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Methionine restriction selectively targets thymidylate synthase in prostate cancer cells

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Abstract

Tumor cells are more sensitive to methionine restriction than normal tissues, a phenomenon known as methionine auxotrophy. Previous studies showed that 5-fluorouracil and methionine restriction act synergistically against a variety of tumors. The purpose of the current studies was to determine the molecular mechanism(s) underlying this synergy. 5-Fluorouracil is known to inhibit thymidylate synthase (TS), a key enzyme that transfers a methyl group from 5,10-methylene-tetrahydrofolate to dUMP during nucleotide biosynthesis. We found that methionine restriction reduced 5,10-methylene-tetrahydrofolate levels by 75% and selectively inhibited TS activity in PC-3 human prostate cancer cells within 24 hr, whereas it did not in normal prostate epithelial cells. The observed fall in TS activity was accompanied by a commensurate reduction in TS protein levels as determined by western blot analysis. In contrast, 5-fluorouracil inhibited TS activity by >90% but increased TS protein levels. This increase was abrogated by methionine restriction. Surprisingly, methionine restriction increased ^{3}H-leucine incorporation in PC-3 cells over the first 24 hr, suggesting that reduction of TS levels was not simply due to global protein synthesis inhibition. Methionine restriction also significantly reduced the ratio of dUMP to dTTP in PC-3 cells, creating an imbalanced nucleotide pool. These results suggest that synergy between methionine restriction and 5-fluorouracil is attributable to multiple factors, including depletion of reduced folates, selective inhibition of TS, and creation of an imbalanced nucleotide pool. Dietary and/or enzymatic methionine restriction combined with 5-fluoruracil has great promise as a novel treatment for advanced cancer.

Keywords: Methionine; Folate; Thymidylate synthase; Prostate neoplasms

1. Introduction

Methionine is an essential amino acid that cannot be synthesized from any of the other standard amino acids. Nonetheless, normal mammalian cells proliferate normally in the absence of methionine as long as homocysteine is present in the growth medium [1], and animals fed diets in which methionine has been replaced by homocysteine suffer no ill effects and grow normally [2,3]. Homocysteine is a nonstandard amino acid that has the same structure as methionine except that it lacks the methyl group (Fig. 1). Methionine independence of normal tissues is due to remethylation of homocysteine to methionine by the enzymes 5-methyltetrahydrofolate homocysteine methyltransferase and betaine-homocysteine methyltransferase. Although these enzymes are functional in some tumors [4], most tumors are dependent upon exogenous, preformed methionine and therefore fail to grow even in the presence of homocysteine [5–8]. Dietary methionine restriction causes regression of animal tumors, including human prostate cancer xenografts in nude mice [9,10] and inhibits metastasis in animal models [3,11]. Methioninase, an enzyme that degrades methionine and homocysteine, also inhibits growth of solid tumors and leukemia in animals [12–17]. One clinical trial of chemotherapy combined with short-term methionine restriction by total parenteral nutrition showed preliminary evidence of activity against gastric cancer [18]. In addition, a recent clinical trial showed that dietary methionine restriction is safe and feasible in adults with metastatic cancer, and results in significant reduction of plasma methionine levels [19].

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Abbreviations: TS, thymidylate synthase; FBS, fetal bovine serum.

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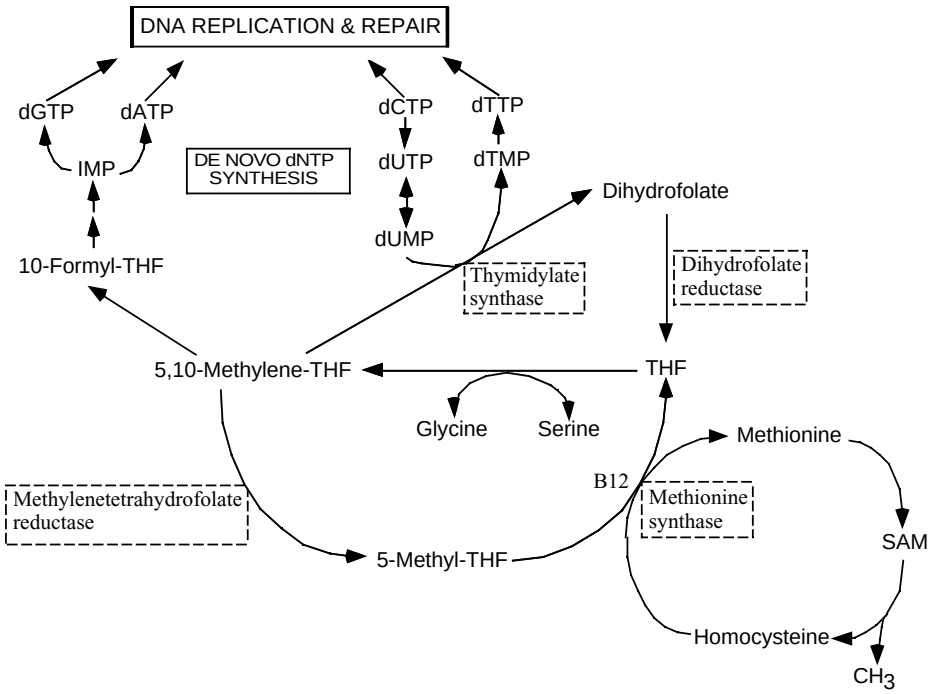

Fig. 1. Schematic overview of folate-derived one carbon (methyl) metabolism in nucleotide and methionine biosynthesis.

