Modulation of cell cycle and gene expression in pancreatic tumor cell lines by methionine deprivation (methionine stress): implications to the therapy of pancreatic adenocarcinoma

Demetrius M. Kokkinakis, XiaoYan Liu, and Russell D. Neuner

Department of Pathology and the Cancer Institute, University of Pittsburgh, Pittsburgh, Pennsylvania

Abstract

The effect of methionine deprivation (methionine stress) on the proliferation, survival, resistance to chemotherapy, and regulation of gene and protein expression in pancreatic tumor lines is examined. Methionine stress prevents successful mitosis and promotes cell cycle arrest and accumulation of cells with multiple micronuclei with decondensed chromatin. Inhibition of mitosis correlates with CDK1 down-regulation and/or inhibition of its function by Tyr^{15} phosphorylation or Thr^{161} dephosphorylation. Inhibition of cell cycle progression correlates with loss of hyperphosphorylated Rb and up-regulation of p21 via p53 and/or transforming growth factor-β (TGF-β) activation depending on p53 status. Although methionine stress–induced toxicity is not solely dependent on p53, the gain in p21 and loss in CDK1 transcription are more enhanced in wild-type p53 tumors. Up-regulation of SMAD7, a TGF-β signaling inhibitor, suggests that SMAD7 does not restrict the TGF-β-mediated induction of p21, although it may prevent up-regulation of p27. cDNA oligoarray analysis indicated a pleiotropic response to methionine stress. Cell cycle and mitotic arrest is in agreement with up-regulation of NF2, ETS2, CLU, GADD45α, GADD45β, and GADD45γ and down-regulation of AURKB, TOP2A, CCNA, CCNB, PRC1, BUB1, NuSAP, IFI16, and BRCA1. Down-regulation of AREG, AGTR1, M-CSF, and EGF, IGF, and VEGF receptors and up-regulation of GNA11 and IGFBP4 signify loss of growth factor support. PIN1, FEN1, and cABL up-regulation and LMNB1, AREG, RhoB, CCNG, TYMS, F3, and MGMT down-regulation suggest that methionine stress sensitizes the tumor cells to DNA-alkylating drugs, 5-fluorouracil, and radiation. Increased sensitivity of pancreatic tumor cell lines to temozolomide is shown under methionine stress conditions and is attributed in part to diminished O^6-methylguanine-DNA methyltransferase and possibly to inhibition of the cell cycle progression. [Mol Cancer Ther 2005;4(9):1338–48]

Introduction

Pancreatic adenocarcinoma is the fourth leading cause of death in the United States, with an estimated incident of 30,000 victims annually. Most patients who are diagnosed with this tumor die within a year and the 5-year survival is <5%. Only a very few tumors are as aggressive as pancreatic cancer, and unlike many other cancers, early discovery does not always lead to improved survival (1). Frequent mutations in p53 (75%), p16/RB1 (90%), BRCA2 (50%), and K-*ras* (90%; ref. 2) are likely to yield a tumor that is resistant to a variety of conventional chemotherapies and to radiation (3, 4). Interestingly, pancreatic tumors are sensitive to the restoration of p53 (5) but not to restoration of p16/RB1 or silencing of K-*ras* (6, 7), which only result in suppression of growth without significant effect on apoptosis. Clinically, the coexpression of RB1 and p53 pathway-related proteins, such as $p21^{WAF1/CIP1}$ and BAX, render a significantly higher survival rate in patients with ductal carcinoma of the pancreas (8). Research in this laboratory (9) has identified that the two prominent factors in the resistance of pancreatic neoplasms to alkylating chemotherapeutic agents are (*a*) the high level of O^6-methylguanine-DNA methyltransferase (MGMT) in the tumor and (*b*) the widespread mutation of p53 that prevents p53-mediated cell cycle arrest in response to DNA damage (10). In wild-type (wt) p53 tumors, prolonged suppression of MGMT with O^6-benzylguanine and concomitant activation of p53/p21 pathway to induce a G_1-S arrest results not only in elevated killing due to an increase of BAX to BCL2 ratio but also in an enhancement of the efficacy of mismatch repair via the up-regulation of mismatch repair components. The above observations set the stage for an effective treatment of pancreatic malignancies having high levels of MGMT and a functional p53/p21 system. However, most pancreatic tumors are p53 dysfunctional and do not arrest in G_1-S in response to DNA damage. Efforts to restore p53 in these tumors in a clinical setting are cumbersome and not likely to become available in the near future. Compounds that restore the binding affinity of mutant p53 to DNA, such as

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Note: R.D. Neuner is currently at the Department of Molecular and Cellular Biology, University of Massachusetts, Amherst, MA 01003.

Requests for reprints: Demetrius M. Kokkinakis, Division of Basic Research, Hillman Cancer Center, University of Pittsburgh Cancer Institute, UPCI Research Pavillion, Room G12.e, 5117 Centre Avenue, Pittsburgh, PA 15213-1863. Phone: 412-623-1110; Fax: 412-623-4747. E-mail: kokkinakisdm@upmc.edu

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CP-31398, are promising but not yet suitable for clinical applications (11, 12). In addition, prolonged inactivation of MGMT with currently available MGMT inactivators, such as O^6-benzylguanine, is problematic due to nonspecificity resulting in enhanced toxicity and mutagenesis in normal tissue. An alternative approach to induce cell cycle and mitotic arrest and at the same time eliminate MGMT activity specifically in the tumor can be achieved by depleting plasma methionine (13). Application of methionine stress *in vivo* has shown that it has no deleterious effects on normal tissue even under moderate levels of genotoxic stress (14). Furthermore, methionine stress has a broad antitumor effect that involves the induction/activation of tumor suppressor genes, such as transforming growth factor-β (TGF-β), MDA-7, Rb, p21, and p27, to silence prosurvival pathways, such as phosphatidylinositol 3-kinase (PI3K), RAS, nuclear factor-κB, and CDK1, and at the same time induce proapoptotic pathways propagated by activation of tumor necrosis factor–related apoptosis-inducing ligand and death receptor 6 (15). Methionine stress could synergize with alkylating and perhaps other chemotherapeutic agents in all pancreatic tumors regardless of p53 status by reducing their high MGMT levels and by causing cell cycle arrest via the up-regulation of p21 and GADD45, activation of Rb, and down-regulation of CDK1. In terms of cell cycle kinetics, methionine stress can restore cell cycle/mitotic arrest in mutant p53 tumors and prevent immediate transition from G_1 to S and G_2, thus enhancing BAX and mismatch repair–mediated death. Furthermore, the mutational variability of pancreatic tumors that is believed to contribute to their resistance to single therapeutic treatments will be overcome by the pleiotropic action of methionine stress. An important consideration in choosing methionine stress is that it has been tested in primates and the results are supportive for its timely use in clinical trials (16).

Materials and Methods

Cell Lines and Culture

The pancreatic adenocarcinoma cell lines CAPAN1, CAPAN2, CFPAC1, MiaPaCa2, BxPc3, and Panc1 were obtained from the American Type Culture Collection repository (Manassas, VA). The mouse embryo fibroblast line NIH 3T3 was used as control (13).

Plasmids and Transient Transfection of CAPAN1

Human plasmid pCMV-p53 (Clontech Laboratories, CA) full-length p53 cDNA clone (Open Biosystems, Huntsville, AL) that express the human wt-p53 tumor suppressor protein under the constitutive cytomegalovirus promoter was used for restoration of the p53 function in CAPAN1 (that harbor endogenously mutated p53) by transient transfections. Cells were transiently transfected immediately after trypsinization and replated using the improved transfection technique for adherent cells with Effectene reagent (Qiagen, Valencia, CA). Briefly, the cells were incubated with lipid-DNA complexes at 37°C and 5% CO_2. p53 expression plasmids (10 μg) or empty vector (10 μg) were used. Forty-eight hours after transfection, cells were exposed to methionine stress.

