Amino Acids

Evaluation of polyamines as marker of melanoma cell proliferation and differentiation by an improved High Performance Liquid Chromatographic method

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Abstract:	The differentiation therapy is focused on the identification of new agents able to impair the proliferative and metastatic potential of cancer cells through the induction of differentiation. Although several markers of cell differentiation on tumor cells have been identified, their causal relationship with neoplastic competence has not been characterized in sufficient detail to propose their use as new pharmacological targets useful for the design of new differentiation agents. Polyamines level in cancer cells and in body fluids was proposed as potential marker of cell proliferation and differentiation. The main advantage of this marker is the possibility to evaluate the antineoplastic activity of new drugs able to induce cell differentiation and consequently to inhibit tumor growth and metastasis. The presented report shows a simply and highly reproducible reverse-phase high performance liquid chromatographic (HPLC) method for the determination of ortho-phtalaldehyde (OPA) derivatives of polyamines: putrescine (PUT), cadaverine (CAD),

	spermidine (SPD) and spermine (SPM). The novelty of this method is the fluorescence response for OPA-derivate of SPM, generally low in other procedures, that has been significantly improved by the use of a fully endcapped packing material with minimal silanol interactions. The limits of detection for PUT, CAD, SPD and SPM were 0.6, 0.7, 0.8, and 0.4 pmoles/mL, respectively. The analysis time was ≤ 20 min, and the relative recovery rate was of about 97%. In order to verify the usefulness of this method, it has been validated in a murine melanoma cell line (B16-F10) treated with two theophylline derivatives (namely 8-chlorotheophylline and 8-bromotheophylline). These two compounds increased the activity of tissue transglutaminase (TG2) and the synthesis of melanin, two recognized markers of melanoma cell differentiation, and significantly reduced the levels of intracellular polyamines.
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Evaluation of polyamines as marker of melanoma cell proliferation and differentiation by an improved High Performance Liquid Chromatographic method

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Abstract

The differentiation therapy is focused on the identification of new agents able to impair the proliferative and metastatic potential of cancer cells through the induction of differentiation. Although several markers of cell differentiation on tumor cells have been identified, their causal relationship with neoplastic competence has not been characterized in sufficient detail to propose their use as new pharmacological targets useful for the design of new differentiation agents. Polyamines level in cancer cells and in body fluids was proposed as potential marker of cell proliferation and differentiation. The main advantage of this marker is the possibility to evaluate the antineoplastic activity of new drugs able to induce cell differentiation and consequently to inhibit tumor growth and metastasis.

The presented report shows a simply and highly reproducible reverse-phase high performance liquid chromatographic (HPLC) method for the determination of *ortho*-phtalaldehyde (OPA) derivatives of polyamines: putrescine (PUT), cadaverine (CAD), spermidine (SPD) and spermine (SPM). The novelty of this method is the fluorescence response for OPA-derivate of SPM, generally low in other procedures, that has been significantly improved by the use of a fully endcapped packing material with minimal silanol interactions. The limits of detection for PUT, CAD, SPD and SPM were 0.6, 0.7, 0.8, and 0.4 pmoles/mL, respectively. The analysis time was ≤ 20 min, and the relative recovery rate was of about 97%. In order to verify the usefulness of this method, it has been validated in a murine melanoma cell line (B16-F10) treated with two theophylline derivatives (namely 8-chlorotheophylline and 8-bromotheophylline). These two compounds increased the activity of tissue transglutaminase (TG2) and the synthesis of melanin, two recognized markers of melanoma cell differentiation, and significantly reduced the levels of intracellular polyamines.

Keywords: polyamines, OPA-derivatization, melanoma cells, differentiation therapy.