The selective antitumor activity of methionine restriction is not due to an *absolute* difference between benign and malignant tissues, since neither can survive for long in the *complete* absence of methionine. Rather, tumors are *relatively* more sensitive to methionine restriction than normal tissues are, just as many tumors are relatively more sensitive to chemotherapy and radiation therapy. In contrast, restriction of other essential amino acids is either very toxic or ineffective [20]. Methionine restriction therefore does not represent indiscriminate ''starvation''.

The molecular mechanisms underlying methionine dependence of cancer cells have not been fully elucidated, but they probably relate to one or more of the specialized functions of methionine that distinguish it from other amino acids. Methionine is the immediate precursor of *S*-adenosylmethionine (SAM), the major methyl donor for methylation of DNA, RNA, and other molecules (Fig. 1). Other investigators have suggested that methionine dependence of tumors is due to elevated rates of transmethylation in cancer cells compared to corresponding normal tissues [21,22].

Folate serves as a bridging molecule for nucleotide and methionine biosyntheses, since folate-derived methyl groups are required for biosynthesis of methionine and nucleotides. In normal tissues, such as liver and kidney [23], methionine restriction and concomitant reduction of SAM diverts methyl groups from 5-methyl-tetrahydrofolate to methionine biosynthesis, thereby leading to irreversible conversion of 5,10-methylene-tetrahydrofolate to 5-methyl-tetrahydrofolate (Fig. 1) [24]. This process is referred to as the ''methyl folate trap'' [25]. This diversion of folate to methionine synthesis reduces normal *de novo* nucleotide biosynthesis, since folate-derived methyl groups are essential for both purine and pyrimidine synthesis. Thymidylate synthase (TS; EC 2.1.1.45) is an S-phase enzyme that catalyzes the reductive methylation of dUMP by 5,10-methylene-tetrahydrofolate, generating dTMP and dihydrofolate [26]. Folate deficiency causes accumulation of dUMP, which incorporates into DNA instead of thymine [27–29]. This leads to excessive uracil incorporation into DNA, which causes point mutations, single- and double-stranded DNA breaks, micronucleus formation, and DNA hypomethylation.

TS is an attractive target for anticancer drug design in light of its critical role in dTMP synthesis. Inhibitors of TS, such as the fluoropyrimidines 5-FU and 5′-fluoro-2′-deoxyuridine (FdUrd) and the antifolate methotrexate are widely used as cancer chemotherapy agents [30,31]. Newer antifolates, such as nolatrexed (AG337, Thymitaq), pemetrexed (LY231514), and raltitrexed (Tomudex), are under development [31]. In growing cells, fluoropyrimidines (e.g. 5-FU and FdUrd) are metabolized to 5′-fluoro-2′-deoxyuridylic acid (FdUMP), which inhibits TS via formation of a covalent complex containing the nucleotide analog, 5,10-methylene-tetrahydrofolate, and TS [32]. This ternary complex is quite stable, resulting in prolonged inhibition of the enzyme and depletion of dTMP pools. The resulting imbalanced nucleotide supply compromises DNA replication, resulting in DNA strand breaks and apoptotic cell death, which in this context is referred to as ''thymineless death'' [33,34].

Clinical efficacy of 5-fluorouracil is enhanced by pretreatment of patients with folinic acid [35,36]. Folinic acid enhances the inhibitory effect of 5-fluorouracil on TS by increasing levels of 5,10-methylene-tetrahydrofolate,

thereby stabilizing the complex between chemotherapy drug and enzyme. The multi-step process of converting folinic acid to 5,10-methylene-tetrahydrofolate is coupled to methylation of homocysteine to form methionine. The efficacy of folinic acid therefore depends upon *de novo* methionine synthesis. Methionine deprivation would therefore be expected to accelerate conversion of folinic acid to 5,10-methylene-tetrahydrofolate by increasing conversion of homocysteine to methionine [23,37–39].

Based on the above considerations, Machover *et al.* [37] and Mini *et al.* [40] hypothesized that methionine depletion would increase the rate at which folinic acid is converted to 5,10-methylene-tetrahydrofolate in leukemia cells in culture, thereby enhancing the efficacy of folinic acid and 5-fluorouracil. In studies by Machover *et al.*, methionine depletion was accomplished with recombinant methioninase, an enzyme that cleaves methionine. Counter to their hypothesis, however, 5,10-methylene-tetrahydrofolate and tetrahydrofolate levels did not increase in those studies [37]. Nonetheless, methionine depletion did enhance the efficacy of folinic acid combined with 5-fluorouracil, which was attributed to decreased TS activity rather than the expected increase in 5,10-methylene-tetrahydrofolate [37].

