Suppression by L-Methionine of Cell Cycle Progression in LNCaP and MCF-7 Cells but not Benign Cells

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Abstract. *Background/Aim: Methionine inhibits proliferation of breast and prostate cancer cells. This study aimed to determine cell cycle effects of methionine and selectivity for cancer cells. Materials and Methods: MCF-7 (breast), LNCaP (prostate), and LS-174 (colon) cancer cells (wild-type p53), DU-145 (prostate) and SW480 (colon) cancer cells (mutated p53), and immortalized, non-tumorigenic MCF-10A (breast), BPH-1 (prostate), and NCM-460 (colon) epithelial cells were used. Cell cycle effects were assessed by flow cytometry and cell cycle-related gene expression by microarray analysis and QRT-PCR. Results: L-Methionine at 5 mg/ml for 72 hours (non-apoptotic) arrested cell cycle in LNCaP, DU145, and MCF-7 cells, but not in untransformed cells, nor in LS-174 cells. LNCaP and MCF-7 cells were arrested at G_1, but DU-145 at S. Methionine up-regulated CDKIs and down-regulated CDKs. Conclusion: L-Methionine selectively inhibits proliferation of breast and prostate cancer cells, but not non-tumorigenic cells, and may thus have therapeutic benefits. p53 status appeared to determine the cell cycle stage at which methionine acts.*

The efficacy of conventional chemotherapy for patients with advanced cancer is limited, pointing to the need to identify new therapeutic strategies. Recombinant proteins have been increasingly used for therapies in the recent years (1). Alternatively, it is possible to create novel therapeutic agents through metabolic engineering of amino acids (2, 3). Identification of the key amino acids that may have a therapeutic potential is an important step for such metabolic engineering of amino acids.

It has been previously observed that L-methionine possesses inhibitory effects on cell proliferation of both breast and prostate cancer cell lines, concomitant with post-translational modification of the tumor suppressor p53 (4). These results suggest an anticancer potential of this important amino acid and point to the possibility of developing new therapeutic agents using methionine analogs. Methionine is an essential amino acid that plays fundamental roles in protein synthesis and a number of other biochemical and cellular processes (5-9). In addition, methionine acts as a precursor of glutathione, an important tripeptide that reduces reactive oxygen species (10), thereby protecting cells from oxidative stress (11). It is also involved in DNA and protein methylation by serving as the methyl-group donor, thereby playing an important role in regulation of gene expression and protein functions (12). Methionine is also required for the biosynthesis of the polyamines, spermine and spermidine, which are necessary for a number of cellular activities including cell proliferation (13). Furthermore, methionine analogs are known to be capable of inhibiting protein synthesis (14, 15). The present study was designed to gain a better understanding of the inhibitory effects of methionine on cell cycle progression.

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Key Words: Methionine, prostate, breast, colon, p53.

Materials and Methods

Cell lines. Three types of cells were selected in this study: (a) Wild-type p53 expressing cancer cells, including MCF-7 breast cancer cells, LNCaP prostate cancer cells, and LS-174 colon cancer cells; (b) inactive (mutated) p53-containing DU-145 prostate cancer cells and SW480 colon cancer cells, and (c) immortalized, but

nontumorigenic epithelial cells, including MCF-10A breast cells, BPH-1 prostate cells, and NCM460 colon cells. These cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA), except NCM460 cells which were obtained from In Cell (San Antonio, TX, USA).