Cultures and Methionine Stress Conditions

All cell lines were adapted to DMEM (BioWhittaker, Walkersville, MD) with 10% fetal bovine serum (Media Tech, Inc., Herndon, VA) in 6.5% CO_2 atmosphere at 37°C until confluent. Cultures were subsequently adapted to DMEM-A (Media Tech) supplemented with an additional 584 mg/L L-glutamine (Sigma, St. Louis, MO) to a final concentration of 8 mmol/L, 10 mL/L penicillin/streptomycin (Sigma), and 100 mL/L dialyzed fetal bovine serum (Life Technologies, Grand Island, NY). Methionine stress was induced by transferring cell cultures to methionine-free DMEM-B (Media Tech) supplemented with 27 mg/L homocysteine thiolactone hydrochloride (Sigma), 62.5 mg/L L-cysteine dihydrochloride (Sigma), and L-glutamine to a final concentration of 8 mmol/L, penicillin/streptomycin, and dialyzed fetal bovine serum (full medium B). Medium was replaced in all cultures every 36 hours. The conditions applied here to induce methionine stress are different than those employed previously (13) due to the employment of commercial culture medium and the omission of high concentrations of cobalamin and folic acid, which were shown to be nonessential for overcoming methionine stress in tumors.

MGMT Assay

The biochemical assay for MGMT activity was used to determine levels of this protein in cells cultured under control or methionine stress conditions (13).

Synergy between Methionine Stress and Temozolomide

Pancreatic tumor cell lines were cultured in medium A as described above and allowed to reach 50% confluence. The following experiments were done to determine the efficacy of temozolomide. Control: Cells were treated with 0, 50, 100, 200, 500, and 1,000 μmol/L temozolomide for 30 minutes in medium A in the absence of serum and subsequently transferred to full medium A for 96 hours. O^6-benzylguanine/temozolomide: Cells in medium A were treated with O^6-benzylguanine (30 μmol/L) for 24 hours and then treated with 0, 50, 100, 200, 500, and 1,000 μmol/L temozolomide for 30 minutes in medium A in the absence of serum and transferred to full medium A for 96 hours. Methionine stress/temozolomide: Cells were transferred to full medium B for 72 hours and then treated with 0, 50, 100, 200, 500, and 1,000 μmol/L temozolomide for 30 minutes in medium B in the absence of serum and supplemented with full medium B for another 48 hours. O^6-benzylguanine/methionine stress/temozolomide: Cells were transferred to full medium B supplemented with O^6-benzylguanine (30 μmol/L) for 24 hours, then transferred to full medium B without O^6-benzylguanine for another 48 hours, then treated with 0, 50, 100, 200, 500, and 1,000 μmol/L temozolomide for 30 minutes in medium B in the absence of serum, and subsequently supplemented with full medium B for another 48 hours. At the end of these treatments, cells were trypsinized and colony formation was evaluated using the soft agar colony-forming assay. Briefly, 10,000 cells per well were mixed with full medium A containing 0.5% agar and plated on six-well plates (three wells per condition). The

plates were then transferred to a 37°C incubator with 5% CO_2. After 12 to 14 days of incubation, colonies were scored. Colony formation for each condition was calculated in relation to values obtained for untreated controls.

Flow Cytometry

Cell cultures in methionine-efficient medium and in homocysteine medium were harvested every 24 hours washed to remove floaters, then trypsinized, washed with PBS twice, and centrifuged (800 rpm) at 4°C. The cell count was adjusted to 2×10^5 to 3×10^5 cells/mL in PBS per sample. 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 Epics XL (Fullerton, CA). EXPO32 software was used for optimum analysis of cell cycle (G_1, G_2, and S phases).

Affymetrix Chip Hybridization

RNA was isolated from CAPAN1 and CFPAC1 cultures at the earliest time that cells were fully arrested under methionine stress and were nearly in a homogeneous state as determined by flow cytometry. That state was achieved at days 4 and 6 after initiation of methionine stress for CAPAN1 and CFPAC1, respectively. RNA was analyzed as described previously (14). mRNA expression of >22,000 genes and expressed sequence tags was done for pairs of CAPAN1 and CFPAC1 tumor cells grown in methionine or homocysteine medium with the use of U133A (22,000 genes) chips from Affymetrix in duplicate. Briefly, biotin-labeled cRNA (15–20 μg) was fragmented by incubating in a buffer containing 200 mmol/L Tris acetate (pH 8.1), 500 mmol/L potassium acetate, and 150 mmol/L magnesium acetate at 95°C for 35 minutes. The fragmented cDNA was hybridized with a pre-equilibrated Affymetrix chip at 45°C for 14 to 16 hours. The hybridizations were washed in a fluidic station with nonstringent buffer (6× saline-sodium phosphate-EDTA, 0.01% Tween 20, 0.005% antifoam) for 10 cycles and stringent buffer (100 mmol/L MDS, 0.1 mol/L NaCl, 0.01% Tween 20) for four cycles and stained with streptavidin phycoerythrin. This was followed by incubation with biotinylated mouse anti-avidin antibody and restained with streptavidin phycoerythrin. The chips were scanned in an Agilent ChipScanner (Affymetrix, Inc., Santa Clara, CA) to detect hybridization signals. Baseline analyses were done using Oligoarray Suite 5.0 to identify statistically significant gene expression alterations between samples derived from cells grown in methionine and those maintained in homocysteine containing medium. Because samples were analyzed in triplicates, these results were further screened for consistent P by the Student's t tests ($P < 0.05$) to eliminate random sampling errors.

Western Blot Analysis

Cultures used in this experiment were used to extract protein at the same time intervals used for harvesting RNA (i.e., when cells were fully arrested under methionine stress; 4–6 days from the introduction of methionine stress). Cells were washed twice with cold PBS and lysed (while still attached) for 30 minutes in 100 μL ice-cold radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 8.0) and 150 mmol/L NaCl containing 0.1% SDS, 1% NP40, 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 × g for 10 minutes at 4°C. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Aliquots of cell extracts containing 50 μg 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 TBS-T: 1 mol/L Tris (pH 7.4), 5 mol/L NaCl, 0.05% Tween 20] and then in Blotto A containing the following antibodies: (*a*) the rabbit polyclonal β-tubulin (1:300), GADD45γ (1:100), GADD45α (1:500), p21 (1:200), p27 (1:200), p38 (1:500), ASM (1:200), SMAD7 (1:200), TGFBR2 (1:200), goat polyclonal SPHK2 (1:200), mouse monoclonal p53 (1:200), and SMAD4 (1:200) antibodies from Santa Cruz Biotechnology (Santa Cruz, CA); (*b*) the rabbit polyclonal P-AKT (Ser^{473}; 1:1,000) and P-AKT (Thr^{308}; 1:1,000), P-Rb (Ser^{780}; 1:1,000), P-Rb (Ser^{795}; 1:1,000), P-Rb (Ser^{807}/Ser^{811}; 1:1,000), CDC2 (1:1,000), P-CDC2 (Tyr^{15}; 1:1,000), P-CDC2 (Thr^{161}; 1:1,000), or mouse monoclonal Rb antibodies from Cell Signaling Technology (Beverly, MA); and (*c*) mouse monoclonal MDA-7 (1:500) was donated by Dr. Sunil Chada (Introgen Therapeutics, Inc., Houston, TX) and β-actin (1:1,000) was purchased from Sigma. After washing in TBS-T buffer (4 × 10 minutes, room temperature), filters were incubated for 1 hour at room temperature in Blotto A containing 1:4,000 dilution of peroxidase-conjugated anti-rabbit secondary antibody (Amersham Biosciences Corp., Piscataway, NJ) or Blotto A containing 1:4000 dilution of peroxidase-conjugated anti-mouse secondary antibody (Amersham Biosciences). After washing in TBS-T, enhanced chemiluminescence was done according to the manufacturer's recommendation. Protein expression was quantitatively analyzed via laser scanning densitometry using NIH Image version 1.61 software. All results were normalized to β-actin and β-tubulin.