Multiple cell culture and animal studies, including those by Machover *et al.* [37] also showed synergy between dietary and/or enzymatic methionine restriction combined with 5-fluorouracil even in the *absence* of folinic acid [16,41,42]. However, the mechanisms underlying this observed synergy remain unclear. Based on the "methyl *folate* trap" argument outlined above, one would expect 5,10-methylene-tetrahydrofolate levels to become *depleted* rather than enhanced by methionine restriction in the *absence* of a source of exogenous folate, namely folinic acid. Consistent with that possibility, Machover *et al.* [37] found that the combined level of 5,10-methylene-tetrahydrofolate plus tetrahydrofolate fell by approximately 50% in leukemia cells in culture in response to partial methionine depletion. The observed reduction was attributable to a 60–70% reduction of tetrahydrofolate, since 5,10-methylene-tetrahydrofolate levels appeared to be unaffected.

We undertook the current studies to test the hypothesis that deprivation of methionine in the absence of folinic acid does in fact reduce intracellular folate levels in methionine-dependent prostate cancer cells, resulting in an imbalanced nucleotide pool. We also determined whether methionine restriction affected levels or enzymatic activity of TS. We found that methionine restriction affected multiple aspects of folate and nucleotide metabolism.

2. Materials and methods

2.1. Cell culture

Human prostate cancer PC-3 cells (American Type Culture Collection) were maintained in RPMI-1640 (Life Technologies, Inc.) supplemented with 10% FBS (HyClone Laboratories) at 37° in 5% CO_2. Methionine restriction experiments were performed in methionine-free RPMI-1640 (Life Technologies, Inc.) supplemented with 10% FBS and 100 μM homocysteine (Sigma Chemical Co.). Folate restriction experiments were performed in folate-free RPMI-1640 medium (Life Technologies, Inc.) supplemented with 10% FBS. Primary culture prostate epithelial cells (PrEC) were purchased from BioWhittaker, Inc. and Clonetics Products. PrEC was maintained in the prostate epithelial basal medium and methionine restriction was performed in prostate epithelial cell labeling medium without methionine supplemented with 100 μM homocysteine, which were obtained from the company.

2.2. Reagents

Antibody for TS was obtained from Lab Vision Corporation. 5,10-Methylene-tetrahydrofolate was purchased from Schircks Laboratories. [5-^{3}H]-dUMP was from Amersham (Amersham Co.).

2.3. Western blot analysis

Aliquots of samples with 50 μg of protein, determined by the Bradford assay (BioRad), were mixed with loading buffer (final concentrations of 62.5 mM Tris–HCl (pH 6.8), 2.3% SDS, 100 mM dithiothreitol, and 0.005% bromophenol blue), boiled, fractionated in a 10% SDS–PAGE, and transferred onto a 0.45-μm nitrocellulose membrane by electroblotting (BioRad). The membranes were blocked with 2% fat-free milk in PBS, and then probed with first antibody (0.05 μg/mL IgG) in PBS containing 0.1% Tween 20 (PBST) and 1% fat-free milk. The membranes were then washed four times in PBST and incubated with horseradish peroxidase-conjugated $F(ab')_2$ of secondary antibody (BioRad) in PBST containing 1% fat-free milk. After washing four times in PBST, the membranes were visualized using the ECL western blotting detection system.

2.4. Thymidylate synthase assay

TS assay was performed as previously described [43]. Briefly, 25 μL of cell extract containing 50 μg protein, 5 μL 6.5 mM 5,10-methylene-tetrahydrofolate, and 10 μL of Tris–HCl buffer were combined at room temperature. The assay was initiated by addition of 10 μL [5-^{3}H]-dUMP 1 uM (1.0 mCi/mL, Amersham Pharmacia Biotech), incubated for 30 min at 37°, and stopped by addition of 50 μL ice-cold 35% trichloroacetic acid and 250 μL of 10% neutral activated charcoal. After centrifugation, 150 μL of the supernatant were counted by liquid scintillation. TS activity was proportional to the amount of tritium released from [5-^{3}H]-dUMP into solvent upon dTMP formation.

2.5. Primer design and synthesis for TS quantitative real-time PCR

Oligonucleotide primers for TS were designed using Molecular Beacon program (PREMIER Biosoft International). Primers were sense: 5′-GCAGATCCAACACATC-CTC-3′; and antisense: 5′-AAACACCCTTCCAGAACAC-3′. The nucleotide position for the amplification product as given by the GenBank accession number (AB077208) is 105–253. Oligonucleotide primers for β-actin were designed using Baylor College of Medicine Primer Selection program (http://searchlauncher.bcm.tmc.edu/seq-util/seq-util.html). Primers were sense: 5′-AGCACGGCATCG-TCACCAACT-3′; and antisense: 5′-TGGCTGGGGTGTT-GAAGGTCT-3′. The nucleotide position for the amplification product as given by the GenBank accession number (X00351) is 256–435. Primers were carefully designed to cross exon/intron regions, avoid the formation of primer–dimer, hair pin and self-complementarity. Synthetic oligonucleotide primers were obtained from Invitrogen (Life Technologies).

