DETERMINATION OF S-ADENOSYLMETHIONINE AND S-ADENOSYLHOMOCYSTEINE IN TWO PROSTATIC ADENOCARCINOMA CELL LINES

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Abstract: Studies of processes leading to cancer tumour progression are one of the major foci of current cancer research. Therefore, it is important to shade the light on the metabolic pathways involved in cancerogenesis. In the present study we have chosen as a model two prostatic adenocarcinoma cell lines (PC-3 and LNCaP) for the comparison of the metabolites involved in tumour growth. Applying the HPLC-MS method, we determined and compared the concentration levels of S-(5'-adenosyl)-L-homocysteine (SAH) and S-(5'-adenosyl)-L-methionine (SAM), that are involved in the tumour growth processes. SAM/SAH ratio, a methylation index describing the aggressiveness of tumour cells, was determined here. The methylation index was 0.4 and 1.2 for PC-3 and LNCaP cells, respectively, pointing out that the PC-3 cells are more aggressive than LNCaP cells. This finding is of great importance for our following studies.

Key Words: SAM, SAH, methylation index, PC-3, LNCaP, metallothionein

INTRODUCTION

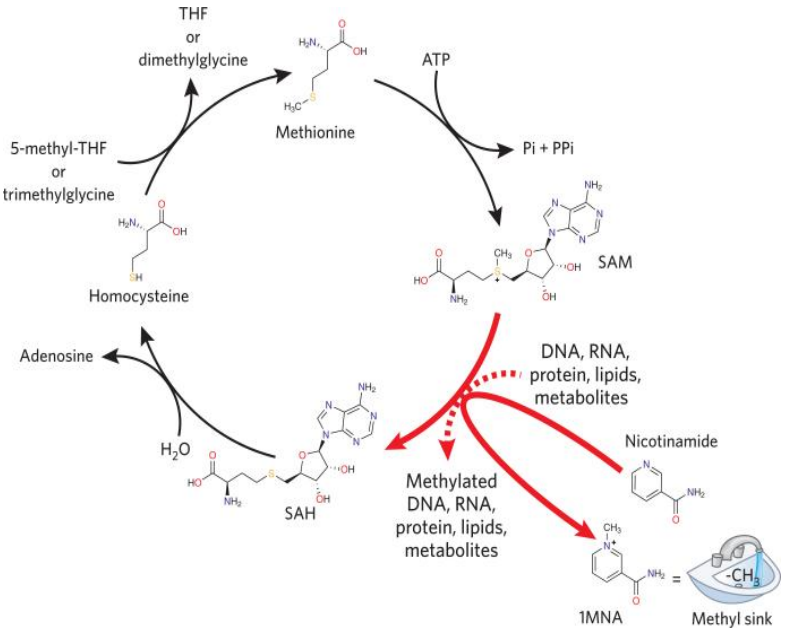
Prostate cancer is the most common noncutaneous cancer in men in the Czech Republic. An estimated prevalence in years 2012-2017 is 50.4% (Ferlay et al. 2013). The processes leading to prostate cancer progression are still poorly understood. Therefore the studies of metabolomic pathways involved in the tumour growth are of a high importance and could subsequently lead to the discovery of new approaches to tumour growth inhibition. Metallothionein (MT), the cysteine rich metalloprotein, is one of the potential markers for tumour disease development. There is important evidence that increased content of heavy metals and MTs in tumour tissues is connected to the increased invasiveness and metastasizing of a tumour (Gumulec et al. 2014).

