Relation of Exposure to Amino Acids Involved in Sarcosine Metabolic Pathway on Behavior of Non-Tumor and Malignant Prostatic Cell Lines

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BACKGROUND. Sarcosine (N-methylglycine) was previously delineated as a substantial oncometabolite of prostate cancer (PCA) and its metabolism seems to be significantly involved in PCA development and behavior.

METHODS. We focused on investigation whether the exposure of prostate cells (PNT1A, 22Rv1, and PC-3) to sarcosine-related amino acids (glycine, dimethylglycine, and sarcosine) affects their aggressiveness (cell mobility and division rates, using real-time cell based assay). The effect of supplementation on expression of glycine-N-methyltransferase (GNMT) mRNA was examined using qRT-PCR. Finally, post-treatment amino acids patterns were determined with consequent statistical processing using the Ward’s method, factorial ANOVA and principal component analysis (P < 0.05).

RESULTS. The highest migration induced sarcosine and glycine in metastatic PC-3 cells (a decrease in relative free area about 53% and 73%). The highest cell division was achieved after treatment of 22Rv1 and PC-3 cells with sarcosine (time required for division decreased by 65% or 45%, when compared to untreated cells). qRT-PCR revealed also significant effects on expression of GNMT. Finally, amino acid profiling shown specific amino acid patterns for each cell line. In both, treated and untreated PC-3 cells significantly higher levels of serine, glutamic acid, and aspartate, linked with prostate cancer progression were found.

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CONCLUSIONS. Sarcosine-related amino acids can exceptionally affect the behavior of benign and malignant prostate cells. *Prostate* © 2016 Wiley Periodicals, Inc.

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**INTRODUCTION**

The amino acid sarcosine is currently studied as a potential biomarker for the early stages of prostate carcinoma (PCAs). Even though the linkage of sarcosine with PCA development and its potential in a diagnosis of early stages of tumors was described [1,2], its usage as a marker remains still unclear [3,4]. Hence, it is necessary to study the fate of sarcosine and other amino acids, which act as the intermediate products of tumor metabolism in PCA. Formation and oxidation of sarcosine occurs in mitochondria and are provided by two basic pathways (schematically depicted in Fig. 1, where sarcosine can be simultaneously produced from dimethylglycine (Dmg) or glycine. The first pathway involves repeated methylation of phosphatidylethanolamine (PE) by S-adenosylmethionine (SAM) to form phosphatidylcholine (PC) with the resulting intermediate product betaine. This reaction forms Dmg and regenerates methionine from homocysteine. The Dmg is subsequently converted to sarcosine via dimethylglycine dehydrogenase (DMGDH) [5]. The second metabolic pathway creates sarcosine during the transformation of the methyl group of S-adenosylmethionine catalyzed by the enzyme glycine N-methyltransferase (GNMT) [6,7]. These two reactions ultimately produce 5,10-methylenetetrahydrofolate and are dependent on oxidized flavine cofactors (e.g., flavine adenine dinucleotide) [5].

GNMT acts as an essential enzyme that influences synthesis of sarcosine [8]. Due to properties of GNMT, its excessive production causes conversion of glycine