Introduction

Putrescine (PUT), cadaverine (CAD), spermidine (SPD) and spermine (SPM) are naturally occurring polycationic alkylamines, that are absolutely required for eukaryotic cell growth (Figure 1). These biogenic amines, commonly called polyamines, have specific roles on cell cycle, apoptosis, immune system, neurological as well as pulmonary functions (Moschou and Roubelakis-Angelakis 2014; Hesterberg et al. 2018; Igarashi and Kashiwagi 2019). Their levels are regulated by multiple pathways, such as synthesis from amino acid precursors, cellular uptake mechanisms, as well as stepwise degradation and efflux (Thomas and Thomas 2001; Wallace et al. 2003).

Interestingly, the intracellular content of polyamines, as well as their metabolic pathway are frequently dysregulated in cancer cells, thus providing a unique set of targets for therapeutic intervention (Casero et al. 2018). In fact, polyamines biosynthesis in tumor cells is highly upregulated, and this evidence correlates with the increase in tumor cell proliferation rate compared to the normal counterpart. To this matter, the intracellular content of polyamines is generally considered an important marker of cell proliferation (Bachrach et al. 2001). In addition, polyamines leave cells and accumulate in tumr tissue and their level increases in body fluids of cancer patients. Therefore, assays of urinary and blood polyamines have been proposed to detect cancer and to determine the success of chemotherapy in patients after surgical resection (Russell et al. 1975; Russell 1983). Since drugs that inhibit the synthesis of polyamines can reduce cancer growth, they have been used for therapeutic purposes (Bachrach 2004). However, Identification of more effective molecules is required. Recently, drugs able to induce differentiation in cancer cells, reducing their proliferative and metastatic potentials, have attracted considerable attention and proposed for differentiation therapy approacches (Thiele et al., 2000). In this regard, having a sensitive and reliable analysis method, capable of evaluating the intracellular levels of biogenic amines in tumor cells and in body fluids, appears to be an

essential requirement in the choice of anticancer agents with greater antiproliferative potential *in vivo* (Lentini et al. 2007). Direct determination of polyamines presents a challenging analytical problem, since they are usually hydrophobic, poor chromophores and often occur at low concentration in biological fluids. However, their analysis after the labelling with *ortho*-phtalaldehyde (OPA) and β-mercaptoethanol combined with fluorescence detection after HPLC chromatographic separation is particularly effective (Beninati et al. 1988; Venza et al. 2001; Frank and Powers 2007; Raza and Al-Shabanah 2010).

Tissue transglutaminase (type 2 tranglutaminase; TG2) catalyzes the post-translational modification of cellular proteins by polyamines, which results in the control of cell differentiation in different tumors (Tabolacci et al. 2019). Therefore, also the study of the effect of new drugs on TG2 activity could represent a further method to predict their antitumor potential. In addition, melanin protein represent a differentiation marker unique to melanocytes, which is abnormally upregulated in neoplastic melanocytes (Jimbow et al. 1993).

Theophylline (Theo, 1,3-dimethylxanthine) represents a well-known inducer of differentiation in melanoma cells (Lentini et al. 2000; Cordella et al. 2019). Therefore, we investigated the potential of two Theo derivatives with respect to the control compound Theo as inducers of differentiation in melanoma cells, namely 8-chloroteophylline (1,3-dimethyl-8-chloroxanthin; 8-Cl-Theo) and 8-bromotheophylline (8-Br-Theo), analyzing the intracellular concentration of polyamines, the activity of TG2 and the melanin content. The first studied compound is 8-Cl-Theo, a drug related to classical methylxanthines (Theo and caffeine), able to block adenosine receptors (Halpert et al. 2002). 8-Br-Theo is a drug used as a weak diuretic in combination with other drugs (Mitsias and Vovolis 2011; Shah et al. 2014).