Results

Methionine stress was effective in retarding growth of pancreatic tumor cells in culture. Extensive cell death was evident in CAPAN1, MiaPaCa2, and BxPc3 by the presence of detaching cells (Fig. 1A). Accumulation of large cells with multiple micronuclei and decondensed chromatin was evident in CFPAC1 as well as in CAPAN1 and CAPAN2 (data not shown). In contrast, the growth of NIH 3T3 fibroblasts was not affected by the stress (Fig. 1A and B). The cell cycle of pancreatic tumor cells was diversely affected by methionine stress (Fig. 2). Cells with a higher DNA content accumulated in cultures of CAPAN1, CAPAN2, and especially CFPAC1, whereas a G_1 arrest was shown in cultures of Panc1 and MiaPaCa2. The cell

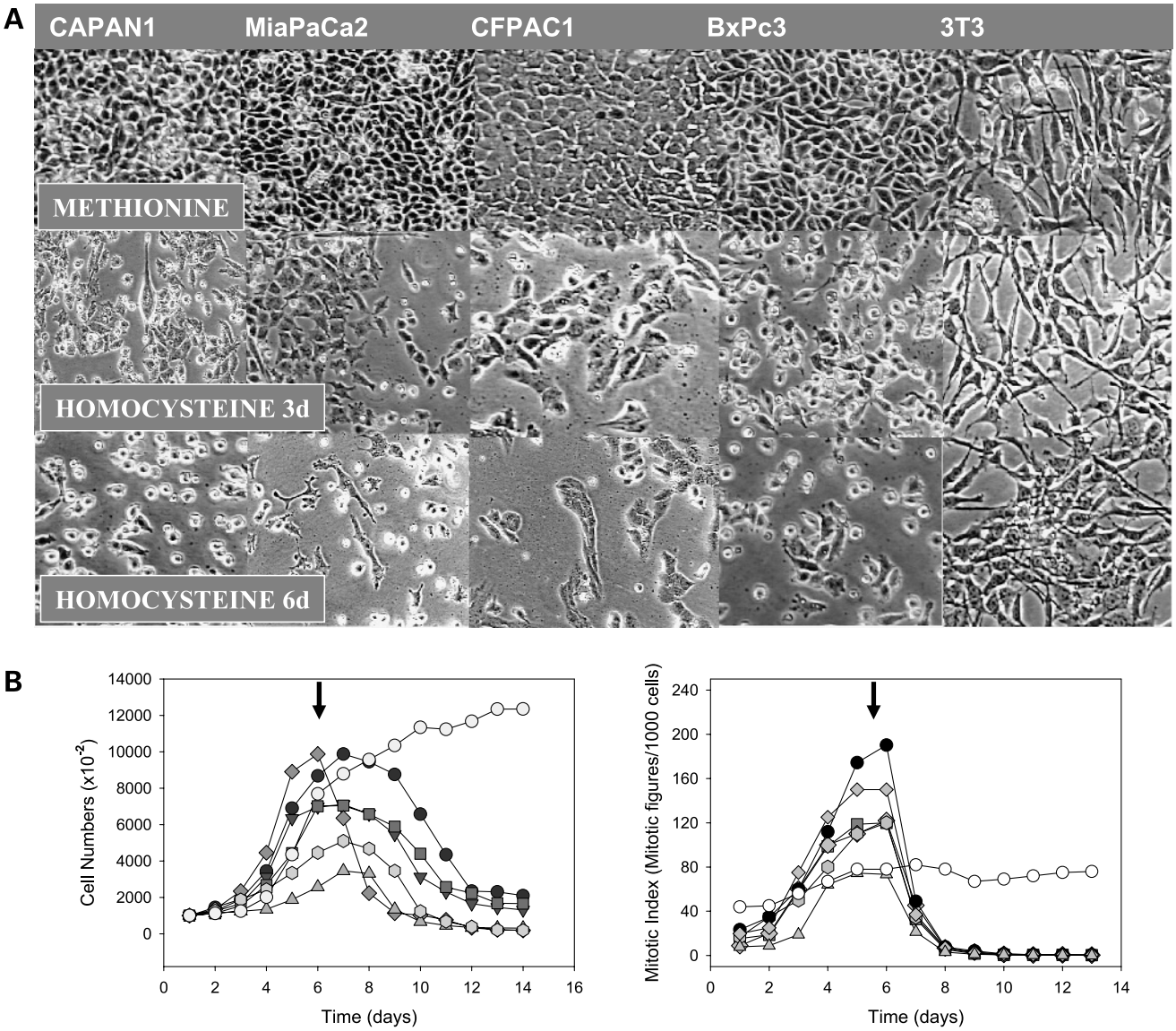

Figure 1. Effect of methionine withdrawal (methionine stress) on pancreatic tumor cell cultures. CAPAN1, CAPAN2, Panc1, MiaPaCa2, CFPAC1, and BxPc3 pancreatic adenocarcinoma tumor and NIH 3T3 (control) cells were cultured in methionine-efficient medium containing 10% dialyzed serum until they were confluent. At that time, methionine was withdrawn and replaced with homocysteine. **A,** with the exception of NIH 3T3, loss of mitotic activity is evident in all cultures following transfer from methionine to homocysteine on the third day. Cell death and detachment is prominent at days 4 and 6 in CAPAN1, MiaPaCa2, and BxPc3. At d 6, there is also accumulation of multinucleated cells in CFPAC1, which persist for several days (contrast, ×200). **B,** reduction of cell numbers and loss of mitotic activity under methionine stress conditions imposed on confluent cell cultures after 6 d in methionine-efficient medium (*arrows*) is observed in CAPAN1 (*inverted triangles*), CAPAN2 (*triangles*), Panc1 (*squares*), MiaPaCa2 (*closed circles*), CFPAC1 (*hexagons*), and BxPc3 (*diamonds*) but not in NIH 3T3 (*open circles*), the growth of which is not affected by the stress. Representative of the mean of three independent determinations. Live cell numbers were determined by trypsinization of cultures in T75 flasks and the counting of trypan blue excluding cells. Mitotic indices were determined from mitotic figures of cells span on slides.

cycle was not affected in NIH 3T3 by methionine stress. Apoptosis was not frequent because dying cells did not display fragmented nuclei, and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay did not reveal evidence of extensive apoptosis in CAPAN1 and CFPAC1 (data not shown).