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Fig. 1. Scheme of metabolic pathway of sarcosine biosynthesis and degradation to its non-methylated precursor—glycine and its connection to other important metabolic pathways. Enzymes in scheme are numbered and depicted as follows: 1, DMGDH, dimethylglycine dehydrogenase; 2, GNMT, glycine-N-methyltransferase; 3, SARDH, sarcosine dehydrogenase; 4, SHMT1/2, serine hydroxymethyltransferase; 5, MTHFR, 5,10-methylenetetrahydrofolate reductase; 6, MTR, methionine synthase; 7, BHMT, betaine-homocysteine methyltransferase; 8, CHDH, choline oxidase; 9, MAT, methionine adenosyltransferase; 10, SAM-dependent methyltransferase; 11, PEMT, phosphatidylethanolamine methyltransferase; 12, AHCY, S-adenosylhomocysteine hydrolase; 13, CBS, cystathionine β-synthase; 14, CTH, cystathionase; 15, GCL, glutamate-cysteine ligase; 16, GSS, glutathione synthetase; 17, CDO1, cysteine dioxygenase 1; 18, CSAD, cysteine sulfinic acid decarboxylase; 19, GOT1, glutamate oxaloacetate transaminase I. Dmg stays for dimethylglycine, SAM for S-adenosyl-methionine, SAH for S-adenosyl-homocysteine, PE for phosphatidylethanolamine, PC for phosphatidylcholine, and THF for tetrahydrofolate.
to sarcosine and elevates the sarcosine levels in urine. This makes sarcosine interesting in the field of non-invasive cancer biomarkers. Thus, sarcosine appears to be not only a non-proteogenic amino acid but also an important metabolite in oncogenesis. According to Sreekumar et al., the elevated levels of sarcosine correlated with progression of prostate cancer and metastatic process [9] and accordingly, it has been revealed that supplementation of sarcosine to prostate cancer cell lines induced a selection of invasive phenotype in culture [10]. Nevertheless, the explanation for these phenomena remains unclear. Moreover, the metabolic fate of sarcosine in prostate cancer cells has not been fully resolved yet.

Therefore, the insight into the metabolic pathways of sarcosine and the mechanisms of its regulation are required to be investigated. This study is aimed to evaluate the effects of treatment of three prostatic cell lines (a “non-tumor” PNT1A, a primary tumor-derived 22Rv1 and metastasis-derived PC-3) with three sarcosine-pathway-related amino acids (glycine, dimethylglycine, and sarcosine) on the cell cancer- geneous status. The evaluation was carried out using as a combination of assays determining the effects of these amino acids on the expression of GNMT mRNA, cell migration, invasiveness, their division and growth capabilities.

**EXPERIMENTAL SECTION**

**Chemical Compounds**

All standards and other chemicals were purchased from Sigma–Aldrich (St. Louis, MO) in ACS purity, unless noted otherwise.

**Prostatic Cell Lines**

Three human prostatic cell lines were used in this study: (i) the PNT1A human cell line established by immortalization of normal adult prostatic epithelial cells by transfection with a plasmid containing SV40 genome with a defective replication origin. The primary culture was obtained from the prostate of a 35-year-old male post mortem; (ii) 22Rv1 which is a human prostate carcinoma epithelial cell line derived from a xenograft that was serially propagated in mice after castration-induced regression and relapse of the parental, androgen-dependent CWR22 xenograft. (iii) The PC-3 human cell line established from a grade 4 androgen independent and unresponsive adenocarcinoma from 62-year-old Caucasian male and derived from metastatic site in bone. All cell lines used in this study were purchased from Health Protection Agency Culture Collections (Salisbury, UK).

**Culture Conditions**

PNT1A and 22Rv1 cells were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS). PC-3 cells were cultured in Ham’s F12 medium with 7% fetal bovine serum (FBS). All media were supplemented with penicillin (100 U/ml) and streptomycin (0.1 mg/ml), and the cells were maintained at 37°C in a humidified incubator (Sanyo, Moriguchi, Japan) with 5% CO2. The treatment with amino acids was initiated after cells reached ~60–80% confluency. Cells were then harvested and washed four times with PBS, pH 7.4.