2.6. cDNA synthesis and quantitative real-time PCR

Total RNA (5 μg) was treated with DNase1 (Invitrogen) and incubated at 70° for 10 min. The RNA was then reverse-transcribed in the presence of 10 mM dithiothreitol (DTT), 50 ng of random hexamers, 0.25 mM each of the four deoxytriphosphate nucleotides and 200 U of Superscript™ II Reverse Transcriptase in a total volume of 20 μL according to the manufacturer's protocol (Invitrogen). Residual RNA was removed by adding 1 μL of *Esherichia coli* RNase H (Invitrogen; 222 U/μL) and the reaction incubated at 37° for 20 min. Quantitative PCR was carried out by adding 5 μL of template cDNA to a final 25 μL reaction volume containing 3 mM $MgCl_2$; 0.4 μM each forward and reverse primers and 2.5 μL of LC-FastStart DNA Master SyBr Green 1 (Roche). Real-time PCR was done using the iCycler iQ instrument (BioRad Laboratories) using optimized PCR reaction conditions. Amplification of TS and β-actin was carried out as follows: a 3 min hot start at 95°, followed by 40 cycles of denaturation at 95° for 30 s, annealing at 56° for 20 s and a 72° extension for 30 s. Each assay included a negative control and the experiment was done in duplicate. The fluorescence emitted by the reporter (SyBr Green) dye was detected online in real-time, and the threshold cycle (C_t) of each sample was recorded as a quantitative measure of the amount of PCR product in the sample. The C_t value is the fractional cycle number at which the fluorescence generated by the reporter dye exceeded a fixed level above baseline. The TS signal was normalized against the relative quantity of β-actin and expressed as $\Delta C_t = (C_{tTS} - C_{t\beta\text{-actin}})$. The change in TS signal relative to the reference signal (one sample) was expressed as $\Delta\Delta C_t = (\Delta C_{tcontrol} - \Delta C_{tsample})$. Relative changes in expression was then calculated as $2^{[-\Delta\Delta C_t]}$.

2.7. 5,10-Methylene tetrahydrofolate assay

Intracellular 5,10-methylene-tetrahydrofolate was measured by the standard TS assay as described above. Fifty micrograms control PC-3 cell extract was used as the source of TS for each assay. Folate extracts from four million cells in 100 μL (containing unknown amounts of 5,10-methylene-tetrahydrofolate) were added to the standard reaction mixture. Release of tritium into the solvent in this assay therefore reflected 5,10-methylene-tetrahydrofolate levels rather than TS activity. Folate extraction was performed as previously described [37]. Briefly, cells were suspended in cold buffer (50 mM Tris–HCl (pH 7.4), 50 mM sodium ascorbate, and 1 mM EDTA) to a density of 4×10^7 cells/mL. Cells were lysed in a boiling water bath for 3 min and centrifuged at 14,000 *g* for 5 min at 4°. The supernatant was used immediately for the assay or frozen at −70° until used.

2.8. Measurement of total cellular protein synthesis

2×10^5 of PC-3 cells were seeded per well in 6-well plates. The next day, 3 mL of either complete medium or methionine-free medium containing 1 μL of ^{3}H-leucine (1.0 mCi/mL, Amersham Pharmacia Biotech) were added into each well at 1, 3, 6, 24 hr before harvest of the total cellular protein. The cells were then washed with PBS twice and lysed in 100 μL of lysis buffer (20 mM Tris–HCl, pH 8.0; 137 mM NaCl; 10%, w/v glycerol; 10 mM NaF; 1% Triton X-100; 1 mM Na_3VO_4; 2 mM EDTA; 1 mM PMSF; 20 μM leupeptin; and 0.15 U/mL aprotinin). The total cellular protein was then concentrated by TCA precipitation. The samples containing 10% TCA were incubated on ice for 30 min and spun at 14,000 *g* for 5 min. The precipitated protein was dissolved in 50 mL of 0.1 M NaOH. Radioactivity was determined by liquid scintilation counter.

2.9. Preparation of cellular dNTP extract for HPLC analysis [44]

5×10^5 cells from each sample were mixed with 10 μL 0.6 M trichloroacetic acid. The lysate was incubated at 4° for 30 min. After centrifugation, the acidic supernatant was transferred to a microcentrifuge tube. An equal volume of ice cold 80% 1,1,2-trichlorotrifluoroethane and 20% tri-*n*-octylamine was added to the lysate. The mixture was vortexed for 15 s and then centrifuged at 14,000 *g* for 5 min at 4°. The aqueous supernatant was removed and centrifuged at 14,000 *g* for 5 min at 4°. Samples were stored at −70° until used.

2.10. HPLC analysis

Chromatographic analyses were performed with a Waters 625 LC System (Waters Corporation) consisting of a Waters 625 Fluid Handling Unit with a Rheodyne 9125-080 Manual Injector and 20 μL sample loop, 625E Powerline Controller, and 484 Tunable UV Detector. Component separation was achieved using a reversed phase SS Exsil ODS column (5 μM particle size, 4.6 mm × 250 mm, SGE Incorporated). The column was maintained at ambient temperatures. The methodology of Cross *et al.* [44] with some modification was used to separate the nucleotides. Briefly, two buffers comprised the mobile phase—Buffer A consisting of 0.2 M $(NH_4)H_2PO_4$ in 1.0 M KCl at pH 5.35, and Buffer B consisting of 0.2 M $(NH_4)H_2PO_4$ in 1.25 M KCl and 10% methanol at pH 5.0. pH was adjusted with NaOH solution and Buffer B was titrated after the addition of methanol. UV detection was at 250 nm. Solvent flow rate was maintained at 0.8 mL/min during the elution gradients. The elution gradients were as follows: 100% Buffer A for 8 min followed by a 13 min linear gradient to 75% Buffer A and 25% Buffer B. At 22 min, a 2 min linear gradient to 15% Buffer A and 85% Buffer B started. 15% Buffer A and 85% Buffer B was maintained until the end of the run at 40 min. Afterwards, the column was regenerated with 100% Buffer A at 1.0 mL/min for 15 min.