Methionine stress had a profound effect on gene expression as shown by cDNA oligoarray analysis in CAPAN1 and CFPAC1 (Table 1; Supplementary Data).[1] In CAPAN1, 94 genes of known function were up-regulated and 103 down-regulated by a factor of ≥2.5 from 20,611 genes and glutathione *S*-transferases examined. In CFPAC1, the

[1] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

number of genes that were up-regulated or down-regulated by ≥2.5-fold were 81 and 109, respectively. The expression of a greater number of genes was significantly altered by methionine stress, but to a <2.5-fold, in both these tumor lines. Excluding genes with products involved in metabolism or have a structural or unknown function, the genes that were up-regulated by ≥2.5-fold in CAPAN1 in response to methionine stress were *GADD45B, CLU, CDK5, SMAD7, CSF1, BRCA1, TGFBRAP1, TFIP11, TREX1, ETS2, GDF15, MYCBP, PIN1, G0S2, IGFBP4, SPHK2, NF2, S100A3, IER3, IFI16, RAD51C, GNA11, MAT2A, CDKN2C, NEUGRIN, BAG5, TXN, BAG3*, and *BID*. Genes of similar function that were down-regulated by at least 2.5-fold in this tumor line were *FOS, NEK2, FOSB, CCNB2, LGALS1, KIF20A, KNTC2, KLF6, TOP2A, DUSP2, GAS1, CDCA8, FANCA, CDT1, DUSP1, PTPLA, DUSP6, NUSAP1, TP73L, HMGB2, MGMT, SERPINA3, TYMS, EREG, CSNK1E, CDC2L6, PRC1, IRS2, FANCL, IFITM1, ITGB4, AREG, PLK1, MKI67, TBL3, AURKB, 10-Sep, UBE2C, STC1, RHOB, APRIN, CDC2, MAD2L1, PLCB3, NAP1L1, CCNB1, MSH6, NR4A1, GRP58, CCNG1, CDC42EP3, BUB1, ERCC1, EGR1, QARS, SGK*, and *CCNA2*. The majority of the above genes were also affected in CFPAC1 in a similar manner as in CAPAN but not always to the same extent or order (Supplementary Data).[1] Excluding genes with products involved in metabolism or had a structural or unknown function, those that were up-regulated by >2.5-fold in CFPAC1 in response to methionine stress were *S100A8, PI3K, S100A3, CSF2, SOCS1, SERPINB4, SPHK2, SERPINA5, TREX1, CSF3, CXCL6, HSPA5BP1, IFIT2, SGK, TNC, FGB, BAP1, G0S2, SOD2, SERPINB3, PTX3, GBP1, PTGS2, S100A9, ICAM1, PIM2, SPINK1, BRCA1, CSPG2, IFNGR1, PIK3C2B, IGFBP4, IL8, CDC25B, MAT2A, IRF1, CSF1, BID*, and *TIEG*. Genes that were down-regulated by at least 2.5-fold in CFPAC1 were *MYT1, KNTC2, DUSP2, GAS1, TP73L, 10-Sep, STC1, EGR3, ARHGEF12, PIN1, TGFBRAP1, CLU, INSIG1, LCK, CSNK1E, NEK2, MGMT, MST1R, ISG20, MKI67, F2R, CDCA8, KIF20A, EGFR, AGTR1, GRP58, PLK1, NR4A1, IL18, FANCA, TNFSF11, AREG, CDC2, RNASEH1, INPP4B, KLF6, PRC1, APRIN, UBE2C*, and *CCNB2*. All four of the above gene groups are listed in order of descending change under methionine stress.

The effect of methionine stress on the CDK1, Rb, PI3K, p53/p21, TGF-β, MDA-7, and ceramide pathways was determined by Western blot analysis (Fig. 3). As expected, p53 was highly expressed in mutant p53 lines CAPAN1, MiaPaCa2, and Panc1 but not in CFPAC1. Methionine stress caused down-regulation of the CDK1 protein in the majority of cell lines tested (CAPAN1, CAPAN2, CFPAC1, and the CAPAN1A clone in which wt-p53 function was restored). The most profound effect on suppression of transcription was seen in p53-efficient cells. Inhibition of CDK1 function by phosphorylation of Tyr^{15} by methionine stress was marked in p53 mutant tumor lines CAPAN1, CFPAC1, MiaPaCa2, and Panc1. Loss of activity due to down-regulation of Thr^{161} phosphorylation was also evident in CAPAN1, CAPAN2, and CFPAC1. The profound effect of methionine stress on CDK1 may be mediated to some extent by wt-p53, which negatively regulates transcription of CDK1 and cyclin B1 while enhancing the CDK1 inhibitors p21, GADD45, and 14-3-3σ (17–19). The presence of wt-p53 had also an effect on the

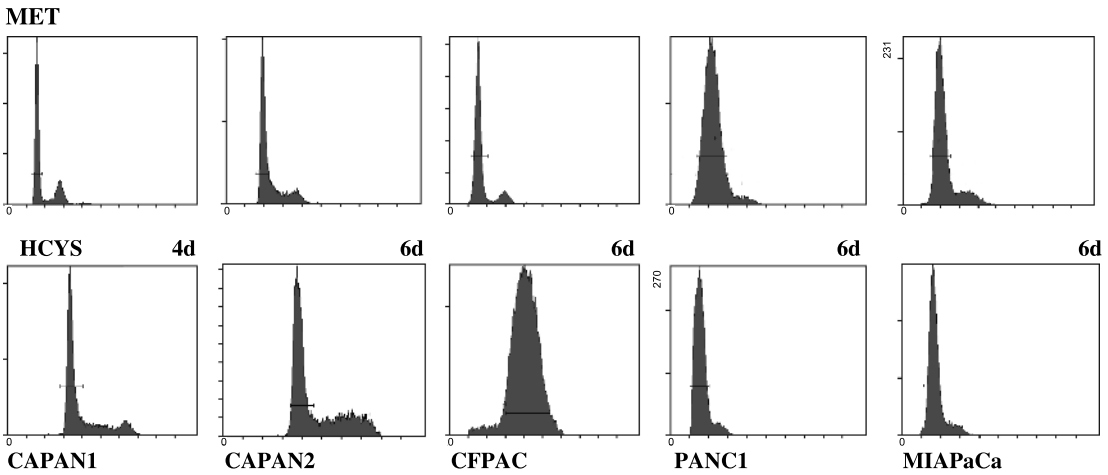

Figure 2. Effect of methionine stress on the cell cycle of pancreatic tumor cells. Two major types of response are observed. The first type is prominent in cell cultures of CAPAN1 and CAPAN2 and particularly in CFPAC1. Withdrawal of methionine results in loss of mitotic activity and accumulation of cells with two to four times the DNA content of controls. Cells are either blocked in G_2 (CAPAN1 and CAPAN2) or contain multiple micronuclei (CFPAC1, and to a lesser extent CAPAN1 and CAPAN2). A second type of response is observed in Panc1 and MiaPaCa2 in which cells are blocked in G_1 rather than in G_2. Prominent mitosis in these two lines is observed only in methionine medium. Composition of cultures based on cellular DNA content as determined by flow cytometry is represented by the mean of three determinations as follows: CAPAN1 (methionine, G_1: 81.5, S: 3.8, G_2: 9.9; homocysteine, G_2: 71.7, MNC: 11.7), CAPAN2 (methionine, G_1: 69.8, S: 7.7, G_2: 10.9; homocysteine, G_2: 44.3, MNC: 58.5), CFPAC1 (methionine, G_1: 82.1, S: 4.7, G_2: 8.8; homocysteine, G_1: 4.2, G_2: 5.5, MNC: 79.8), Panc1 (methionine, G_1: 87.7, S: 5.4, G_2: 6.51; homocysteine, G_1: 92.4, S: 1.1, G_2: 12.1), and MiaPaCa2 (methionine, G_1: 76.5, S: 7.1, G_2: 10.3; homocysteine, G_1: 89.2, S: 2.2, G_2: 8.2). *MET,* methionine; *HCYS,* homocysteine; *MNC,* multinucleated cells having a DNA content greater than that present in G_2.