**Cell Content Quantification**

The suspension of 10,000 cells was added to each well of standard microtiter plates (E-plates 16). After addition of medium (200 μl), plates were incubated for 2 days at 37°C to ensure cell growth. To determine the effects on cell viability, the amino acids (sarcosine, glycine, and dimethylglycine) in concentration 0–3 mmol/l were used. Plates were incubated for 24 hr; then, media were removed and replaced by a fresh medium, three times a day. Further, a medium was replaced by 200 μl of fresh medium containing 50 μl of MTT (5 mg/ml in PBS) and incubated in a humidified atmosphere for 4 hr at 37°C, wrapped in aluminum foil. After the incubation, MTT-containing medium was replaced by 200 μl of 99.9% dimethyl sulfoxide to dissolve MTT-formazan crystals. Then, 25 μl of glycine buffer (pH 10.5) was added to all wells and absorbance at 570 nm was immediately determined (VersaMax microplate reader, Molecular Devices, Sunnyvale, CA).

**In Vitro Wound-Healing Assay**

The cells were pipetted into 16-well plate to reach the confluence ~80%. After seeding of cells on the bottom of a plate, a pin was used to scratch and remove cells from a discrete area of the confluent monolayer to form a cell-free zone. After that, cells were re-suspended in a fresh medium enriched with sarcosine, glycine, and dimethylglycine (1.5 mmol/l). After 24 hr, the pictures of cells were taken and compared with pictures obtained in 0 hr, using TScratch software (CSElab, Zurich, Switzerland).
Growth and Proliferation Assay Using Real-Time Cell-Based Assay

The real-time cell-based assay (RTCA) was carried out using the xCELLigence system (Roche Applied Science and ACEA Biosciences, San Diego, CA). After seeding the total number of cells (10,000) in 100 μl medium to each well in E-plate, the attachment, proliferation and spreading of the cells was monitored every 15 min. After 24 hr, amino acids (1.5 mmol/l) or MiliQ water (control measurements) were added and cell impedance was monitored for 250 h. For evaluation of exposure, a “doubling time” function, describing the cell division rate, was employed.

Isolation of RNA and Reverse Transcription

High pure total-RNA isolation kit (Roche, Basel, Switzerland) was used for isolation of cellular RNA. The medium was removed and samples were twice washed with 5 ml of ice-cold PBS. Cells were scraped off, transferred to clean tubes and centrifuged at 20,800 g for 5 min at 4°C. After this step, lysis buffer was added and RNA isolation was carried out from 22Rv1, PNT1A, and PC-3 according to manufacturer’s instructions. Isolated RNA was used for cDNA synthesis. RNA (500 ng) was transcribed using transcriptor first strand cDNA synthesis kit (Roche) according to manufacturer’s instructions. Prepared cDNA (20 μl) from total-RNA was diluted with RNase-free water to a total volume of 100 μl and 5 μl of this solution was directly analyzed by q-PCR.

Quantitative Polymerase Chain Reaction (q-PCR)

q-PCR was performed using the TaqMan gene expression assay system with the Lightcycler 480 II RT-PCR system (Roche, Basel, Switzerland) and the amplified DNA was analyzed by the comparative Ct method using β-actin as a housekeeping gene. The primer and probe sets for β-actin (assay ID: Hs99999903_ m1) and GNMT (Hs00219089-m1) were selected from TaqMan gene expression assays (Life Technologies, Carlsbad, CA). q-PCR was performed under the following amplification conditions: total volume of 20 μl, initial incubation 50°C/2 min followed by denaturation 94°C/10 min, then 40 cycles 94°C/10 sec, 60°C/1 min.

Preparation of Cell Lines for Determination of Patterns of Cellular Amino Acids

The harvested cells were frozen in liquid nitrogen to disrupt their structure. The frozen sample was homogenized using ultrasonic homogenizer SONOPLUS mini20 (Bandelin Electronic, Berlin, Germany). Subsequently, 1 ml of 0.2 M phosphate buffer (pH, 7.0) was added and the sample was homogenized for 5 min. The homogenate was further centrifuged using Microcentrifuge 5417R under the following conditions at 4°C for 15 min. Finally, the supernatant was filtered through a membrane filter (0.45-μm nylon filter disk; Millipore, Billerica, MA) and analyzed.