A series of standards containing varying amounts of dUMP and dTTP ranging from 1.0 to 0.02 nmol was analyzed using the above methodology. The different quantities and their correlating absorption areas existed in a linear relationship. Using the least squares method, a linear equation was generated. This linear equation was used to calculate the quantity of dUMP or dTTP represented by the absorption peaks in the chromatograms generated from our experimental samples.

2.11. Cell growth assay

Tumor cell growth was estimated by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay as previously described [45]. Briefly, PC-3 cells were harvested by exposure to 0.25% trypsin/0.02% EDTA (w/v) and seeded into 96-well microculture plates at a density of 2500 cells/well in RPMI 1640 medium supplemented with 10% FBS. After incubation in 5% CO_2 at 37° overnight, the cells were incubated with fresh medium containing either 0, 10, or 100 μM methionine with or without 5-FU for 3 days. Thereafter, 20 μL of MTT (2.5 mg/mL in phosphate-buffered saline, PBS) was added to each well, and the cells were further incubated for 2 hr at 37° to allow complete reaction between the dye and the enzyme mitochondrial dehydrogenase in the viable cells. After removal of residual dye and medium, 100 μL dimethylsulfoxide were added to each well, and the absorbance at 570 nm was measured with a microplate reader (BioRad).

3. Results

3.1. Methionine restriction reduced intracellular folate levels

We first measured intracellular 5,10-methylene-tetrahydrofolate levels in prostate cancer cells in order to investigate whether methionine restriction diverted folate to methionine synthesis, as it does in normal liver [24] and kidney cells [23] as a result of the "methyl folate trap" [25]. Methionine deprivation reduced the level of 5,10-methylene-tetrahydrofolate by 75% within 24 hr. The effect was maintained for up to 72 hr (Fig. 2A). As a control, we also measured intracellular 5,10-methylene-tetrahydrofolate levels in response to folate depletion for 24 hr. As expected, 5,10-methylene-tetrahydrofolate fell by 67% in PC-3 cells cultured in folate-free medium (Fig. 2B).

3.2. Methionine restriction inhibited TS activity

We next determined whether methionine restriction inhibited TS in prostate cancer cells, as it is known to

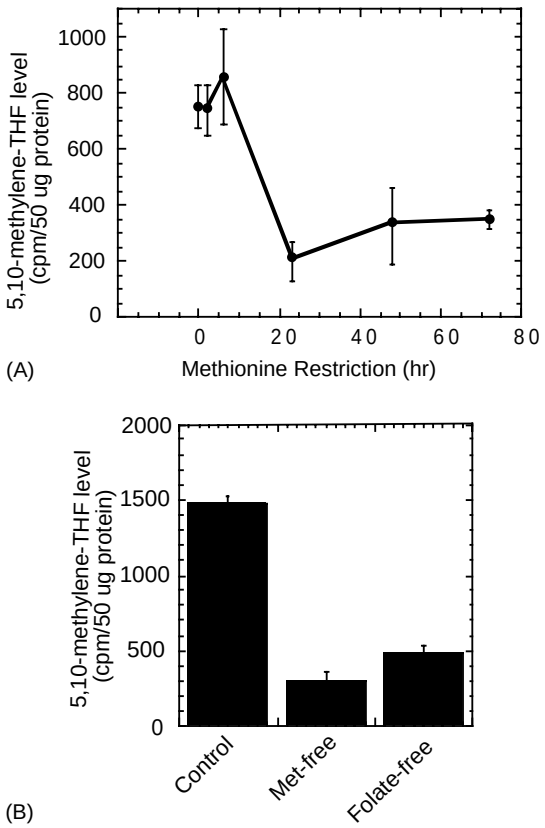

Fig. 2. Effect of methionine restriction on 5,10-methylene-tetrahydrofolate levels in PC-3 cells. (A) Kinetics of 5,10-methylene-tetrahydrofolate depletion in cells grown in methionine free medium for up to 72 hr. (B) 5,10-Methylene-tetrahydrofolate depletion in PC-3 cells after 24 hr in methionine free medium as compared to folate free medium. 5,10-Methylene-tetrahydrofolate levels were measured as described in Section 2. Values are mean ± SD, N = 5.