Table 1. Changes in expression of selected genes in CAPAN1 and CFPAC1 at 4 and 6 days after application of methionine stress

Gene	Symbol	Chromosome	CAPAN1 methionine	CAPAN1 homocysteine	M/H	CFPAC1 methionine	CFPAC1 homocysteine	M/H
Growth arrest and DNA damage inducible, β	GADD45B	chr19p13.3	47.4	2,605.4	0.02	546.5	978.9	0.56
Clusterin	CLU	chr8p21-p12	31	610.2	0.05	NE	NE	NE
SMAD, mothers against DPP homologue 7	SMAD7	chr18q21.1	30.1	337.2	0.09	410.8	968.3	0.63
Breast cancer 1, early onset	BRCA1	chr17q21	31.6	316.5	0.11	125.5	427.1	0.29
TGF, β-receptor-associated protein 1	TGFBRAP1	chr2q12.1	72.9	648.9	0.11	NE	NE	NE
v-ets erythroblastosis virus E26 oncogene homologue 2	ETS2	chr21q22.3|21q22.2	105	807.1	0.13	615.9	414.2	1.49
Protein (peptidyl-prolyl cis/trans isomerase) 1	PIN1	chr19p13	102	683.8	0.15	NE	NE	NE
Insulin-like growth factor-binding protein 4	IGFBP4	chr17q12-q21.1	185.7	1,113.8	0.17	914.2	2,643.7	0.35
Sphingosine kinase 2	SPHK2	chr19q13.2	139.3	832.8	0.17	62	360.5	0.17
Neurofibromin 2	NF2	chr22q12.2	131.7	561.4	0.23	364.6	687.5	0.53
IFN, γ-inducible protein 16	IFI16	chr1q22	75.6	271.7	0.28	2,023.9	2,653	0.76
Guanine nucleotide-binding protein (G protein)	GNA11	chr19p13.3	75	265.1	0.28	553.9	639.5	0.87
Methionine adenosyltransferase II, α	MAT2A	chr2p11.2	3,776.7	12,193.4	0.31	1,368.7	3,525.9	0.39
Cyclin-dependent kinase inhibitor 2C (p18)	CDKN2C	chr1p32	278.4	896.5	0.31	148.2	288.2	0.51
BH3-interacting domain death agonist	BID	chr22q11.1	1,192.2	2,777.6	0.43	640.1	1,499.7	0.43
Cyclin D1	CCND1	chr11q13	792	1,404.2	0.56	1,037	1,319.3	0.79
TGF-β-inducible early growth response	TIEG	chr8q22.2	1,195.6	1,670.1	0.72	2,002.6	4,534.4	0.44
BCL2 antagonist of cell death	BAD	chr11q13.1	646.4	889.9	0.73	594.7	963.9	0.62
BCL2 antagonist/killer 1	BAK1	chr6p21.3	525.2	708.7	0.74	640.9	857.1	0.75
WEE1 homologue (Schizosaccharomyces pombe)	WEE1	chr11p15.3-p15.1	463.7	201.2	2.30	709.4	606.6	1.17
BUB1 budding uninhibited by benzimidazoles 1	BUB1	chr2q14	727.9	234	3.11	320	140.6	2.28
Cyclin B1	CCNB1	chr5q12	1,510.3	453.4	3.33	791.5	336.9	2.35
Cell division cycle 2, G_1-S and G_2-M	CDC2	chr10q21.1	417	121	3.45	616.1	196.1	3.14
Ras homologue gene family, member B	RHOB	chr2p24	2,306.3	618.9	3.73	5,804.2	4,537.3	1.28
Aurora kinase B	AURKB	chr17p13.1	824.7	209.1	3.94	296.5	178	1.67
Antigen identified by monoclonal antibody Ki-67	MKI67	chr10q25-qter	143.7	33.6	4.28	308.7	51	6.05
Amphiregulin (schwannoma-derived growth factor)	AREG	chr4q13-q21	2,295.2	518.4	4.43	2,725.8	822.8	3.31
Fanconi anemia, complementation group L	FANCL	chr2p16.1	459.7	95.8	4.80	263.3	230.4	1.14
Protein regulator of cytokinesis 1	PRC1	chr15q26.1	615.6	118.7	5.19	762	252.1	3.023
Cell division cycle 2-like 6 (CDK8-like)	CDC2L6	chr6q21	578.7	110.8	5.22	284	255.7	1.11
Thymidylate synthetase	TYMS	chr18p11.32	2,251.8	404.7	5.56	360.2	231.7	1.55
O^6-methylguanine-DNA methyltransferase	MGMT	chr10q26	336.2	57.8	5.82	404.8	43.3	9.34
Nucleolar and spindle-associated protein 1	NUSAP1	chr15q15.1	726.2	104.9	6.92	523.5	303	1.73
Cell division cycle associated 8	CDCA8	chr1p34.3	513.5	55.1	9.32	342.8	61.3	5.59
Topoisomerase (DNA) IIα 170 kDa	TOP2A	chr17q21-q22	1,280.4	92.3	13.87	1,457	748.1	1.95
Cyclin B2	CCNB2	chr15q22.2	1,235.3	30.1	41.04	759.6	304.8	2.49
Colony-stimulating factor 2 (granulocyte-macrophage)	CSF2	chr5q31.1	NE	NE	NE	53.5	727.1	0.07
Interleukin-6 (IFN, β2)	IL6	chr7p21	NE	NE	NE	1,819.7	3,530.2	0.52
Angiotensin II receptor, type 1	AGTR1	chr3q21-q25	NE	NE	NE	2,314.6	583	3.97

NOTE: All changes listed are significant ($P < 0.05$). NE, not expressed: relative expression of <200 in both methionine and homocysteine sets.