Cell culture. MCF-7 cells (16) were cultured in Minimum Essential Medium (MEM; Eagle, Invitrogen, Grand Island, NY, USA) containing 2 mM L-glutamine (Mediatech-Cellgro, Herndon, VA, USA), 1.5 mg/l sodium bicarbonate, 0.1 mM non-essential amino acids, and 1 mM sodium pyruvate supplemented with 10% fetal bovine serum (FBS) (vol/vol) (HyClone Lab Inc., Logan, UT, USA) and 10 mg/ml insulin (17, 18) LNCaP (10), DU-145 (19), and BPH-1 (20) cells were cultured in RPMI 1640 media (Mediatech-Cellgro, Herndon, VA, USA) supplemented with 10% FBS, 2 mM L-glutamine, antibiotic-antimycotic solution (1X; Mediatech-Cellgro), and MEM vitamin solution (1X; Mediatech-Cellgro, Herndon, VA, USA). MCF 10A (CRL-10317 ATTC Number) was grown in DMEM-H/F12 medium with 1% penicillin-streptomycin-fungizome (Invitrogen Corp., Carlsbad, CA, USA), 10 mg/ml insulin, 100 ng/ml cholera toxin (List Biological Laboratories, Inc., Hornby, Canada), 20 ng/ml epidermal growth factor (Invitrogen), 500 ng/ml hydrocortisone, and 5% FBS at 37˚C in 5% CO_2 humidified atmosphere. SW480 and LS174 cells were grown in MEM supplemented with 10% FBS, 1× antibiotic/antimycotic solution in, 100 μM non-essential amino acids and 100 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid buffer solution (all from Invitrogen). NCM 460 cells were cultured in M3:10 nutrient mix containing 10% fetal bovine serum and antibiotics (In Cell). All cells were cultured at 37˚C in a humidified atmosphere containing 5% CO_2.

L-Methionine (analytical grade; Sigma M5308) was obtained from Sigma (St. Louis, MO, USA) and incorporated in the respective media at final concentration 5.0 mg/ml, and cells were incubated for 72 h at this concentration of methionine.

Flow cytometric analysis. Approximately 6×10^5 cells/wells were initially seeded in triplicate in 6-well plates such that the final cell density reached 50% confluence at the time when the experiments were completed. After being cultured overnight, cells were treated with L-methionine (5 mg/ml) for 72 h. This concentration was chosen because it has been previously observed (4) that methionine at this concentration causes optimal growth inhibitory effects but has no apparent toxic effect on cell death. The adherent and non-adherent of cells treated with or without L-methionine were harvested, washed in cold PBS, fixed with 80% ethanol for 8 h at 4˚C, and stained with propidium iodide buffer (50 μg/ml propidium iodide, 0.1% sodium citrate, and 0.1% Triton X-100) overnight at 4˚C. Approximately 10,000 cells were analyzed using a Becton Dickinson FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). The percentage of different check-point cells was quantified using Cell Quest software (Becton Dickinson, San Jose, CA, USA). Experiments were carried out in triplicate.

RNA isolation and microarray analysis. Cells were washed three times with ice-cold PBS and harvested by trypsin. Total RNA was extracted using the RNease Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The concentration and purity of total RNA was determined spectrophotometrically at 260 nm and 280 nm. The quality of RNA was further evaluated by agarose gel electrophoresis. Biotin labeled cRNA was generated from high quality total RNA using the Illumina TotalPrep RNA Amplification kit (Ambion, Austin, TX, USA). Briefly, 350 ng of total RNA with high 260/280 absorbance ratio (>1.8) (21) and RIN number were reverse transcribed with an oligo primer bearing T7 promoter. The first-strand cDNA, produced in the reaction, was used to make the second-strand cDNA. The purified second-strand cDNA along with biotin UTPs was used to generate biotinylated, antisense RNA of each mRNA in an *in vitro* transcription (IVT) reaction. Size distribution profiles of the labeled cRNA samples were evaluated by a bioanalyzer. Purified labeled cRNAs (1.5 μg) were hybridized to the Sentrix Human-6 v2 expression Beadchip at 55˚C overnight. Signals were developed with Streptavidin-Cy3. An Illumina BeadArray Reader (Illumina: San Diego, CA, USA) was used to scan the chips.