Ion-Exchange Chromatography

Amino acids including sarcosine were determined using ion-exchange chromatography with Vis detection after post-column derivatization with ninhydrin (AAA-400, Ingos, Prague, Czech Republic), following conditions employed in our previous study [3].

Quantification of Total Protein in Cell Lines

Total protein was determined using the SKALAB CBT 600T kit (Skalab, Svitavy, Czech Republic), on automatic spectrophotometer BS-400 (Mindray, Shenzhen, China), following the manufacturer’s instructions.

Statistical Analysis

Prior all analyses, data were standardized. Correlation analysis followed by hierarchical clustering using the Ward’s method was exploited to reveal dependencies between variables. To analyze the effects of cell line, amino acid used for treatment and the concentration of amino acid used for treatment, factorial ANOVA was used. To reveal dependences in complex data, principal component analysis was employed. Unless noted otherwise, the threshold for significance was P < 0.05. For analyses Software Statistica 12 (StatSoft, Tulsa, OK) was employed.

RESULTS

Cytotoxicity of Sarcosine, Glycine, and Dimethylglycine on Prostate Cells

In the first step, prostatic cell lines were tested for their susceptibility to applied amino acids using MTT assay. Figure 2A–C illustrates that treatment with glycine (Gly), sarcosine (Sar), and dimethylglycine (Dmg), respectively, resulted in low or no inhibition of cell lines growth, observed particularly by the highest applied concentration or above the used concentration range (2.0–3.5 mmol/l). The found data were further employed to design the experimental workflow. Because the undesired cytotoxicity can affect performance of further analyses, concentrations not exceeding 1.5 mmol/l were utilized for subsequent treatments only.
An Effect of Sarcosine, Glycine, and Dimethylglycine on Growth Properties of Prostate Cells

The growth of the cells were tested using wound-healing assay (Fig. 3A), which is an easy, low-cost and well-developed method to measure cell migration in vitro [11]. After formation of a new artificial gap on a confluent cell monolayer and subsequent supplementation with 1.5 mmol/l of sarcosine, glycine, or dimethylglycine, it was found that the cell migration was induced distinctly by sarcosine and glycine in PC-3 (relative free area of 53% and 73%, respectively) and 22Rv1 cells (57% and 87%), which is shown in Figure 3B. Contrary to that, dimethylglycine treatment suppressed migration in 22Rv1 and PC-3 (150% in 22Rv1 and 202% in PC-3), whereas the treatment of PNT1A led to increase in cell migration (80%). Using the real-time cell-based assay, we also focused on investigation of the effects of sarcosine, glycine, and dimethylglycine on the division rates of the tested prostate cells. As shown in Figure 3C, the most significant effects were achieved after cultivation with exogenously added sarcosine, which resulted in elevation of a division rate of 22Rv1 and PC-3 cell lines (time required for their division was 65% and 45%, respectively). Furthermore, the 22Rv1 cells were also boosted by treatment with glycine and dimethylglycine.

An Effect of Treatment of Prostate Cells With Sarcosine, Glycine, and Dimethylglycine on Expression of mRNA of Cellular GNMT

The results shown in Figure 4A demonstrate that relative expression of GNMT mRNA differs among the tested cell lines. The lowest GNMT mRNA expression was identified in non-malignant PNT1A cells, followed by metastatic PC-3 and primary tumor 22Rv1 cells, which corresponds to the known fact that GNMT over-expression is associated with the cancer cells more than with the non-tumor ones [12]. In the case of PC-3 cells, glycine treatment induced significant down-regulation of GNMT mRNA. Similar effect was observed in 22Rv1 cells, where 0.1 and 0.5 mmol/l of glycine stimulated expression of GNMT, however, higher applied concentrations resulted in down-regulation as in PC-3 cells. The highest increase in GNMT mRNA expression by glycine was found in PNT1A cells; nevertheless, the higher concentration (1.0 and 1.5 mmol/l) led to a decrease in GNMT mRNA expression (Fig. 4B). Contrary to relatively low effects of glycine, sarcosine...
supplementation mostly induced down-regulation of GNMT gene among all tested cell lines (PNT1A > PC-3 > 22Rv1) (Fig. 4C). Dimethylglycine treatment stimulated expression of GNMT mRNA in PNT1A cells, whereas this amino acid inhibited expression of mRNA of this enzyme in primary tumor (22Rv1) and metastatic cell lines (PC-3) (Fig. 4D). Taken together, the results demonstrate that supplementation of cells with even low concentrations of sarcosine and its pathway-related amino acids is able to trigger overexpression of GNMT (particularly in the case of dimethylglycine in PNT1A cells) or its down-regulation during treatment of PC-3 cells with glycine, sarcosine and dimethylglycine or, 22Rv1 cells supplemented with sarcosine and dimethylglycine.

