatgatgtttgg/cgtgctccactaggaccttc,
 CCNB1 (215 bp) gcctgagcctattttggtg/tgactgctgctcttctca,
 AURKB (192 bp) aactcctaccctggcccta/cgttaagatgctgggtgtcc,
 BCAT1 (177 bp) aagatgggaggaattaccg/tggaggagttgcccagttctt,
 BRCA1 (151 bp) gcttagcaaggagccaacat/tctgagcatgagcagttctg,
 RNASEL (131 bp) cagatctgcaaccacaaaa/agccgtccaaggtctctca,
 TOP1 (131 bp) gaccacctcacaacgattc/ctggtgaacgactctgctg,
 TOP1MT (193 bp) gagcaacccccagtagctt/agctgctcctgcagctctc,
 RAD52 (180 bp) tttggaggagctgacagc/ccctcaatgtgacacacctc,
 RAD54 (150 bp) gcaagctgacattacatctca/cctgaagattgcaaaatcc,
 HGF (169 bp) aacacagcttttgcctctg/tgcaggtcatgcatcaac, and
 OGG1 (216 bp) tcagaatccaaggtgtgc/agatgagcctccacctg.

Western Blot Analysis

Protein was extracted from live cells in culture at 48 and 96 hours after MDS. Protein expression was measured as described previously (35). Cultures used in this experiment were harvested at the same time intervals used for harvesting RNA. Cells were washed twice with cold PBS and lysed (while still attached) for 30 minutes in 100 μ L ice cold radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 8.0) and 150 mmol/L NaCl containing 0.1% SDS, 1% NP40 and 0.5% sodium deoxycholate, 0.1 mg/mL phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 1 mg/mL aprotinin]. Cell debris was removed by centrifugation at 14,000 $\times g$ for 10 minutes at 4°C. Protein was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Aliquots of cell extracts containing 50 μ g total protein were resolved in 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Perkin-Elmer Life Sciences, Inc., Boston, MA). Filters were blocked for 1 hour at room temperature in Blotto A [5% nonfat dry milk powder in 1 mol/L Tris (pH 7.4), 5 mol/L NaCl, 0.05% Tween 20] and then in Blotto. The following antibodies were used: (a) rabbit polyclonal β -tubulin (1:300), p21 (1:200), p27 (1:200), p38 (1:500), mouse monoclonal p53 (1:200), and SMAD4 (1:200) antibodies from Santa Cruz Biotechnology (Santa Cruz, CA); (b) rabbit polyclonal P-AKT (Ser⁴⁷³; 1:1,000), P-AKT (Thr³⁰⁸; 1:1,000), P-Rb (Ser⁷⁸⁰; 1:1,000), P-Rb (Ser⁷⁹⁵; 1:1,000), P-Rb (Ser⁸⁰⁷/Ser⁸¹¹; 1:1,000), CDC2 (1:1,000), P-CDC2 (Tyr¹⁵; 1:1,000), P-CDC2 (Thr¹⁶¹; 1:1,000), or mouse monoclonal Rb antibodies from Cell Signaling Technology (Beverly, MA); and (c) mouse monoclonal MDA7 (1:500) provided by Dr. Sunil Chada and β -actin (1:1,000) from Sigma.

Activation Status of NF- κ B Assessed by p65/p50 Localization with Double Immunofluorescence Assay

Control and MDS MEWO cells were cultured on Lab-Tek chamber slides (Nunc, Inc., Naperville, IL). Slides were blocked with 3% bovine serum albumin in PBS (pH 7.4) with 0.25% Tween 20 for 30 minutes. Slides were then incubated overnight at 4°C in primary antibodies, mouse anti-p65 and rabbit p50 (Santa Cruz Biotechnology), diluted 1:100 with the blocking buffer. After washes in PBS (pH 7.4) with 0.25% Tween 20 (3 \times 15 minutes), the cells were overlaid with secondary antibodies, Cy2-conjugated anti-mouse IgM and Cy3-conjugated anti-rabbit IgG (Rockland Immunochemicals, Gilbertsville, PA) diluted 1:1,000 in blocking buffer. After three washes in PBS (pH 7.4) with 0.25% Tween 20 (3 \times 15 minutes) and one wash in PBS (1 \times 15 minutes), the slides were mounted with aqueous mounting medium with antifade and 4',6-diamidino-2-phenylindole (VectaShield, Vector, Burlingame, CA) and visualized with a Zeiss Axioskop II (Thornwood, NY) epifluorescence microscope. Images were processed by pseudocoloring with RGB values corresponding to each of the fluorophore emission and the area of colocalization (yellow) of p65 (green) and p50 (red) subunits was quantitated.

Cell Survival Assays

Experiments here were designed to determine the effect of MDS alone or in combination temozolomide, cisplatin, or BCNU on MEWO, M103, WM35 and E.DERM survival. Doses of these agents that have no significant effect on growth and survival of the above lines when grown in methionine efficient medium were selected. Cells were grown to 50% confluency and then cultured for an additional 24 hours in methionine medium supplemented with 30 μ mol/L *O*⁶-benzylguanine to deplete MGMT protein. The cells then were transferred to methionine-deficient medium for 96 hours. At 96 hours after MDS treatment, cells were processed according to the following two methods: (a) Cells were treated with 30 μ mol/L cisplatin, 30 μ mol/L BCNU, or 200 μ mol/L temozolomide for 30 minutes, washed, and placed on methionine medium for 24 hours. (b) Cells were placed in methionine medium for 24 hours before treatment with the same cytotoxic agents, then treated with the cytotoxic agents for 30 minutes, washed, and transferred in methionine medium again. Controls were treated with *O*⁶-benzylguanine and MDS for the same time intervals but not with the cytotoxic agents. For colony formation, 5,000 cells were plated in agar and allowed to grow for 14 days. Surviving fractions were determined by measuring the colonies divided by the number of cells. Data were corrected for plating efficiency and presented as mean \pm SD of three independent determinations. Reduction of survival due to treatment with cytotoxic agents was determined by comparisons of treated versus nontreated controls.

Determination of Methionine and Homocysteine in Biological Samples

Methionine and total homocysteine were determined by high-performance liquid chromatography 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 μ L concentrated cell culture medium was mixed with 75 μ L penicillamine (internal standard) and 25 μ L of 60 mg/mL Tris (2-carboxy-ethyl)-phosphin-HCl. Tubes were capped, vortexed for 60 seconds, and allowed to stand for 10 minutes at room temperature. The samples were cooled on ice, and 500 μ L ice-cold 0.3 N perchloric acid was added. The tubes were tightly capped, vortexed for 30 seconds, and centrifuged for 5 minutes at 10,000 rpm. A 100 μ L aliquot of the supernatant was transferred into an ESA 542 autosampler operating at 5°C and 20 μ L were injected onto a 80 \times 4.6 mm ESA HR-80 C-18 3- μ m high-performance liquid chromatography column (ESA) equilibrated at 30°C. The sample was eluted isocratically with a mixture 10% acetonitrile in 0.15 mol/L phosphate buffer (pH 2.9) containing 1.0 mmol/L SDS. HCYS, penicillamine, and MET were eluted at 5.1, 6.7, and 8.6 minutes, respectively, and were identified and quantitated by the ratios of the detector's response at the operating potentials. Attached cells were briefly washed with PBS, lysed in hypotonic phosphate buffer by thaw-freezing thrice, sonicated briefly, centrifuged to remove debris, and then analyzed by the same method.

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