Microarray data analysis. The Illumina BeadArray technology is based on randomly arranged beads, with each bead binding many (usually over 30) identical copies of a gene-specific probe. This redundant design yields higher confidence and more robust estimations. To take advantage of this unique feature of Illumina BeadArray, the Bioconductor lumi package (22) was used to undertake the preprocessing of Illumina data with default settings. Each array was VST (variance-stabilizing transforms) transformed (23), followed by quantile normalization across all samples. Probes with intensity lower or equal to background levels were filtered. A total of 15,814 probes were used for further analysis. To identify differentially expressed genes, routines implemented in Illumina Bioconductor package were applied to fit linear models to the normalized expression values (24). The variance used in the *t*-score calculation was corrected by an empirical Bayesian method for better estimation under small sample size. To control the effects of multiple testing and reduce false positives, *p*-values were further adjusted based on the false discovery rate (FDR) (25). Genes with an FDR adjusted *p*-value of <0.01 and a fold-change of >1.5 were referred to as 'differentially expressed genes'.

Quantitative reverse transcription-PCR (QRT-PCR). Reverse transcription was carried out with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) on the total RNA isolated from LNCaP and MCF-7 cells that had been treated with or without L-methionine using the RNeasy kit from Qiagen (Valencia, CA, USA). PCR sequences were CCAGATT AACCATCCCAGTC (forward) and AGGCTCGGCCATT CTTTA (reverse) for *CDKN2C* primer, CCGGGAGAAAGATGTCAAAC (forward) and GGTTAACTCTTCG TGGTCCA (reverse) for *CDKN1B* primer, and GTCACTGTCTTGTA CCCTTG (forward) and GCGTTTGGAGTGGTAGAAATC (reverse) for *CDKN1A* primer. PCR products were separated on a 1% agarose gel. Expression of a housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used to normalize the PCR reactions. For real time-PCR, cDNA was mixed with primers and SYBR Green PCR Master Mix according to the manufacturer's instructions (Applied Biosystems). Real-time PCR was carried out by an ABI7900-HT sequence detection system from Applied Biosystems, using commercially available relative quantitation of mRNA levels, and was plotted as fold increase compared with untreated samples. *GAPDH* was used for normalization. ΔC_t values (target gene C_t minus *GAPDH* C_t) for each triplicate sample was averaged. $^{\Delta\Delta}Ct$ was calculated and mRNA amplification was determined by the formula, $2^{-\Delta\Delta}C_t$. The *p*-value was estimated using the Student's *t*-test by comparing PCR measurements (adjusted by *GAPDH*) of control and treated groups.

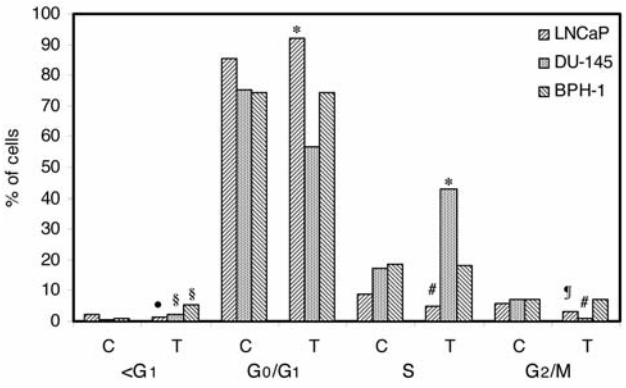

Figure 1. *Flow cytometric analysis of propidium iodide-stained cells showing the effects of L-methionine treatment (5 mg/ml for 72 h) on cell cycle progression of LNCaP and DU145 prostate cancer cells and non-tumorigenic BPH-1 prostate epithelial cells. Data are expressed as means of three independent cultures. C: Untreated control cells; T: cells treated with methionine. (•0.05<p<0.1; §p≤0.05; #p≤0.01; *p≤0.001; ¶p≤0.0001; two-sided t-test.*