**Analysis of the Effect of Treatment of Prostate Cells With Sarcosine, Glycine, and Dimethylglycine on Patterns of Cellular Amino Acids**

In the next step, the effect of treatment with glycine, dimethylglycine and sarcosine on the spectrum of amino acids in the tested prostate cell lines was analyzed. The correlation heatmap showing the response of cellular amino acid spectra to exposure of cells with sarcosine, glycine, and dimethylglycine is shown in Figure 5A. There was a significant positive correlation of all supplemented amino acids with intracellularly measured ones; $r = 0.62, 0.38$, and $0.48$ at $P < 0.05$ for glycine, dimethylglycine, and sarcosine, respectively. Based on factorial ANOVA, a significant effect of all prediction factors on the amino acid pattern was found as follows: cell line $F(28, 144) = 136.6, P < 0.001$ (Fig. 5C), amino acid used for treatment $F(38, 144) = 28.66, P < 0.001$ (Fig. 5D) and the concentration of treatment $F(76, 286) = 7.2, P < 0.001$ (Fig. 5E). The combined effect of all three variables was significant, $F(304, 930) = 2.7, P < 0.001$, too. Noteworthy, the highest sarcosine levels were found in 22Rv1 (mean in untreated cells $3.12 \mu$mol/mg of total protein), followed by PC-3 ($1.78 \mu$mol/mg of total protein) and PNT1A ($0.84 \mu$mol/mg of total protein), which corresponds to the expression of the GNMT gene. Inasmuch, glycine, sarcosine, and dimethylglycine treatment led to large
changes in amino acid patterns in the cells (for details, see Table S1 in supplementary data). The obtained data indicate that the cell line type is highly specific for its amino acid pattern and this pattern is significantly influenced by amino acid supplementation.

**Characterization of Patterns of Cellular Amino Acids**

The previous analyses did not sufficiently highlight trends and relationships in the complex amino acid profile of the cell lines. Therefore, correlations among the individual amino acids detected in the cells were performed (Fig. 5B). Based on the results found by correlation analyses, amino acids can be divided into three clusters: cluster Lys, His, Dmg, Sar, Cys (cluster 1 in Fig. 5B), which is characterized by minimal correlations between these amino acids. The other two clusters, Phe, Ile, Ala, Arg, Thr (cluster 2 in Fig. 5B) and Tyr, Leu, Gly, Val, Met, Pro, Glu, Ser, Asp (cluster 3 in Fig. 5B) are characterized by strong correlation between those amino acids. A specific correlation pattern is apparent for sarcosine and cysteine, which demonstrate a negative correlation with all other amino acids except themselves.