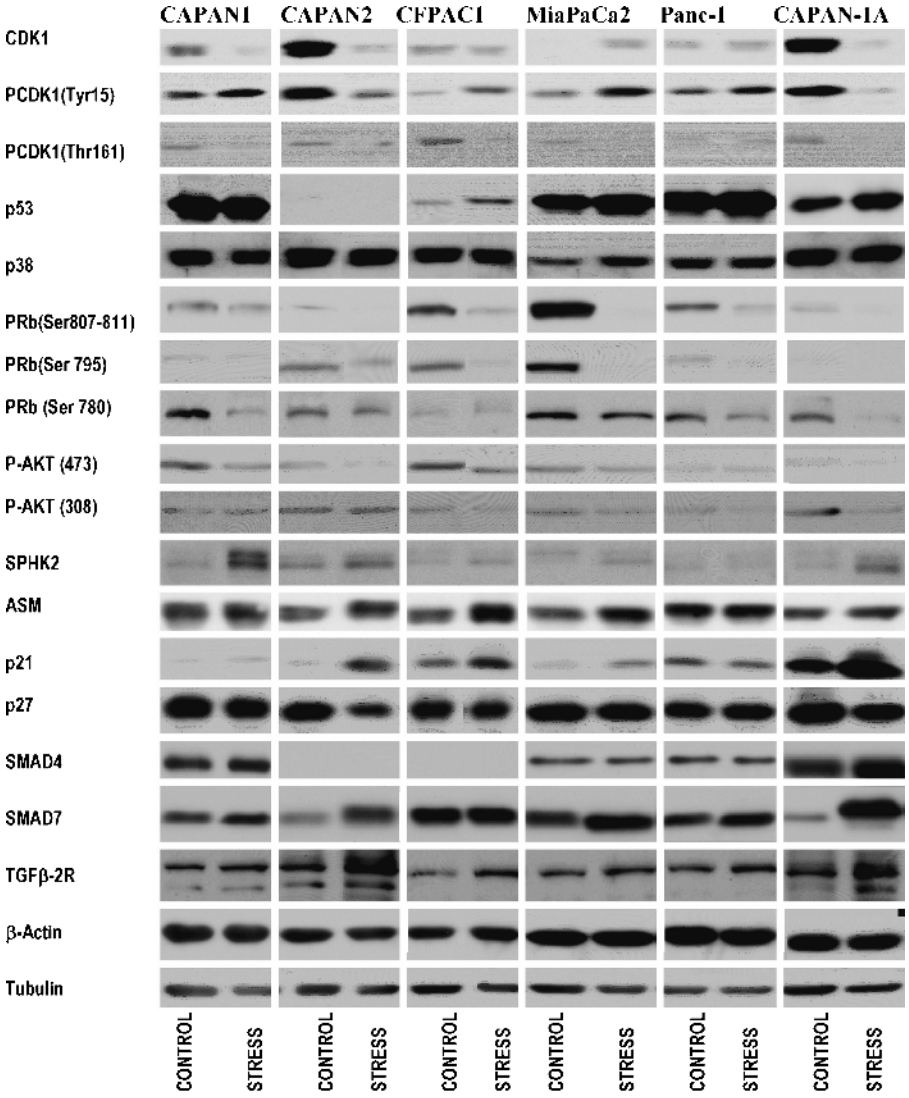

Figure 3. Effect of methionine stress on CDK1, Rb, PI3K, p53/p21, and ceramide signal transduction pathways as determined by Western blot analysis of pancreatic tumor cell lines under methionine stress. Methionine stress causes down-regulation of the CDK1 signal in the majority of cell lines tested (CAPAN1, CAPAN2, CFPAC1, and the CAPAN1 clone in which wt p53 function was restored). A most prominent effect is seen in wt-p53 cells. Inhibition of CDK1 function by phosphorylation of Tyr^{15} under methionine stress conditions is clearly shown in p53 mutants CAPAN1, CFPAC1, MiaPaCa2, and PANC1. Loss of activity due to dephosphorylation of Thr^{161} (possibly by WEE1) is evident in CAPAN1, CAPAN2, and CFPAC1. Methionine stress causes depletion of P-Rb forms P-Ser^{795}, P-Ser^{780}, and P-Ser^{807}/Ser^{811} in CAPAN2, CFPAC1, MiaPaCa2, and PANC1. At the same time, up-regulation of p21 protein expression occurs in all cell lines, except PANC1 in which CDK1 was also unaffected. The p27 was down-regulated in CAPAN2 but it did not respond to methionine stress in the rest of pancreatic tumor lines. Potential up-regulation of the TGF-β pathway is indicated by increases in the expression of the TGF-β2 receptor in all pancreatic tumor cell lines. However, the lack of an effect on SMAD4 and the increase of SMAD7 in p53-efficient CAPAN2 and CAPAN1A may indicate the loss of TIEG function in these two lines. SMAD7 overexpression correlated with failure of CDK1 dephosphorylation and CDK1 inactivation in MiaPaCa2 and PANC and with the up-regulation of p27 in most tumor lines. Phosphorylation of AKT is down-regulated in all cell lines, except PANC1 in which it is already low. This indicates a negative effect of methionine stress on PI3K activity. AMS is up-regulated by methionine stress and shows excessive levels in dying cells (data not shown).

inducibility of TGF-β2 receptor, p21, and SMAD7 by methionine stress. The up-regulation of two proteins with opposite functions, such as p21, which inhibits cell cycle progression, and SMAD7, which inhibits TGF-β signaling, by methionine stress suggests that either SMAD7 does not inhibit all TGF-β-mediated events, such as the induction of p21, for example, or p21 is strictly under the control of p53 in pancreatic tumors. The latter is not supported by the induction of p21 in tumors with mutated p53, which implies that TGF-β signaling to induce p21 may be up-regulated by methionine stress in spite of the high expression of SMAD7.

Notable was the effect of methionine stress on the depletion of P-Rb forms P-Ser^{795}, P-Ser^{780}, and P-Ser^{807}/Ser^{811} in CAPAN2, CFPAC1, MiaPaCa2, and Panc1. Unlike p21, p27 did not respond to methionine stress in pancreatic tumor lines. Potential up-regulation of the TGF-β pathway was indicated by increases in the expression of the TGF-β2 receptor. However, the lack of an effect on SMAD4 and the increase of SMAD7 in p53-efficient CAPAN2 and CAPAN1A may indicate a minor role of TGF-β compared with p53. Phosphorylation of AKT was down-regulated in all cell lines, except Panc1 in which it was already low. This indicates a negative effect of methionine stress on PI3K activity. AMS was up-regulated by methionine stress and showed excessive levels in dying cells. SPHK2 was markedly up-regulated in CAPAN1, CAPAN2, CAPAN1A, and to some extent CFPAC1 and MiaPaCa2.

MGMT levels have been reported previously to be elevated in advanced adenocarcinoma of the pancreas as contrasted to normal pancreatic tissue (20). Human pancreatic cell lines retain high levels of MGMT activity in culture. Thus, in six pancreatic tumor cell lines tested, MGMT levels ranged from 350 to 1,500 (Table 2). Such levels prevented the accumulation of O^6-methylguanine adducts in pancreatic tumors in response to temozolomide treatment (9). Although temozolomide at its maximum tolerated dose in athymic mice (340 mg/m^2) reduced MGMT activity in all pancreatic tumor xenografts tested,

Table 2. Effect of methionine stress on MGMT levels in pancreatic tumor cell lines

Tumor line	MGMT (fmol/mg protein)	
	Control	Under methionine stress*
BxPC3	375 ± 22	30 ± 4
PANC1	692 ± 37	32 ± 3
MiaPaCa2	880 ± 45	29 ± 4
CFPAC1	943 ± 39	38 ± 8
CAPAN1	1,123 ± 55	34 ± 6
CAPAN2	1,456 ± 89	33 ± 9

*Grown in homocysteine medium for 6 days. Average of six determinations ± SD.

enough activity remained to ensure repair of O^6-methylguanine adducts (9). Although MGMT can be reduced following treatment with available MGMT inactivators, such as O^6-benzylguanine, its levels remained low for only 24 hours after treatment and they were fully restored within 72 hours (Table 3). In contrast, methionine stress reduced MGMT levels in all pancreatic tumor lines to <40 fmol/mg protein, and such levels remained low as long as methionine was deprived. Because methionine stress resulted in marked reduction of MGMT mRNA expression (Table 1), the restoration of activity following repletion of methionine required a longer period than the recovery from O^6-benzylguanine treatment. The effect of methionine stress versus that of O^6-benzylguanine on enhancing the efficacy of temozolomide against two resistant, p53-mutated tumors is shown in Fig. 4. Temozolomide by itself is ineffective in suppressing growth of either CAPAN1 or CFPAC1 (Fig. 4). However, in the presence of O^6-benzylguanine, it inhibits growth with an ED_{50} of 365 and 456 μmol/L for CFPAC1 and CAPAN1, respectively. Methionine stress alone increases the sensitivity to temozolomide, yielding ED_{50}s of 105 and 110 μmol/L for CFAPAC1 and CAPAN1, respectively. Pretreatment of cells with O^6-benzylguanine during the first 24 hours of methionine stress treatment increases the effectiveness of methionine stress on the cytotoxicity of temozolomide and further reduces the ED_{50}s to 43 and 48 μmol/L for CFPAC1 and CAPAN1.