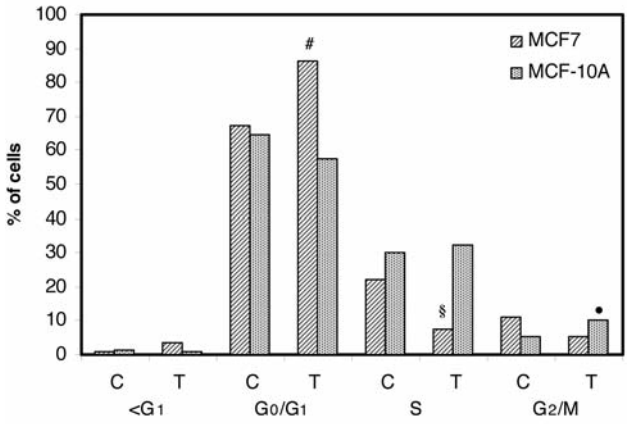

Figure 2. *Flow cytometric analysis of propidium iodide-stained cells showing the effects of L-methionine treatment (5 mg/ml for 72 h) on cell cycle progression of MCF-7 breast cancer cells and non-tumorigenic MCF-10A breast epithelial cells. Data are expressed as means of three independent cultures. C: Untreated control cells; T: cells treated with methionine. •0.05 < p<0.1; §p≤0.05; #p≤0.01; two-sided t-test.*

Results

Effects of L-methionine on cell cycle progression. Effects on cell cycle progression of cell lines exposed to methionine at a concentration of 5 mg/ml for 72 h, selected based on previously published findings (4), were evaluated by flow cytometry. Methionine treatment caused an accumulation of cells in G_1 in both MCF-7 (breast) and LNCaP (prostate) cancer cells (Figures 1 and 2). MCF-7 cells appeared to be more sensitive to methionine than were LNCaP cells, as the percentage increase of cells in G_1 was higher for MCF-7 cells (29%) than for LNCaP cells (8%). However, in DU-145 cells, which contain mutated p53, L-methionine caused considerable accumulation of cells in the S phase; the percentage of cells in the S phase was increased 2.5-fold from 17 to 43% (Figure 1). In contrast, methionine did not have any effect on cell cycle parameters in immortalized, but non-tumorigenic, MCF-10A (breast) and BPH-1 (prostate) epithelial cells (Figures 1 and 2). Treatment with methionine of LS174 colon cancer cells and immortalized, non-tumorigenic NCM460 colon cells, both positive for wild-type p53, caused no significant modifications in cell cycle progression as compared with vehicle-treated cells (Figure 3). In contrast, p53-negative SW480 colon cancer cells accumulated in the S phase after treatment with methionine with the percentage of cells in the S phase increasing from 42 to 58% (Figure 3).

The percentage of cells in sub-G_1 after treatment with methionine was no more than 2% for most cell lines, which was not different from the percentage cells in sub-G_1 in untreated cells. Thus, methionine caused no significant induction of apoptosis in most cell lines tested, with only few exceptions. Methionine slightly, but significantly, increased

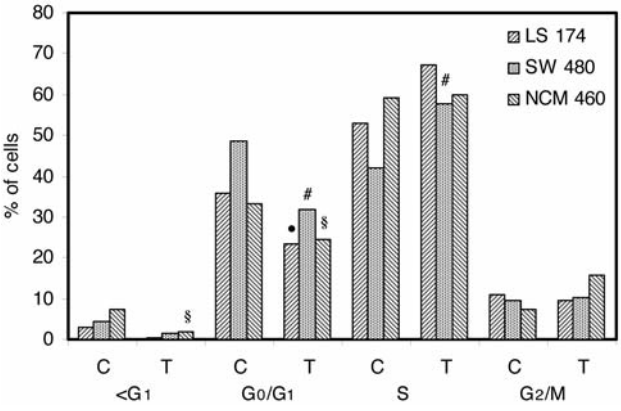

Figure 3. *Flow cytometric analysis of propidium iodide-stained cells showing the effects of L-methionine treatment (5 mg/ml for 72 h) on cell cycle progression of LS-175 and SW480 colon cancer cells and non-tumorigenic NCM461 colon epithelial cells. Data are expressed as means of three independent cultures. C: Untreated control cells; T: cells treated with methionine. •0.05<p<0.1; §p≤0.05; #p≤0.01; two-sided t-test.*

apoptosis rates in BPH-1 cells from 1% in vehicle treated cells to 5.2%, while it reduced apoptosis from 7.4% to 1.6% in benign colon NCM-460 cells which had the highest percentage of cells in sub-G_1.