The focus of our current research is on metallothionein biomarkers and their distribution in biological tissues. In our previous study we have successfully used MALDI-TOF MSI (matrix assisted laser desorption/ionization time-of-flight mass spectrometry imaging) to acquire 2D spatial distribution of chicken metallothionein MT-1 in chicken embryo (Guran et al. 2015). Recently, we have also used MALDI-TOF MSI to determine spatial distribution of human metallothionein MT-1X in PC-3 tumour xenografts (Heger et al. 2016). With respect to these results we discussed other factors, which are linked to MT expression and tumour growth. In closer look onto metabolic processes involved in the production of MT, we have found that the homocysteine produced in methionine cycle from SAH (Figure 1) is further transformed into cysteine (Figure 2), which is, *inter alia*, used in synthesis of cysteine-rich proteins, such as MTs (Hughes et al. 2009, Shlomi and Rabinowitz 2013). One of the most important processes in tumour cells is a methylation of DNA, proteins, peptides and other biomolecules. S-(5'-adenosyl)-L-homocysteine (SAH) and S-(5'-adenosyl)-L-methionine (SAM) are intermediates in methionine cycle and participate in methylation processes. SAH is formed by SAM demethylation with

a help of nicotinamide N-methyl-transferase. This enzyme is overexpressed in some types of tumours and therefore it can enhance cancer aggressiveness by draining methyl groups from SAM. The SAM/SAH ratio is one of the parameters used for describing the aggressiveness of tumour cells. Moreover, the so-called methylation index, expressed as ratio SAM/SAH, is connected with tumour development (Shlomi and Rabinowitz 2013). These findings inspired us to gain some information about SAM and SAH concentration levels in two prostatic adenocarcinoma cell lines (PC-3 and LNCaP). We have used HPLC-ESI-QqTOF MS for determination of SAM and SAH as it is one of the most suitable methods for metabolomics (Wu and Li 2016).

The aims of this study were i) to develop/adapt and validate HPLC-ESI-QqTOF MS method for analysis of SAM and SAH in cells, especially in PC-3 and LNCaP cell lines, ii) use the method to determine concentrations of SAM and SAH in PC-3 and LNCaP cells, and iii) to determine the methylation index (SAM/SAH). The obtained results will be used in future experiments to find whether the concentration levels of MTs are in correlation with methylation index.

Figure 1 Methionine cycle describing how SAM and SAH are involved in methylation processes. THF is tetrahydrofolate, 1MNA is 1-methylnicotinamide. Adapted with permission from Shlomi and Rabinowitz (Shlomi and Rabinowitz 2013).



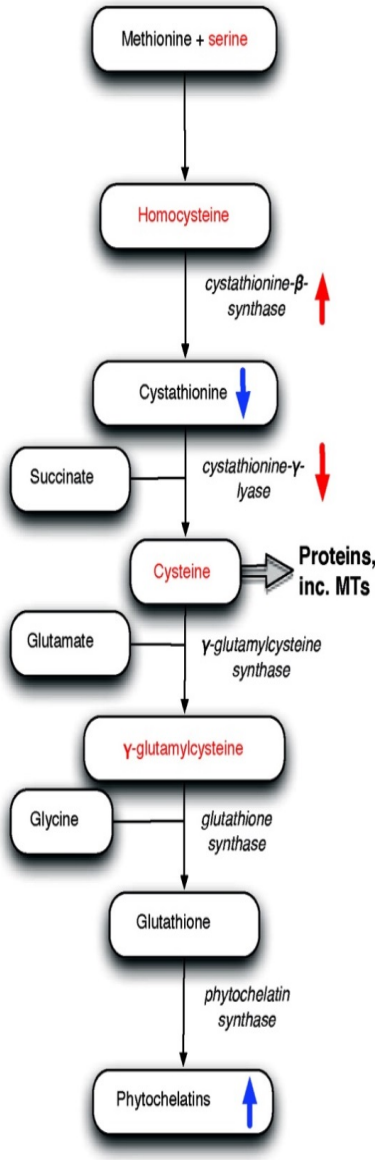


Figure 2 (on right) Metabolic processes resulting from methionine cycle connected with expression of metallothioneins. Adapted with permission from Hughes et al. (Hughes et al. 2009).

MATERIAL AND METHODS

Chemicals

All chemicals used for HPLC-ESI-QqTOF MS were purchased in LC-MS quality from Sigma Aldrich (St. Louis, MO, USA). Other chemicals were purchased from the same company in ACS quality, unless otherwise noted. As SAM standard was used S-(5'-adenosyl)-L-methionine iodide (mass ratio of SAM in this substance is 0.7564). As SAH standard was used S-(5'-adenosyl)-L-homocysteine (crystalline).