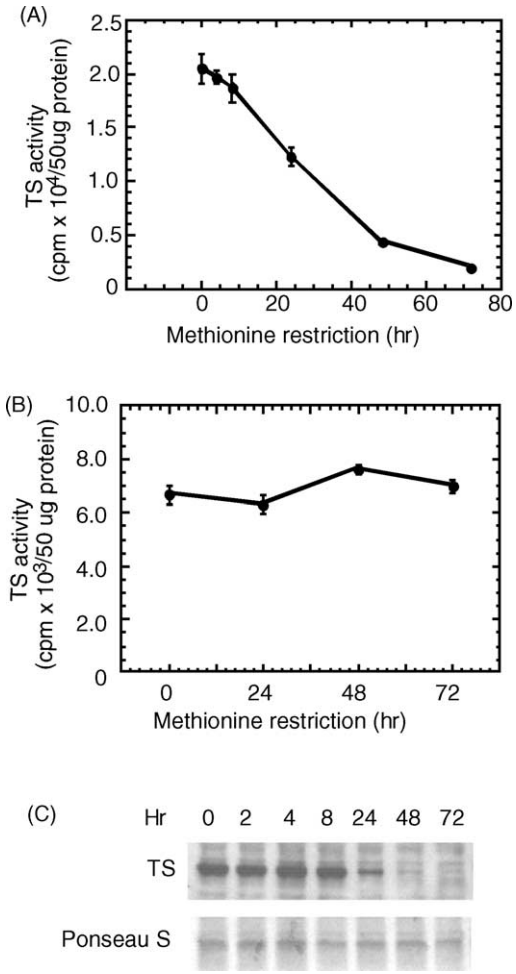

Fig. 3. Selective inhibition of TS activity in PC-3 cells by methionine restriction. TS activity in PC-3 cells (A) and normal prostate epithelial cells (B) grown in methionine free medium for up to 72 hr was measured as described in Section 2. (C) TS protein levels in PC-3 cells grown in methionine-free medium for up to 72 hr measured by western blot analysis as described in Section 2. Values in A and B are mean ± SD, N = 5.

in leukemia cells [37]. We found that methionine restriction inhibited TS activity in PC-3 cells by approximately 40% within 24 hr and by 80% in 48 hr (Fig. 3A). In contrast, TS activity in normal human prostate epithelial cells was unaffected by methionine restriction (Fig. 3B). The observed fall in TS activity in prostate cancer cells in response to methionine restriction was accompanied by a commensurate fall in TS protein levels by 80% within 24 hr. This 80% reduction was confirmed by western blot dilution experiments (not shown). TS protein was almost undetectable within 48 hr (Fig. 3C). TS RNA levels as measured by quantitative real-time PCR also fell by 74% within 24 hr and by 82% within 48 hr, as shown in Table 1.

In contrast to the observed fall in TS abundance, global cellular protein synthesis, as measured by tritiated leucine incorporation, was not significantly affected by methionine restriction within the first 24 hr (Fig. 4). In fact, leucine

Table 1
Quantitative RT–PCR of thymidylate synthase RNA levels in PC-3 cells in response to methionine restriction

	Sample				
	TS C_t value	β-Actin C_t value	ΔC_t	$\Delta\Delta C_t$	$2^{[-\Delta\Delta C_t]}$
Control	18.6	10.9	7.7	−2.85	7.21
24 hr	21.6	11.95	9.65	−0.9	1.86
48 hr	21.0	10.8	10.2	−0.35	1.27
72 hr	22.55	12.0	10.55	0.0	1.00

Levels relative to 72 hr of treatment are listed in the far right column. See Section 2 for experimental details.

incorporation during the first 6 hr of the experiment was actually greater in cells deprived of methionine than it was in control cells (Fig. 4).

As a control, we next measured TS enzymatic activity in PC-3 cells in response to 5-FU. As expected, 5-FU inhibited TS activity by 95% within 8 hr (Fig. 5A), whereas it dramatically *increased* TS protein level as determined by western blot (Fig. 5B). The observed TS protein accumulation in response to 5-FU was largely abrogated by concurrent methionine restriction (Fig. 5C).

3.3. Methionine restriction disrupted nucleotide balance

We next used HPLC to measure the effect of methionine restriction on nucleotide levels in PC-3 cells, since TS plays a central role in nucleotide biosynthesis. The ratio of dUMP to dTMP rose from 0.48 ± 0.07 at baseline (Fig. 6A and Table 2) to 1.75 ± 0.61 after 24 hr of methionine restriction (Fig. 6B and Table 2) and remained at about the same level for up to 48 hr (Table 2). 5-FU treatment was used as a positive control for TS inhibition, and, as expected, resulted in a dramatic increase in dUMP/dTTP ratio (Fig. 6C and Table 2).

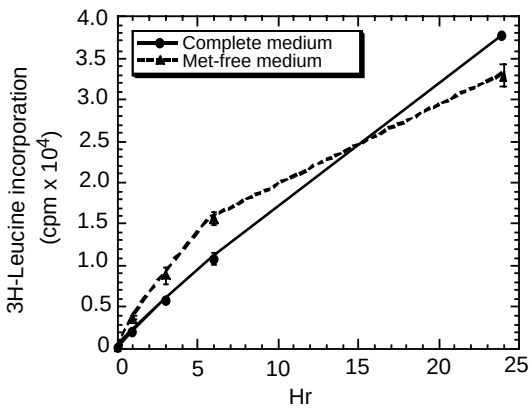

Fig. 4. Effect of methionine restriction on total cellular protein synthesis in PC-3 cells. Protein synthesis as determined by rate of ^{3}H-leucine incorporation was measured as described in Section 2. Values are mean ± SD, N = 5.