Nevertheless, the correlation analysis did not allow to interpret complex multidimensional relationships between the amino acids’ levels after the treatment of cells with sarcosine, glycine, and dimethylglycine—so called “amino acid patterns” of cell lines. Therefore, the principal component analysis was used. The component analysis allowed us to detect the structure in relationships between amino acid levels, and thus helped us to reveal characteristic patterns for the respective cell lines—non-tumor, primary tumor, and secondary/metastatic tumor cells. A two-factor model was employed with the eigenvalue 3.61, thus 49.4% of total variability of data (30.4 and 19.0 for factors 1 and 2, respectively) is explained. First, cases (cell lines, and amino acids used for supplementation) were projected into a factor plane (Fig. 6A). A color-coding by the cell line revealed a significant clustering of cell lines by a factor 2, whereas non-tumor PNT1A cells are clustered rather by positive values of factor 2, metastatic PC-3.
are associated rather with negative values of this factor. Primary tumor 22Rv1 cluster is located between PNT1A and PC-3. Thus, the second factor is considered as “non-malignant—aggressive tumor cluster.” In the next step, cases were color-coded by the amino acid used for treatment of prostate cell lines. This correlated with factor 1 on a factor plane; for each cell line the precursors—notably glycine was associated with more negative values than sarcosine. Thus, this factor was further designated as “precursor-product” (Fig. 6B). When variables (i.e., determined amino acids) were plotted to this factor plane, a similar shift is apparent (Fig. 6C). Whereas an amino acid pattern of the non-tumor PNT1A cell line is associated rather with negative values of factor 1, a metastatic-derived PC-3 amino acid pattern is associated with positive values of this factor, which corresponds also to “precursor” factor in Fig. 6B demonstrating sarcosine connection to prostate cancer.

DISCUSSION

The metabolic abnormalities of prostate cancer cells have not yet been fully elucidated [13]. Amino acids play an important role in cellular physiology, since they are involved in a number of fundamental metabolic processes [14,15]. Thus, we have focused on determination of response of the prostate cell biomolecules involved in a sarcosine metabolic pathway to supplementation of these cells with amino acids with emphasis on sarcosine, a widely discussed biomarker of PCa.

Only the high concentrations of sarcosine, glycine, and dimethylglycine are toxic to the tested prostate...
cells. Their cytotoxic concentrations are in conformity with those described by Stachlewitz et al., who have found that only the high concentrations of glycine (units of mM) are able to prevent increases in Ca\(^{2+}\) in cells, thereby inhibiting cell proliferation [16]. Likewise, we have found in our previous study, focused on possible effects of sarcosine on PC-3 cells, that only the high concentrations of sarcosine are able to inhibit cell growth, as a result of disruption of redox equilibrium [14]. Importantly, throughout our study, supplementation of prostate cells with tested amino acids did not exceed the concentrations of 1.5 mmol/l.

Sarcosine metabolism is suspicious to be an important part of malignant transformation of prostate cells. According to the changes in the cell mobility and division rates, sarcosine and glycine, but not dimethylglycine, can stimulate the migration of malignant cell lines (22Rv1 and PC-3). These findings are in agreement with a study carried out by Sreekumar et al., who have found that direct addition of sarcosine imparted an invasive phenotype to benign prostate cells and the number of motile prostate cells was significantly higher upon sarcosine treatment \((P = 6.997^{-6}, n = 10)\) [9]. Similar, to that, Khan et al. demonstrated that addition of sarcosine to prostatic GNMT knockdown cells partially rescued their invasive properties, while addition of a sarcosine isomer, alanine, failed to rescue the invasive phenotype [17]. Notably, both studies highlighted the role of glycine, inducing invasion in the cells, however, to a lesser degree than sarcosine. It is plausible that this phenomenon is linked with the conversion of glycine to sarcosine catalyzed by GNMT.