The present work allows evidence of a new more sensitive method developed for the reverse-phase HPLC determination of intracellular polyamines using OPA and β-mercaptoethanol as pre-column derivatizing agents. This analytical technique offers advantages of great simplicity and rapidity, high degree of versatility and specificity. The method was tested by analysis of cell

lysates from primary cultures of mouse epidermal keratinocytes (MEK) as a control and human (SKMEL-110) and murine (B16-F10). In addition, this technique has been applied in determining the potential role of 8-Cl-Theo and 8-Br-Theo in the differentiation of melanoma B16-F10 cells, evidenced by decreased intracellular levels of polyamines. This multi-parameter analysis could represent as a new way to screen for new differentiative agents.

Materials and Methods

Reagents

All solutions were prepared with ultra-pure water, generated by Elix® 5 Water Purification Systems (Millipore, Billerica, MA, USA) water-deionizer. Chromatographic grade acetonitrile, tetrahydrofuran, methanol, chloridric acid, perchloric acid (PCA) came from Mallinckrodt Baker (Phillisburg, NJ, USA). Tris(hydroxymethyl)aminomethane, boric acid, OPA, β-mercaptoethanol, PUT, CAD, SPD, SPM, diaminooctane (DAO), trichloroacetic acid (TCA), and all reagents were from Sigma Aldrich (St. Louis, MO, USA). Dulbecco's minimum essential medium (D-MEM), fetal calf serum (FCS), trypsin/EDTA 2.5%, 200 mM glutamine, penicillin/streptomycin (10,000 UI/ml) were from Gibco (Grand Island, NY, USA).

Cell cultures

Highly metastatic B16-F10 murine and SKMEL-110 human melanoma cells (obtained from I.J. Fidler, University of Texas, M.D. Anderson Cancer Center, Houston, TX, USA) were propagated under standard culture conditions. Mouse epidermal keratinocytes (MEK) were prepared from BALB/c mice as described (Hennings et al. 1980). Cell viability was tested by the Trypan Bleu (0,25%) exclusion test at different interval times (24, 48 and 72 h). Protein was measured by the method of Bradford using bovine serum albumin (BSA) as standard.

Polyamine extraction

Cell lysates were obtained after treatment of cells with 7% PCA at 4°C for 30 min. DAO was added as an internal standard (20 µM final concentration) and samples were centrifuged (14,000×g for 15 min). Pellet was used for protein determination, whereas the supernatant was treated with 10N potassium hydroxide (2N final concentration) for PCA neutralization. Potassium perchlorate was removed by centrifugation. The supernatant was filtered and the pH adjusted to 9.0 with 1N HCl.

HPLC and chromatographic conditions

Determination of polyamines was performed by AKTABASIC 10 HPLC apparatus (Pfizer, New York, NY, USA), equipped with a 100 µl sample loop. Reverse-phase separations were conducted at room temperature in a LC-18 Supelcosyl column (150mm x 4,6mm, 3 µm), equipped with a guard column Discovery HS C18 (2cm x 4mm, 3 µm) (Supelco, Bellefonte, PA, USA). The derivatives were separated on two mobile phases. A (95% 350 mM sodium citrate, pH 4.0; 5% tetrahydrofuran) and B (45% 350 mM sodium citrate, pH 4.0; 40% acetonitrile; 15% tetrahydrofuran). The elution procedure consisted in a linear gradient from 50% to 100% of buffer B in 5 min, and an isocratic elution for 15 min, at a flow rate of 1.0 mL/min. Detection was accomplished using a spectrofluorimeter (Jasco FP-1520, Easton, MD, USA). Fluorescence detector was set at λ_{ex} 330 nm and λ_{em} 445 nm. The areas of the chromatographic peaks have been integrated with the software UNICORN start 1.1.

OPA derivatization and injection of sample into the HPLC column

The OPA solution (4 mg/mL of methanol) was diluted with 1M sodium borate buffer (pH 9.0) and β-mercaptoethanol (for a final volume of 5 mL: 4.74 mL sodium borate buffer, 0.25 mL OPA solution, 10 µL β-mercaptoethanol). The OPA reagent may be stored at -20°C for five

days. Samples were mixed with OPA reagent in a ratio 1:1. OPA-derivatized sample (100 µL) was injected into the HPLC column. The overall time of mixing of OPA with sample and injection was exactly 30 sec (see Table 1 for the fluorescence decay with the time).