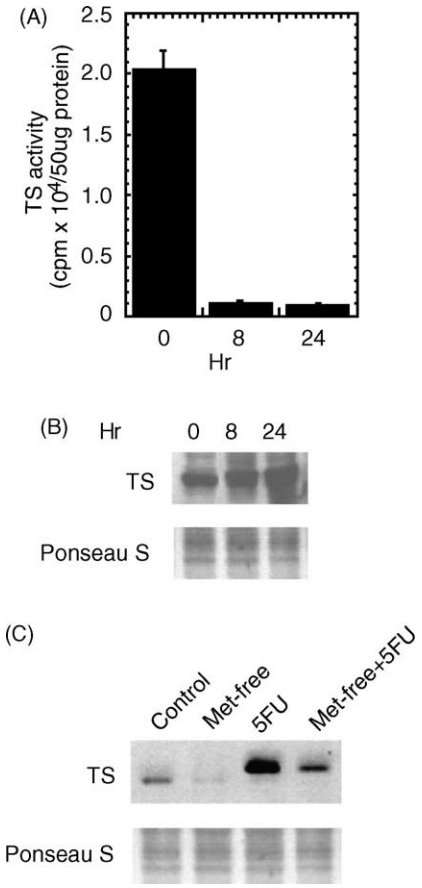

Fig. 5. Abrogation of TS up-regulation by methionine restriction in PC-3 cells following 5-FU treatment. TS activity (A) and abundance (B) following methionine restriction for up to 24 hr were measured with the standard TS enzyme assay and western blotting, respectively, as described in Section 2. (C) TS protein abundance under control conditions or after methionine restriction, 5 μM 5-FU, or methionine restriction + 5 μM 5-FU for 24 hr measured by western blot analysis. Values in A are mean ± SD, N = 5.

3.4. Methionine restriction enhanced PC-3 cells growth inhibition by 5-FU

Treatment of PC-3 cells with 5-FU alone (2.5 μM) for 3 days in medium containing 100 μM methionine inhibited growth by 25% as compared to control conditions (Fig. 7). Reduction of methionine levels in the medium to 10 μM in combination with 2.5 μM 5-FU inhibited growth by an additional 22% (57% reduction compared to control, Fig. 7). This level of depletion is achievable *in vivo* by dietary restriction in adults with metastatic cancer [19]. Further reduction of methionine in the medium combined with 5-FU inhibited growth by yet an additional 22% (total 79% growth reduction, Fig. 7). These highly restrictive conditions are also achievable *in vivo* in selected cancer patients treated with a restrictive diet alone and may be achievable in the majority of patients treated in the future with recombinant methioninase [46]. Results of these

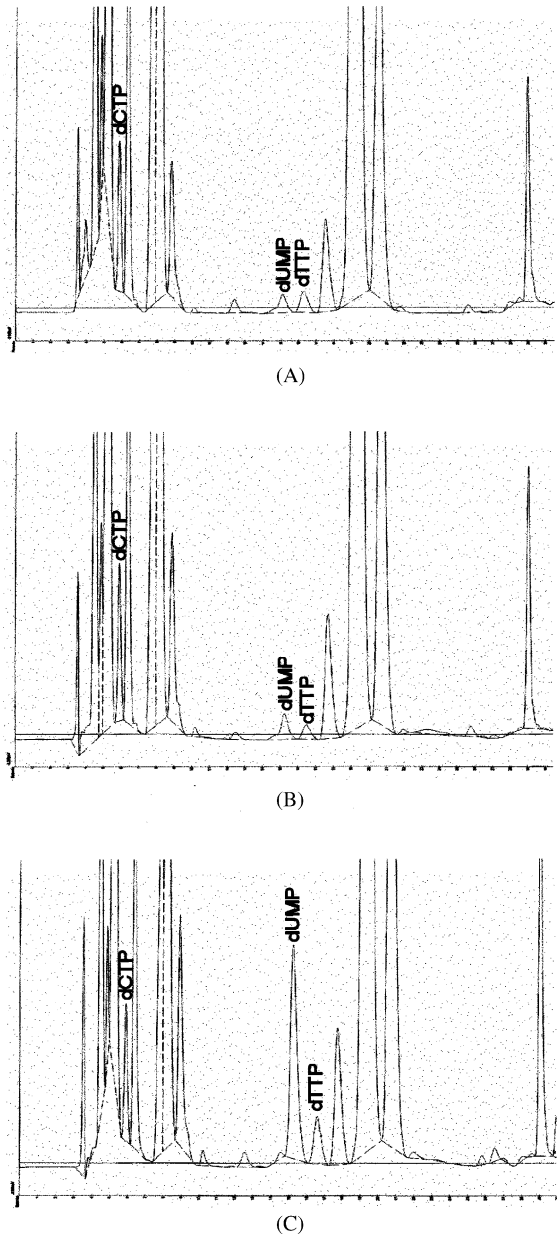

Fig. 6. Disruption of nucleotide balance in PC-3 cells in response to methionine restriction. HPLC nucleotide chromatograms under control conditions (A), following 24 hr of methionine restriction (B), or following 24 hr treatment with 5 μM 5-FU (C). Relevant nucleotide peaks are labeled.