Table 3. Comparison of MGMT recovery kinetics following O^6-benzylguanine treatment or recovery from methionine stress in CAPAN1 tumor cells

Time (h)	O^6-benzylguanine–treated MGMT (fmol/mg protein)	Methionine stress treated
0	1,123 ± 55*	34 ± 13†
12	9 ± 1	34 ± 12
24	64 ± 15	29 ± 22
36	464 ± 65	35 ± 29
72	1,234 ± 91	878 ± 113

*CAPAN1 cells grown in methionine medium and treated with 30 μmol/L O^6-benzylguanine at time 0 for 6 hours, then washed thrice, and harvested at the time intervals indicated.

†CAPAN1 cells under methionine stress for 6 days, then transferred into methionine medium at time 0, and harvested at the time intervals indicated. Average of six determinations ± SD.

Discussion

All pancreatic tumors tested here are methionine dependent (respond to methionine stress) under conditions that have no effect on NIH 3T3 fibroblasts. Response to methionine stress includes inhibition of cell cycle progression and

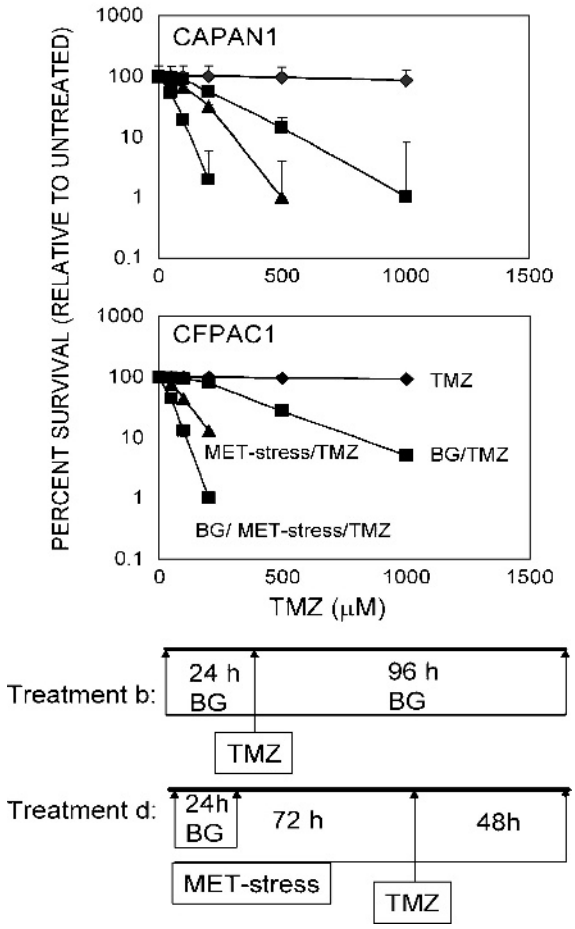

Figure 4. Enhancement of the efficacy of temozolomide (*TMZ*) against pancreatic tumor cell lines with methionine stress (*MET-stress*). CFPAC1 and CAPAN1 were treated with (*a*) single dose of temozolomide for 30 min at the concentrations shown; (*b*) continuous O^6-benzylguanine (*BG*) treatment starting 24 h before a single 30-min temozolomide treatment at the concentrations shown and for 96 h after temozolomide; (*c*) methionine stress for 72 h followed with a single temozolomide treatment for 30 min at the concentrations shown and further methionine stress for an additional 48 h; or (*d*) methionine stress plus O^6-benzylguanine (30 μmol/L) for 24 h followed by methionine stress alone for an additional 48 h and temozolomide for 30 min at the concentrations shown and then kept for 48 h under methionine stress. At the end of these treatments, ability to grow was tested using a clonogenic assay (see Materials and Methods). Colonies of ≥50 cells were counted and expressed as percent of control (no temozolomide). *Points,* mean of three determinations; *bars,* SD.

mitosis and increased cellular death. In CFPAC1, CAPAN1, and CAPAN2, inhibition of mitosis and mitotic death is manifested by the accumulation of cells with multiple nuclei having decondensed chromatin (21). In contrast, MiaPaCa2 and Panc1 are blocked in G_1, and on exit, they may undergo asymmetrical division (mitotic slippage) yielding two distinct G_1 populations with different DNA content and viability depending on the loss or gain of chromosomes. Mitotic catastrophe or failure to complete a successful mitosis is an important mode of death in pancreatic tumor cell lines and could proceed by mechanisms that are fundamentally different than apoptosis (21). A common event in response to methionine stress in all pancreatic lines tested that is likely to be associated with inhibition of mitosis is the loss of CDK1 by down-regulation of gene expression and/or and the inhibition of its activity via phosphorylation at Tyr^{15} and/or dephosphorylation at Thr^{161}. Consistent with the accumulation of multinucleated CFPAC1 cells under methionine stress is the marked down-regulation in gene expression of spindle components CDCA8, FLJ12649, FLJ90806, PRC1, and KIF4A (22, 23). Down-regulation of BUB1, which directly phosphorylates CDC20, thus inhibiting APC/C, is also in agreement with mitotic anomalies (23). Similarly, depletion of NuSAP and especially Aurora B2 kinase under methionine stress could be associated with multinuclearity and increased ploidy in CAPAN1 and CFPAC1 in a manner similar to that shown in other tumor lines (24, 25).

A common response to methionine stress in pancreatic as well as other tumor cell lines tested previously (15) is the loss of Rb phosphorylation. This observation is highly significant for pancreatic neoplasms in which Rb is rarely mutated and thus maintain its ability to control cell cycle progression (26, 27). Loss of Ser^{780} phosphorylation occurs in CAPAN1 and Panc1. Overall, hyperphosphorylated species are lost to the greatest extent (MiaPaCa2 and Panc1) and this may be related to a stricter block in G_1-S transition in these lines. The mechanism of G_1-S arrest by methionine stress is not quite understood but may involve up-regulation of NF2 (28). A 5-fold increase in NF2 expression (CAPAN1) could be linked with inhibition of cell proliferation and G_1 arrest. NF2 down-regulates expression of cyclin D1, inhibits CDK4 activity, and dephosphorylates pRb via its product known as merlin. However, due to the absence of an effect on cyclin D1 by methionine stress in pancreatic tumor lines, the antiproliferative role of NF2 may be constricted to the dephosphorylation of Rb possibly via the inhibition of CDK4 by p18 (CDKN2C). Other possible mechanisms controlling G_1-S cell cycle arrest also include overexpression of ETS2 and the associated down-regulation of c-MYC, two events that trigger the up-regulation of p27, p21, p15, and GADD45α, GADD45β, and/or GADD45γ (29). In relation to cell cycle progression, we have confirmed coexpression of genes involved in such progression (30), including CDCA1 to CDCA8, CDC2, CDC7, CDC23, MCAK, MKI67, and TOP2A, in pancreatic tumor cell lines and we have further shown that the expression of most of these genes is affected negatively by methionine stress at least in CAPAN1 and CFPAC1. Of significant importance in cell cycle inhibition by methionine stress is the up-regulation of n-clusterin by 20- and 4-fold in CAPAN1 and CFPAC1, respectively. Overexpression of n-clusterin is in accordance with a G_2-M cell cycle checkpoint (31, 32).