Cell proliferation

Melanoma cells were seeded and grown in 6-well tissue culture plates in complete culture medium supplemented as reported above, and treated with Theo, 8-Cl-Theo and 8-Br-Theo (1 mM) for 24, 48 and 72 hours. Cells were harvested and counted with a Neubauer modified chamber.

Transglutaminase activity assay and melanin content evaluation

TG2 assay was performed as described previously (Chung and Folk, 1972). Briefly, cells were cultured as described in previous paragraph and in the presence of [^{14}C]-methylamine (46.6 mCi/mmol, 0.5 µl/mL DMEM). Then, cells were harvested, counted, and washed twice in PBS. Cell proteins were precipitated in 10% TCA, washed extensively, solubilized in 0.1 N NaOH at 37 °C. Radiolabelled amine incorporation into cell protein was measured with a scintillation counter (Beckman LS-5000TD, CA, USA).

Determination of intracellular melanin content in both control and treated cells was performed as previously described (Lotan and Lotan 1980).

Statistical analysis

Results were expressed as the mean ± SD of at least three different determinations. Data were analyzed using the Student's unpaired t-test. Differences were considered to be significant at $p < 0.05$. The intra-assay coefficient of variation (cv) was below 3% as determined by replicate analysis of a standard solution (n=10) and a control cell lysate (n=10), obtained from mouse

epidermal keratinocytes (MEK) in a single run. The inter-assay (CV) was between 2% and 10% as determined by replicate analysis of the same MEK cell lysate sample stored at -70°C and analyzed ten separate time over a period of one month. Straight line equations and linear regression coefficients (R) were calculated by UNICORN 4.11 software by Amersham Biosciences.

Results

Polyamines derivatization

Under all conditions of derivatization tested, maximum fluorescent response was observed at 30-sec of incubation time (see Methods section). Longer periods of incubation resulted in progressive loss in fluorescence for all amines (Table 1). In particular, fluorescence of SPM was reduced by almost 40% in about 120 sec. A final concentration of OPA of 100 µg/mL was found to provide the greatest fluorescent intensity for all polyamines and DAO concentrations tested. The optimal condition of pH for the derivatization mixture was found at 9.0 (data not shown). Due to the minimal silanol interactions, and the high ionic strength of buffers, our separation shows a permanence of OPA-SPM derivative into the column of only 300 sec. Therefore, the method is able to recover most of the fluorescence for this polyamine. The rapid degradation of SPM as OPA-derivative has been ascribed to the presence on this amine of unreacted secondary amino groups, which is a limiting factor for the chromatographic analysis with HPLC packing material, leading to longer retention times (Corbin et al. 1989). In our method, the reduction of the retention time for OPA-derivatives of SPD and SPM was achieved by the use of a column packed with an ion-free resin (Supelcosyl) and buffers with high ion-strength (see Methods section).

Retention time reproducibility

The chromatogram of a standard mixture containing 0.5 nmoles of PUT, CAD, SPD, SPM and DAO is shown in Figure 2A. Our method provides an excellent separation of polyamines under consideration. Following HPLC conditions described above, the retention times were: 5,08 ± 0.07 min for SPM (peak 1), 7.11 ± 0.08 min for SPD (peak 2), 10.03 ± 0.05 min for PUT (peak 3), 11.31 min ± 0.04 min for CAD (peak 4) and 15.63 ± 0.09 min for DAO (peak 5) (p<0.005). These values recurred in repeated analysis and indicated that polyamine retention times were reliable and reproducible.