Cell culture and harvesting

The PC-3 human cell line, established from a grade 4 androgen-independent prostatic adenocarcinoma, was purchased from the Health Protection Agency Culture Collection (Saltsbury, UK). The cells were maintained as monolayer culture in the Ham's F-12 medium containing 7% fetal bovine serum (FBS) and penicillin (100 U/ml) and streptomycin (0.1 mg/ml). Cells were grown at 37 °C in a humidified incubator Galaxy® 170 R (Eppendorf, Hamburg, Germany) with 5% CO_2. Then, the cells at ~80% confluence were harvested using Trypsin/EDTA. One million cells were pelleted by centrifugation at 200 × g for 10 min at 4 °C. Finally, pellets were frozen and stored at -80 °C until further use.

The LNCaP human cell line was established from a lymph node metastatic lesion of prostatic adenocarcinoma. Cells were maintained as monolayer culture in the RPMI-1640 with 10% FBS. Medium was supplemented with penicillin (100 U/ml) and streptomycin (0.1 mg/ml), and the cells were maintained at 37 °C in a humidified incubator Galaxy® 170 R (Eppendorf, Hamburg, Germany). Afterward, the cells at ~80% confluence were harvested, washed four times with PBS (pH 7.4) and Trypsin/EDTA. Cells were pelleted by centrifugation at 200 × g for 10 min at 4 °C and counted using Countess IIFL Automated Cell Counter (Life Technologies, Carlsbad, CA). Finally, pellets were frozen and stored at -80 °C until further use.

SAH and SAM extraction

A following extraction protocol was adapted from Stevens et al. (Stevens et al. 2010) and slightly modified by not using a vacuum centrifuge for extract concentration. Methanol with acetic acid (1M) in a volume ratio 80:20 was selected as extraction solvent. According to the literature, this mixture yields good extraction recoveries for both SAM and SAH (Stevens et al. 2010). Firstly, 600 µl of the extraction solvent were added to the frozen pellets. Then the sample was slowly thawed on ice. Subsequently, the cells were shock-frozen in liquid nitrogen and thawed on ice again. The freeze/thaw cycle was performed three times and the sample was vortexed between each cycle. The sample was centrifuged at 9000 × g for 5 min at 4 °C and the supernatant was transferred to a 1.5 ml glass vial. The pellet was washed twice with 200 µl of extraction solvent and all supernatants were combined.

Evaluation of SAM and SAH extraction recoveries

The following protocol for evaluation of extraction recoveries for SAM and SAH was adapted from Stevens et al. (Stevens et al. 2010). Cell pellets were spiked in quadruplicate with three different concentration levels and extracted as was described in previous paragraph. The concentrations of spikes were selected for each analyte based on its intracellular concentration range found in literature. If c is a mean concentration of each analyte found in literature, then the following concentrations were spiked: c, 0.5c, and 2c.

HPLC-ESI-QqTOF MS method

Cell extracts were separated on C18 reverse phase column Phenomenex Kinetex EVO (5 µm particles; 150 × 4.6 mm) in HPLC system consisted of two pumps ESA Model 584 and an autosampler ESA Model 542 (ESA Inc., Chelmsford, USA). Flow rate was 0.5 ml/min. Injected sample volume was 20 µl. Mobile phase A consisted of water with 0.1% formic acid. Mobile phase B consisted of methanol with 0.1% formic acid. The following gradient was programmed: 0 min 0% B; 4 min 30% B; 8 min 40% B; 8.1 min 100% B; 13 min 100% B; 13.1 min 0% B; 18 min 0% B; 18.1 min STOP. HPLC was coupled with ESI-QqTOF mass spectrometer Bruker Maxis Impact (Bruker Daltonik GmbH, Bremen, Germany). The following parameters of mass spectrometer were used: End plate offset potential 500 V; capillary potential 3500 V; nebulizer gas (N_2) pressure 0.3 MPa; dry gas (N_2) flow rate 12 l/min; drying temperature 300 °C. Mass range was set from 50 to 3000 *m/z*. Prior to HPLC-MS analysis a mass spectrometer was calibrated using ESI-TOF Tuning mix (Sigma Aldrich, St. Louis, MO, USA).