**Fig. 6.** Fingerprinting of prostate cells according to amino acid patterns, principal component analysis. (A) Projection of cases on a two-factor plane, color coded according to the cell line. Notice apparent clustering of cell lines by factor 2, thus this factor is considered “non-tumor–tumor.” (B) Identical projection as (A), color coded by amino acid used for treatment. Notice the clustering for each cell line by Factor 1 so Sar precursors are more to the right from Sar for each cell line, so this factor is considered as “precursor–product.” (C) Projection of variables (cellular amino acids) on two-factor plane identical as (A) and (B). See differential distribution of amino acids between cell lines (PNT1A, 22Rv1, and PC-3)—“left-to-right” shift on factor 1.
Song et al. have reported that GNMT activity is connected with the progression of prostate cancer [12]. In agreement with their data, we have shown that GNMT mRNA expression is low predominantly in benign prostate cells, whereas high in the malignant cells. Hence, our results support the finding that sarcosine, generated from glycine by GNMT could be exploited as a PCA biomarker [3,9]. The GNMT enzyme is expected to play a substantial role in modulating prostate cancer cells invasion [18], and thus can be involved in promotion of the oncogenic potential of prostate cells with subsequent facilitating conversion of glycine to sarcosine. We demonstrated that dimethylglycine causes inhibition of GNMT mRNA expression in malignant cells, however, benign PNT1A were affected in a different manner. Increased amount of endogenously added dimethylglycine can stimulate sarcosine formation through action of dimethylglycine dehydrogenase (DMGDH), utilizing dimethylglycine as a substrate, instead of GNMT; nevertheless, whether the activity of DMGDH is disturbed in prostate cancer cells is unknown. The data found in the present work further illustrate that applied sarcosine significantly inhibited expression of GNMT in PNT1A cells, and to a lower extent in malignant PC-3 and 22Rv1. Thus, it can be hypothesized that by this kind of blocking, GNMT is not able to catalyze transformation of sarcosine to glycine and sarcosine can be thus accumulated in cancerous cells, as was shown in many studies [2,9,19]. However, plausible mechanism of this phenomenon is still not defined. Treatment of cells with glycine triggered only slight expression differences in GNMT, thus it can be stated that its expression is not glycine inducible. Our results illustrate the divergent significance of GNMT among the prostate cell lines and confirm the role of free amino acid pool as important metabolic factor, influencing PCA cells as was suggested by Fu et al. [15].

It has been shown that amino acids are substantial for cellular physiology [20–22] and our results demonstrate that free glycine and sarcosine can influence the GNMT expression in PCA cells. Jain et al. have revealed increased reliance of cancer cells on glycine mitochondrial metabolic pathway, which involves sarcosine and dimethylglycine [23]. Importantly, recent works have identified that sarcosine and related metabolites or their associated metabolic pathways, are central to cancer metastasis [9], cellular transformation [24,25], or murine embryonic stem cell proliferation [26]. Hence, we focused on patterns of amino acids in prostate cell lines and on the effect of exogenous supplementation with sarcosine-related amino acids on amino acid patterns in these cells. Our profiling revealed unique amino acid patterns, exhibiting exceptional specificity toward prostate cell types. This phenomenon is likely connected with disturbances in carbohydrate, lipid, and protein metabolism during oncogenesis. Considering the Warburg effect [27], metabolic shifts in tumor cells from respiration to fermentation should result in an increased demand for consumption of amino acids and complex metabolic derangements reflected by alteration in amino acid patterns. Noteworthy, statistical processing of amino acid patterns revealed that malignant cell lines (22Rv1 and PC-3) exhibit common features in increased (Glu, Sar, Gly, Asp, Ser) and decreased (Ala, Thr, Arg, Ile, Phe, Dmg) amino acids, when compared to benign PNT1A cells (Fig. 5C).

First, our interest was caught by differences in glutamic acid. This amino acid is a precursor for glutamine in its interconvertible biosynthesis. In cancer cells, glutamine is the primary mitochondrial substrate, maintaining mitochondrial membrane potential and integrity [28]. Moreover, it provides support for the NADPH production required for redox control and macromolecular synthesis [29]. Our results show that oncogenesis results in elevated level of intracellular glutamic acid. This phenomenon is likely connected with the fact that many of the signaling pathways promoting oncogenesis also reprograms the glutamine metabolism. For instance, Myc coordinates the reprogramming of metabolism to depend on glutamine and to sustain cellular viability and the citric acid cycle (TCA) anaplerosis through carbon donation [30,31]. Interestingly, glycine supplementation led to a significant elevation of glutamic acid levels within the cell lines; however, the reason explaining this phenomenon is not yet clear. Taken together, divergence in glutamic acid metabolism supports the idea that interventions into metabolism could be a potential therapeutic approach in PCA management, as was shown by Wang et al. [32].