Linearity

Calibration curves were elaborated for DAO, PUT, CAD, SPD and SPM, plotting the peak-areas versus known concentrations of each OPA-derivatized polyamine (range from 50 to 1000 pmoles). The amount of each amine was obtained from the peak area using the following equations: pmoles DAO = (peak area + 10.285)/0.3551; pmoles PUT = (peak area + 1.0854)/0.1633; pmoles CAD = (peak area + 3.1972)/0.1991; pmoles SPD = (peak area - 1.7786)/0.1328; pmoles SPM = (peak area + 1.2322)/0.1423. The linear regression (R) coefficients were: 0.997 for DAO; 1.0 for PUT; 0.998 for CAD; 0.998 for SPD; 0.999 for SPM. The C.V. for the peak area was below 3% (n=10).

Sensitivity

Injection of known amounts for each of the amines was used to detect the minimum detection levels. A signal-to-noise ratio of approximately 2 was obtained with 2.7, 3.3, 2.5, and 2.9 pmoles for PUT, CAD, SPD and SPM, respectively.

Recovery

A known amount of DAO (20 µM final concentration) was added to cell lysate samples as internal standard. The chromatographic response of DAO allowed quantification of eventual sample loss. The recovery of each polyamine derivatives was found in the range of about 97% (n=10).

Applications in different melanoma cell lines

Cell lysates from B16-F10, SKMEL-110 melanoma cells and MEK as a control after different passages of culture were analyzed. The chromatograms of cell lysates from MEK (Figure 2B), B16-F10 (Figure 2C) and SKMEL-110 (Figure 2D) cells were acquired. A good separation of the polyamines has been obtained, without the presence of overlapping peaks and no interferences from amino acids were found. The baseline is particularly stable and the retention times are within the ranges shown in the standard chromatograms of Figure 2A. Identification of polyamines on the chromatograms was performed adding known amounts of their standards to cell lysates. The corresponding increase of the peak area for each polyamine was the clear proof of their identification. The concentration of polyamines in MEK cells was used as a reference (Piacentini et al. 1988).

The amount of PUT and CAD per mg of protein in cell lysates is given in Figure 3A, whereas the content of SPD and SPM are shown in Figure 3B. The pattern of polyamine levels is different in the three cell lines analyzed. PUT and CAD levels are less than 2 nmoles/mg of protein for all cell lines tested. In particular, PUT and CAD are undetectable in B16-F10 and MEK cells, respectively. SPM content is similar (<15 nmoles/mg protein) for all cell lines, whereas SPD level shows the major changes. In fact, the content of SPD is 40 nmoles/mg protein (MEK); 4 nmoles/mg protein (B16-F10); 80 nmoles/mg protein (SKMEL-110).

Polyamines levels in the analysis of new differentiative agents

In order to underline the usefulness of this method, B16-F10 melanoma cells were treated with 8-Cl-Theo and 8-Br-Theo (Figure 4A). Treatments of B16–F10 melanoma cells with 1 mM Theo and its derivatives reduced cell proliferation in a time-dependent manner (Figure 4B). In particular, the effects of 8-Cl-Theo and 8-Br-Theo are more marked with respect to Theo. This effect is due to also a slight increase in cell toxicity (data not shown). The intracellular concentration of polyamines was determined after 72 h of treatments. PUT concentration was detected only in traces for all samples. SPD and SPM content was significantly decreased with respect to control (Figure 4C.). Exposure to methylxantines caused the reduction of SPM levels by about 35%. SPM intracellular concentration decreased by about 50% after treatments with the two halogenated-theophylline more than the treatment with Theo. Since Theo represents a well-known TG2 inducer, the effects of 8-Cl-Theo and 8-Br-Theo on TG2 activity and melanin synthesis were evaluated after 72 h of treatments. As shown in Figure 4D, TG2 activity increased by about two fold in all treatments. Similar results are obtained for melanin content, that is significantly augmented (Figure 4D).