Effects of methionine on the expression of genes involved in G_1/S transition of the cell cycle. To explore the possible molecular targets of methionine in breast and prostate cancer cell lines in relation to the cell cycle, microarray analysis was applied to 34 genes involved in G_1/S transition of the cell cycle. Treatment of LNCaP with methionine resulted in a

Table I. *Microarray analysis of the effect of L-methionine treatment (5 mg/ml for 72 h) on expression of genes involved in G_1/S transition of the cell cycle in MCF-7 and LNCaP cells affected with a fold-change of >1.5 and a false discovery rate (FDR) with an adjusted p-value <0.01. Data are expressed as mean fold change of three independent cultures. *P-values significant at ≤0.01. EntrezID is the gene identification number in the Entrez Gene searchable database of genes.*

			LNCaP			MCF-7		
Entrez ID	Gene symbol	Gene name	Fold change	*p*-Value*	FDR	Fold change	*p*-Value*	FDR
113130	*CDCA5*	Cell division cycle associated 5	–6.8961	2.09×10^{-11}	4.05×10^{-8}	–1.5928	8.50×10^{-5}	3.01E-03
1033	*CDKN3*	Cyclin-dependent kinase inhibitor 3	–2.9914	1.81×10^{-7}	1.22×10^{-5}	–2.0511	1.39×10^{-5}	9.38E-04
898	*CCNE1*	Cyclin E1	–1.7985	1.44×10^{-7}	1.03×10^{-5}	–1.0471	4.03×10^{-1}	0.7032
6502	*SKP2*	S-phase kinase-associated protein 2 (p45)	–1.7663	6.74×10^{-7}	3.10×10^{-5}	–1.3497	3.04×10^{-4}	7.34E-03
8317	*CDC7*	Cell division cycle 7 homolog (*S. cerevisiae*)	–1.7488	5.09×10^{-5}	7.03×10^{-4}	–1.332	8.18×10^{-3}	0.0693
1019	*CDK4*	Cyclin-dependent kinase 4	–1.7336	1.38×10^{-6}	5.22×10^{-5}	–1.1341	6.39×10^{-2}	0.2552
595	*CCND1*	Cyclin D1	–1.5936	6.87×10^{-4}	5.00×10^{-3}	1.0003	9.98×10^{-1}	0.9995
1026	*CDKN1A*	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	1.3688	2.09×10^{-3}	1.16×10^{-2}	1.7015	2.63×10^{-5}	1.39E-03
1027	*CDKN1B*	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	1.5149	1.22×10^{-5}	2.48×10^{-4}	–1.002	9.72×10^{-1}	0.9919
1031	*CDKN2C*	Cyclin-dependent kinase inhibitor 2C (p18)	1.7502	2.66×10^{-7}	1.58×10^{-5}	1.0603	2.96×10^{-1}	0.6018

significant change ($p<0.01$) in 9 genes out of the 34 genes examined. Among these genes, expression of *CDKN2C* (p18) and *CDKN1B* (p27) were enhanced in LNCaP cells, whereas expression of *CDCA5, CDKN3, CCNE1, SKP2, CDC7, CDK4,* and *CCND1* was down-regulated (Table I). In MCF-7 cells, methionine treatment brought about a significant regulation in Three genes out of the 34 genes evaluated, of which *CDKN1A* (p21) was up-regulated and *CDKN3* and *CDCA5* were down-regulated ($p<0.01$) (Table I).

These changes in expression of key genes up-regulated by L-methionine treatment were confirmed by real-time RT-PCR analysis. Expression of *CDKN2C* (p18) and *CDKN1B* (p27) in LNCaP cells and *CDKN1A* (p21) in MCF-7 cells displayed a 2.42-fold change (p-value=0.01), 2.1-fold change (p-value=0.006), and 2.23-fold change (p-value=0.004), respectively, as compared with vehicle-treated cells. There was strong induction of the mRNA expression of each of these genes after treatment with L-methionine using both microarray analysis and real-time RT-PCR.