With the exception of Panc1, methionine stress causes induction of p21, which is more profound in the p53-efficient lines CAPAN2 and CAPAN1A. However, the cause of p21 up-regulation in these two lines could also be attributed to induction of the TGF-β signaling pathway consistent with a marked increase of the TGF-β2 receptor. Increased p21 expression in CFPAC1 and MiaPaCa2 is probably associated to the up-regulation of TGF-β because both these lines express mutant DNA and do not up-regulate p21 in response to DNA damage. Unlike p21, p27 is highly expressed in pancreatic tumor cell lines and is not affected by methionine stress. Theoretically, TGF-β1 up-regulates p27 levels while suppressing cyclin A and B and inhibiting CDK2 and CDK1. Although methionine stress suppresses cyclin A and B and inhibits CDK1, it has no effect on p27 levels, indicating a possible interference by SMAD7, which blocks p27 expression (33). The absence of an effect of methionine stress on SMAD4 and the increase in inhibitory SMADs (SMAD7) was unexpected and occurred despite the up-regulation of TGF-β2 receptor protein as well as TIEG and cyclin D1 mRNA levels. SMAD7, an inhibitor of TGF-β1 signaling, binds to TGF-β1 receptor and prevents the association, phosphorylation, and activation of SMAD2 (34). Although SMAD7 blocks TGF-β1-mediated growth inhibition in a variety of tumor cell lines, it also up-regulates the expression of SMAD7 (34–37). SMAD7 has been shown to interfere with the ability of TGF-β1 to maintain Rb in a hypophosphorylated state (33). However, SMAD7 does not alter TGF-β-mediated up-regulation of p21, indicating that it does not interfere with this CDK inhibitor. These observations reconcile the up-regulation of both SMAD7 and p21 by methionine stress in both p53-efficient and mutated cells.

Pancreatic tumors are not responsive to apoptosis and this is probably one of the causes for their resistance to chemotherapy. High BCL2 and MCL1 expression in pancreatic tumors prevents the effectiveness of BAK, BAX, and BCL-x_L. Under methionine stress, the expression of BCL-2 antagonists BID, BAD, BAK, and BAG is modestly stimulated and this may tilt the balance toward apoptosis. Nevertheless, inhibition of apoptosis by high BCL-2 levels is in agreement with accumulation of giant, multinucleated cells, which may die by nonapoptotic mechanisms (38). A possible mechanism by which methionine stress may increase apoptotic potential in pancreatic tumors is the overexpression of IFI16. IFI16 inhibits cell proliferation and enhances cell death in association with BRCA1 (39), whereas its absence inhibits p53-mediated transcription, down-regulates $p21^{WAF1}$ expression, and extends the proliferation potential (40). Another candidate for the effect of methionine stress on apoptosis based on cDNA microarray analysis and Western blot verification is the up-regulation of SPHK2 and ASM. Ceramide generation has been shown

to regulate apoptosis by stress stimuli, such as irradiation, Fas, lipopolysaccharide, and integrin signaling; therefore, the up-regulation of ASM in CAPAN2, CFPAC1, and MiaPaCa2 could be related to apoptosis. Loss of cyclin E2 and down-regulation of CDK2 (2.5 times) are two major factors that implicate the inhibitory effect on cell cycle progression by SPHK2 in CAPAN1 (41, 42). Here, we postulate that apoptosis is initiated by methionine stress by various mechanisms, but it is interrupted by BCL-2 overexpression.

Growth of pancreatic tumors is highly enhanced by hormones and growth factors that are secreted by the tumor cells as it has been shown by unusual cell proliferation in confluent cultures of pancreatic tumor cells. The cytostatic effect of methionine stress may be related to marked down-regulation of EGF, IGF, and AGT receptors and VEGF, M-CSF, and IGF-2 and the up-regulation of *GNA11*, a gene involved in signaling of gonadotropin-releasing hormone receptor, which negatively regulates cell growth (43). The growth of some pancreatic tumors may also be stimulated by IL6, the synthesis of which is regulated by *AREG* (44). In support of IL6 involvement in cell proliferation, *ERB-B* (a gene directly regulated by IL6) is suppressed by 2- and 10-fold in CFPAC1 and CAPAN1, respectively.

In spite of the effectiveness of methionine stress to reverse the tumor phenotype in pancreatic cell lines, its most likely anticancer application will be in combination with chemotherapeutic agents and/or radiation. Gene expression analysis indicates that methionine stress may challenge the resistance of pancreatic tumors to 5-fluorouracil, one of the most common drugs currently used for the treatment of such tumors with modest success. Enhancement of the efficacy of 5-fluorouracil against pancreatic tumors by methionine stress is supported by the observed down-regulation of TYMS, DTYMK, and UP, which are involved in 5-fluorouracil metabolism, and FEN1, FANCG, and RAD23B, which are involved in the repair of 5-fluorouracil-induced DNA damage (45). Enhancement of the efficacy to radiation by methionine stress is supported by the observed down-regulation of RhoB expression, the farnesylated form of which inhibits radiation-induced mitotic cell death (46). Synergy between methionine stress and alkylating agents has been shown in the past (14) but has not been fully investigated. A major contributor to such synergy is the marked and prolonged down-regulation of MGMT mRNA (13), which is accompanied by reduction of the protein to levels that have been shown to be below those capable in interfering with the toxicity of either 1,3-bis(2-chloroethyl)-1-nitrosourea or temozolomide (47). Furthermore, methionine stress seems to induce a p53-like response in p53 mutated (dysfunctional) tumors by up-regulating p21 expression and perhaps by recruiting p73. A possible induction of p73-mediated cell cycle arrest and apoptosis by methionine stress is indicated by a 7-fold up-regulation of PIN1 and cABL in CAPAN. PIN1 potentiates p73-mediated apoptosis on oncotoxic drug treatment (48). PIN also alters p73 conformation, which affects p300-mediated stabilization (2- to 4-fold up-regulation) and acetylation of p73, thereby directing it toward activation of apoptotic genes (49). cABL is activated by ATM on genotoxic insult and promotes p73 phosphorylation by p38. Methionine stress enhances the efficacy of temozolomide in a manner markedly more effective than O^6-benzylguanine. Further enhancement of the efficacy of temozolomide by combining methionine stress and O^6-benzylguanine for the first 24 hours of treatment shows that 72-hour methionine stress does not quench MGMT levels to the same extent as O^6-benzylguanine. However, administration of temozolomide 72 hours after initiation of methionine stress is more effective in the killing of CAPAN1 and CFPAC1 in particular compared with treatment at 148 hours after methionine stress where MGMT protein reaches its lowest level. The above observations show that the observed synergy between methionine stress and temozolomide is not based solely on the down-regulation of MGMT. The observation that the efficacy of the methionine stress plus temozolomide to kill pancreatic tumor cells is enhanced by a single bolus treatment with O^6-benzylguanine 72 hours before treatment with temozolomide supports combining of O^6-benzylguanine and methionine stress to deplete residual MGMT levels before temozolomide administration. In the proposed treatment schedule, O^6-benzylguanine eliminates MGMT activity below 10 fmol/mg protein, whereas methionine stress ensures that new protein is not synthesized and MGMT remains low at the time of temozolomide treatment. The combination of O^6-benzylguanine with methionine stress 72 hours before temozolomide provides the most effective method known thus far in enhancing the efficacy of temozolomide and for sensitizing highly resistant pancreatic tumors to alkylating agents.

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Demetrius M. Kokkinakis, XiaoYan Liu and Russell D. Neuner

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