Discussion

The aim of the present study was to develop a simple, rapid and highly sensitive HPLC method that provides adequate and reproducible separation of biogenic polyamines. This was accomplished with the aid of an ultra-pure fully endcapped packing material, characterized by a high pH resistance, due to low silanol and trace metal interactions, ideal for the analysis of basic compounds. The increase in sensitivity, particularly for SPM, has been achieved by means of an ultra-pure, fully endcapped packaging material, characterized by high pH resistance, by means of low interactions between silanols and trace metals, ideal for the analysis of basic compounds.

Endcapping is a well known technique that significantly reduces the peak asymmetry of basic solutes by limiting the possibilities of strong interactions between amine and silanol functions (Gritti et al., 2005). The availability of the procedure able to detect the content of intracellular polyamines, open new perspectives in the diagnosis and the monitoring of the proliferative state of normal and cancerous cells, in view of the stimulatory role of polyamines in cellular growth and differentiation (Beninati et al. 1993).

As previously stated, the limited stability of OPA derivatives is a crucial factor in HPLC methods. Hence, even if the post-column derivatization has been widely used, its application remains difficult. The reaction between OPA, thiols and primary amines, was deeply investigated with the aim to improve the stability of OPA-derivatives (Simons and Johnson 1978). Furthermore, OPA-derivatives of SPM and SPD have unreacted secondary amine group which affects negatively their stability. The chromatographic elution pattern for polyamines with a C18 reverse-phase column shows SPD and SPM as the last molecules eluted, due to the interaction of their OPA-derivatives with residing free silanol groups of most common packing-materials. Considering the time spent into the column by the two polyamines, the rapid degradation of OPA-derivative does not allow good detection levels, especially for SPM. According to this consideration, the reduction of the time of elution for SPD and SPM would improve the OPA fluorescence response for these two polyamines. The special advantages offered by new packing material in HPLC column technology allows a minimal interaction of OPA-derivatives with unreacted silanols. By this type of column, the elution pattern for OPA-derivatives of polyamines is reversed with SPM as first eluted molecule, and PUT as last.

Differentiation therapy may represents an important evolving aspect of cancer research. It is based on the concept that drugs such as natural molecules can inhibit tumor growth through the induction of cellular terminal differentiation (Thiele et al. 2000), avoiding the typical cytotoxicity of chemotherapeutic agents. Several differentiation markers has been identified (Qi and Ding 2018). However, most of them are specific for a single cancer type and often are not

useful for diagnosis (Sharma 2009). Thus, it is necessary to search for more general and sensitive markers that will be suitable for screening and design of new differentiative agents. Interestingly, the cross-linking activity of TG2 (generally low in cancer cells) and free high polyamines levels may play a pivotal role in cancer cell differentiation (Lentini et al. 2013). In fact, TG2 activity increases protein-polyamine conjugates with consequent decrease of free polyamines. Therefore, polyamines levels may be considered an additional differentiation marker. Previous papers show the inverse correlation between TG2 activity and polyamines in differentiation-induced melanoma cells (Lentini et al. 2007; Tabolacci et al. 2010) and in leukemia cells (Tabolacci et al. 2011). The data obtained show that theophylline and its halogenated derivatives, trigger differentiation in B16-F10 melanoma cells as a result of increased TG2 activity and intracellular melanin content, resulting in a significant reduction in polyamine levels.

These results, together with a better understanding of the roles that polyamines play in growth and differentiation in the normal context, as well as their dysregulation in neoplastic disease, are likely to lead to the discovery of more rational targets and better agents for cancer therapy.

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References

Bachrach U (2004) Polyamines and cancer: minireview article. Amino Acids 26(4):307–309. https://doi.org/10.1007/s00726-004-0076-6.

Bachrach U, Wang YC, Tabib A (2001) Polyamines: new cues in cellular signal transduction. News Physiol Sci 16:106–109.

Beninati S, Abbruzzese A, Cardinali M (1993) Differences in the post-translational modification of proteins by polyamines between weakly and highly metastatic B16 melanoma cells. Int J Cancer 53(5):792–797. https://doi.org/10.1002/ijc.2910530515.