Discussion

The present study demonstrated that methionine treatment selectively inhibited cell cycle progression in breast (MCF-7) and prostate cancer (LNCaP) cells, but had no significant inhibitory effects on non-tumorigenic (immortalized) human breast (MCF-10A) and prostate epithelial cells (BPH-1). Methionine treatment caused breast (MCF-7) and prostate cancer cells (LNCaP) with wild-type p53 to accumulate in G_0-G_1, whereas mutant p53 DU-145 prostate cancer cells accumulated in the S phase. Human SW480 colon cancer cells with mutant p53 also accumulated in the S phase in response to methionine treatment, but cell cycle parameters of malignant (LS-175) and benign (NCM460) colon epithelial cells, both with wild-type p53, were not affected by methionine.

These observations are important because they suggest that methionine inhibits cell proliferation selectively in breast and prostate cancer cells, but not in non-malignant breast and prostate epithelial cells. These properties suggest methionine may be a potentially promising anticancer agent, specifically targeting breast and prostate cancer cells. Furthermore, it is important to note that methionine inhibited proliferation of cancer cells that contain wild-type p53 (MCF-7 and LNCaP cells), causing accumulation of cells in G_1, whereas it inhibited proliferation of cancer cells with mutant p53 (DU-145 prostate and SW480 colon cancer cells), causing accumulation of cells in the S phase. This observation may suggest that the nature of the inhibitory effects of methionine on proliferation of cancer cells is modulated by their p53 status (wild-type *versus* mutant). The growth inhibitory effects of methionine are conceivably mediated through *S*-adenosylmethionine (SAMe), a derivative of methionine and a methyl donor for biological transmethylation reactions (6). Consistent with this idea, Qiu *et al*. (26) reported that SAMe was capable of inducing growth arrest *via* the DNA damage-inducible gene 45 beta (*GADD45* beta) in a wild-type *p53*-containing human hepatoma cell line (HepG2) but not in the p53-null cell line Hep3B. It is conceivable that similar mechanisms may play a role in the differential effects of methionine on prostate and breast cancer cells differing in p53 status.

Consistent with the interference with cell cycle progression of methionine treatment, it was observed that the mRNA expression of three key cyclin-dependent kinase inhibitors (CDKIs) that are involved in the G_1/S transition of cell cycle was up-regulated in LNCaP cells, $p21^{Cip1/Waf1}$ (p21) in MCF-7 cells and $p27^{Kip1}$ (p27) and p18INK4c (p18). CDKIs are potent inducers of cell cycle arrest when over-expressed in mammalian cells. In this study, it was also observed that methionine caused down-regulation of a number of other genes involved in cell cycle regulation, including *CDKN3, CDCA5,*

CCNE1, SKP2, CDC7, CDK4 and *CCND1* in LNCaP cells and *CDKN3* and *CDCA5* in MCF-7 cells.

In summary, the present study revealed that: (i) methionine selectively inhibits cell proliferation in both breast and prostate cancer cells, whereas this effect does not occur in non-tumorigenic breast, prostate, and colon-derived cells; (ii) methionine interferes with cell cycle progression at G_1 in both breast and prostate cancer cells with wild-type p53, but affects cell cycle progression in prostate cancer cells and colon cancer cells with mutated p53 at S phase; and (iii) expression of genes involved in the G_1/S transition of the cell cycle is changed in MCF-7 and LNCaP cells. In the absence of induction of apoptosis in both normal and cancer cells, the observed methionine effects suggest the novel therapeutic potential of methionine analogs that lack the potential negative effects of methionine itself on the well-known methionine-dependence of many tumor cells (26, 27). Further research is needed to fully explore the various molecular targets of and pathways affected by methionine.

Acknowledgements

This work was supported in part by a Supplement to NIH Grant No. R01CA116195. The authors thank Dr. Daniel Guimaraes Tiezzi for technical and editorial assistance.

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Received March 10, 2010
Revised April 5, 2010
Accepted April 14, 2010