Table 2
Quantitative analysis of intracellular dUMP and dTTP level in response to methionine restriction in PC-3 cells by HPLC

Treatment	dUMP[a]	dTTP[a]	dUMP/dTTP
Control	57.7 ± 18.1	117.0 ± 22.5	0.48 ± 0.07
Met-free—24 hr	145.4 ± 52.7	82.6 ± 2.3	1.75 ± 0.61
Met-free—48 hr	74.4 ± 15.7	40.6 ± 5.5	1.87 ± 0.56
5-FU—24 hr	1457.3 ± 265.9	191.7 ± 27.0	7.60 ± 0.99
5-FU—48 hr	867.3 ± 150.2	151.0 ± 30.6	5.75 ± 0.32

Data represent means ± SD. Experiments were repeated at least three times.

[a] pmol/500,000 cells.

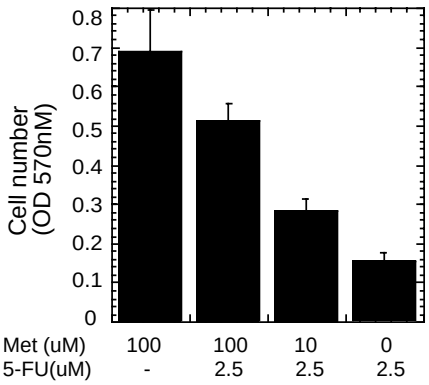

Fig. 7. Enhancement of growth inhibitory effects of 5-FU by methionine restriction. PC-3 cells were grown for 3 days under control conditions (100 μM lacking 5-FU), in the presence of 5-FU alone, or in the presence of 5-FU combined with moderate (10 μM) or severe (0 μM) methionine restriction. Cell number was determined by the MTT assay as described in Section 2. Values represent mean ± SD, N = 5.

growth inhibition studies were similar whether dialyzed serum or nondialyzed serum was used.

4. Discussion

The current results suggest that one mechanism by which methionine restriction induces prostate cancer cell cycle arrest and eventual apoptosis is by depleting 5,10-methylene-tetrahydrofolate, which is a critical precursor for nucleotide biosynthesis. Our data are consistent with those of Machover *et al.* [37], who also showed that methionine restriction reduced 5,10-methylene-tetrahydrofolate levels in cancer cells. In contrast, folinic acid, the formyl derivative of folic acid, potentiates the antitumor activity of 5-fluoruracil by *increasing* 5,10-methylene-tetrahydrofolate, thereby stabilizing the complex between 5-FU and TS. Our studies as well as previous ones [37] therefore suggest that synergy between 5-FU and methionine restriction is due at least in part to diversion of folate to methionine synthesis via the ''methyl folate trap'' [25].

We also found that methionine restriction inhibited TS activity in prostate cancer cells but not in normal prostate epithelial cells, which is a reflection of the greater methionine dependence of cancer cells relative to corresponding normal cells. TS inhibition in cancer cells was accompanied by a commensurate reduction in TS protein levels as measured by western blot analysis, suggesting that enzyme inhibition was largely, if not entirely, due to reduced enzyme abundance rather than enzyme inactivation. Surprisingly, global protein synthesis, as measured by ^{3}H-leucine incorporation, *increased* during the first several hours of methionine restriction, which is consistent with previous studies [47]. This result suggests that the observed fall in TS protein abundance was not simply due to global protein synthesis inhibition but rather to specific down-regulation of TS levels. This down-regulation was probably due to reduced TS mRNA levels, which fell in parallel with TS protein levels (Table 1). However, TS inhibition may also have been due to reduced folate levels in methionine-depleted cells, with consequent generation of ligand-free enzyme that additionally repressed TS mRNA translation or decreased stability of TS polypeptide.

The current results also suggest that synergy between methionine restriction and 5-FU is partially due to blockage of TS up-regulation, which is a major mechanism by which cancer cells become resistant to 5-FU [48,49]. TS inhibition subsequently leads to nucleotide imbalance, which is known to cause cells to undergo a form of apoptosis known as ''thymineless death'' [33,34].

Results of a phase I clinical trial of dietary methionine restriction for adults with advanced cancer from our institution indicated that dietary methionine restriction is safe and feasible for at least several weeks at a time and resulted in significant and clinically relevant declines in plasma methionine levels [19] Nonetheless, it is probably unreasonable to expect patients to remain on a methionine restricted diet or any other severely restrictive diet for prolonged periods of time. Dietary methionine restriction will therefore most likely have the greatest impact when prescribed intermittently in combination with chemotherapy and/or recombinant methioninase, the methionine-degrading enzyme under development by Anticancer Incorporated and their pharmaceutical partners in Asia. In fact, sufficient pre-clinical data already exist to justify a clinical trial of dietary methionine restriction plus chemotherapy in selected tumor types, such as glioblastoma multiforme [12].

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