Figure 6C illustrates that contrary to benign prostate cells, in malignant cells, glutamic acid essentially shares similar distribution with glycine and serine. Serine and glycine are linked biosynthetically. They provide the precursors for the synthesis of proteins, nucleic acids, and lipids [33]. Our results show that cancer cells benefit from higher levels of these amino acids. Serine biosynthesis is a component of glycolysis-diverting pathways, resulting in expression of phosphoglycerate dehydrogenase (PHGDH), which is necessary to sustain cancer growth and oncogenic transformation through production of anaplerotic intermediate of TCA α-ketoglutarate [34]. Serine also supports aerobic glycolysis and lactate production by affecting the activity of pyruvate kinase M2 (PKM2), converting phosphoenolpyruvate to pyruvate and one molecule...
of ATP [35]. Glycine promotes tumorigenesis and its upregulation correlates with cell proliferation and poor prognosis. Its conversion significantly contributes to the biosynthetic requirements of purines, ATP and NADPH in cancer cells [23].

Elevated levels of aspartate in both types of malignant prostate cells are likely related to glutaminolysis, which lyses glutamine to citrate [36]. Such process takes place in all proliferating cells and especially in tumor cells, where the TCA is truncated due to an inhibition of aconitase [37]. Aspartic acid, produced from oxaloacetate during conversion of glutamate to \( \alpha \)-ketoglutarate is utilized as a precursor for synthesis of nucleic acids and serine [38]. Taken together, both malignant prostate cell lines exhibited high dependency on glutamine metabolism, when compared to benign ones.

Our data further indicate that glycine supplementation influences all prostate cell amino acid patterns most effectively, which is likely due to a linkage with serine biosynthesis. Glycine treatment also resulted in elevation of amounts of amino acids connected to glutamine metabolism. Hence, we put evidence that glycine plays substantial role in prostate cells and glycine deprivation (dietetic or enzymatic depletion) may be a new strategy for human cancer therapy as was described earlier [23,33,39]. On the contrary, the highest increase in sarcosine concentrations was found after supplementation with exogenous dimethylglycine, whose role in prostate cancer development is not yet well elucidated. Elevated sarcosine levels during prostate cancer progression [3,9,17,19] are thus likely associated with action of both substrates—glycine and dimethylglycine and catalytic actions of corresponding enzymes—GNMT and DMGDH. One may speculate that inhibition of those enzymes may be used in prevention of prostate cancer.

**CONCLUSION**

The present study illustrates that exogenous supplementation of prostate cells with amino acids, closely related to sarcosine metabolism, can significantly affect the expression of GNMT mRNA in tested cells as well as their growth attributes. The results found indicate that sarcosine production is triggered by dimethylglycine treatment more than by glycine; however, glycine, a well-known cancer-related metabolite, significantly influences the prostate cell amino acid patterns. Above-mentioned data support the studies, which demonstrate that the deprivation of some amino acids can be helpful in management of cancer and furthermore proves the elevated levels of sarcosine in primary and secondary tumor cell lines when compared with non-tumor ones, which corresponds to their GNMT expression levels. We anticipate that sarcosine metabolic pathway is highly important in prostate cancer behavior, and thus further studies, dealing with involved amino acids and their enzymes and also with inhibition of their activity in relation to physical and molecular parameters of prostate cells are strongly required to elucidate this phenomenon. Moreover, it was shown that amino acid patterns unequivocally describe if the prostate cells demonstrate malignant or non-tumor parameters.

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