Statistical evaluation

The HPLC-ESI-QqTOF MS method was calibrated on SAM and SAH standard working solutions prepared in used extraction solution at concentrations 0.04, 0.2, 1, 5 and 25 µM. Each calibration solution was measured five times. Linear regression with least squares approach was used for calculating regression equation and constructing calibration curve. Dixon's Q test was used for identification of

possible outliers among results. The linearity of calibration curve and a significance of intercept *a* ($y = bx + a$) was tested.

For determination of SAM and SAH concentrations in cell extracts, the method of standard additions at constant volume was used. Four standard additions were used for each cell extract. Into five glass vials were added 100 µl of cell extract and 0, 10, 20, 30 and 40 µl of SAM and SAH standards at concentration 500 µM (both) prepared in extraction solution. Finally, each vial was filled up to 1 ml with extraction solution and all solutions were measured three times.

For data analysis was used Microsoft Excel 2010 (Microsoft, Redmond, WA, USA). Statistical significance for all tests was accepted at $p < 0.05$.

RESULTS AND DISCUSSION

In order to analyze SAH and SAM in PC-3 and LNCaP cells, the confirmation of suitability of HPLC-ESI-QqTOF MS method was performed by extensive literature search. Several publications using HPLC and ESI-MS methods for SAM and/or SAH analyses were found (Iglesias González et al. 2015, Klepacki et al. 2013, Stevens et al. 2010). Therefore the method similar to the one used by Stevens et al. (Stevens et al. 2010) was selected for optimization on SAM and SAH standards. In Figure 3A is shown typical chromatogram of SAM and SAH standard calibration mixtures with mass spectra of SAM and SAH. In Figure 3B are shown calibration curves for SAM and for SAH. Basic analytical parameters of method calculated from calibration analysis, with measured extraction recoveries, are shown in Table 1.

*Figure 3 Calibration of HPLC-ESI-QqTOF MS method on SAM and SAH standards. (**A**) A typical chromatogram of SAM (purple line) and SAH (blue line) standard calibration mixture. Under the chromatogram are shown mass spectra of SAM and SAH. (**B**) Calibration curves for SAM (purple points) and SAH (blue points) in a linear range from 0.04 to 25 µM.*

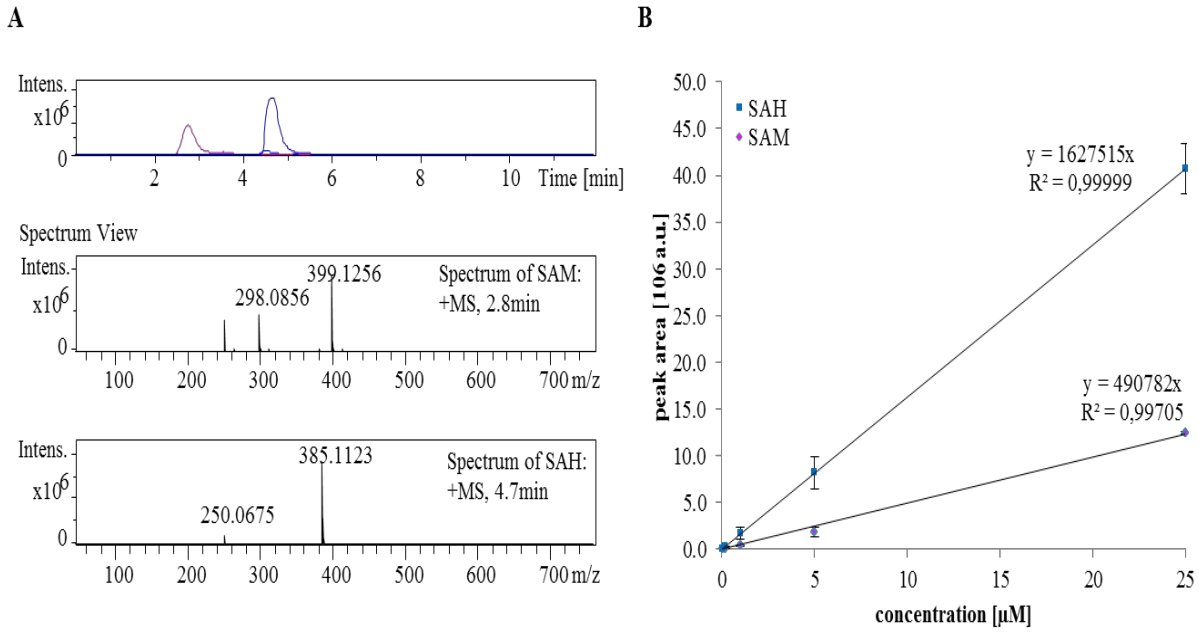


Table 1 Analytical parameters for HPLC-ESI-QqTOF MS method used to determine SAM and SAH concentrations. Number of measurements per one calibration sample was 5.

	MW	Regression equation	Linear dynamic range [µM]	LOD [µM]	LOQ [µM]	Extraction recovery [%]
SAM	398.14	y = 490782x	0.04 – 25.00	0.08	0.27	85 – 103
SAH	384.41	y = 1627515x	0.04 – 25.00	0.01	0.02	83 – 102

Linear range covers the usual concentration range of SAM and SAH in extracts from million cells (Stevens et al. 2010) pointing out that this method is suitable for measuring SAM and SAH in cell extracts. As it is mentioned in material and methods section, the determination of SAM and SAH in cell extracts was done by the method of standard additions at constant volume. In Table 2 are shown determined concentrations of SAM and SAH in PC-3 and LNCaP cells.

Table 2 Determined concentrations of SAM and SAH in PC-3 cells and LNCaP cells by HPLC-ESI-QqTOF MS method and corresponding methylation indexes (SAM/SAH). In total were used three million PC-3 cells and three million LNCaP cells. Number of measurements per one sample with each standard addition was 3.

PC-3 cells	c [μM/10^6 cells]	c [μmol/10^6 cells]	SD [μM/10^6 cells]	SD [μmol/10^6 cells]	RSD [%]
SAM	3.58	0.004	0.19	0.0002	5
SAH	9.58	0.010	0.12	0.0001	1
SAM/SAH	0.4				
LNCaP cells	c [μM/10^6 cells]	c [μmol/10^6 cells]	SD [μM/10^6 cells]	SD [μmol/10^6 cells]	RSD [%]
SAM	18.63	0.019	0.38	0.0004	2
SAH	15.87	0.016	0.43	0.0004	3
SAM/SAH	1.2				

As it is shown in Table 2, the determined concentration of SAM was higher nearly six times in LNCaP cells than in PC-3 cells. The concentration of SAH in LNCaP cells was higher at least 1.5x. The most important information is obtained by calculation of the methylation index, which was 1.2 for LNCaP cells and 0.4 for PC-3 cells indicating that PC-3 cells are probably more aggressive than LNCaP cells. This finding is in correlation with higher metastatic potential of PC-3 than of LNCaP (MuraliKrishna et al. 2005).

CONCLUSION

In this study, the concentration levels of SAM and SAH in PC-3 and LNCaP cells were analyzed by HPLC-ESI-QqTOF MS and corresponding methylation indexes (the ratio SAM/SAH) were calculated. It was found that methylation index for LNCaP cells was 1.2 and for PC-3 cells was 0.4. This means that PC-3 cells are more aggressive than LNCaP cells. These findings are of a high importance for our future experiments focused on correlation between methylation index and expression of metallothioneins. Moreover, in future experiments the UPLC-MS method will be used to improve (decrease) the time of the analyses and those minimize the consumption of chemicals.

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