Beninati S, Martinet N, Folk JE (1988) High-performance liquid chromatographic method for the determination of epsilon-(gamma-glutamyl)lysine and mono- and bis-gamma-glutamyl derivatives of putrescine and spermidine. J Chromatogr 443:329–335. https://doi.org/10.1016/S0021-9673(00)94804-0.

Casero RA Jr, Murray Stewart T, Pegg AE (2018) Polyamine metabolism and cancer: treatments, challenges and opportunities. Nat Rev Cancer 18(11):681–695. doi: 10.1038/s41568-018-0050-3.

Chung SI, Folk JE (1972) Transglutaminase from hair follicle of guinea pig. Proc Natl Acad Sci (Wash) 69:303–307.

Corbin JL, Marsh BH, Peters GA (1989) An Improved Method for Analysis of Polyamines in Plant Tissue by Precolumn Derivatization with o-Phthalaldehyde and Separation by High Performance Liquid Chromatography. Plant Physiol 90(2):434–439.

Cordella M, Tabolacci C, Senatore C, Rossi S, Mueller S, Lintas C, Eramo A, D'Arcangelo D, Valitutti S, Facchiano A, Facchiano F (2019) Theophylline induces differentiation and modulates cytoskeleton dynamics and cytokines secretion in human melanoma-initiating cells. Life Sci 230:121–131. https://doi.org/10.1016/j.lfs.2019.05.050.

Frank MP, Powers RW (2007) Simple and rapid quantitative high-performance liquid chromatographic analysis of plasma amino acids. J Chromatogr B Analyt Technol Biomed Life Sci 852(1-2):646–649. https://doi.org/10.1016/j.jchromb.2007.01.002.

Gritti F, Guiochon G (2005) Effect of the endcapping of reversed-phase high-performance liquid chromatography adsorbents on the adsorption isotherm. J Chromatogr A 1098(1-2):82–94. https://doi.org/10.1016/j.chroma.2005.08.045.

Halpert AG, Olmstead MC, Beninger RJ (2002) Mechanisms and abuse liability of the anti-histamine dimenhydrinate. Neurosci Biobehav Rev 26(1):61–67.

Hennings H, Michael D, Cheng C, Steinert P, Holbrook K, Yuspa SH (1980) Calcium regulation of growth and differentiation of mouse epidermal cells in culture. Cell 19(1):245–254. https://doi.org/10.1016/0092-8674(80)90406-7.

Hesterberg RS, Cleveland JL, Epling-Burnette PK (2018) Role of Polyamines in Immune Cell Functions. Med Sci (Basel) 6(1):E22. https://doi.org/10.3390/medsci6010022.

Igarashi K, Kashiwagi K (2019) The functional role of polyamines in eukaryotic cells. Int J Biochem Cell Biol 107:104–115. https://doi.org/10.1016/j.biocel.2018.12.012.

Jimbow K, Lee SK, King MG, Hara H, Chen H, Dakour J, Marusyk H (1993) Melanin pigments and melanosomal proteins as differentiation markers unique to normal and neoplastic melanocytes. J Invest Dermatol 100:259S–268S. doi: 10.1111/1523-1747.ep12470103.

Lentini A, Abbruzzese A, Provenzano B, Tabolacci C, Beninati S (2013) Transglutaminases: key regulators of cancer metastasis. Amino Acids 44(1):25–32. https://doi.org/10.1007/s00726-012-1229-7.

Lentini A, Forni C, Provenzano B, Beninati S (2007) Enhancement of transglutaminase activity and polyamine depletion in B16-F10 melanoma cells by flavonoids naringenin and hesperitin correlate to reduction of the in vivo metastatic potential. Amino Acids 32(1):95–100. https://doi.org/10.1007/s00726-006-0304-3.

Lentini A, Vidal-Vanaclocha F, Facchiano F, Caraglia M, Abbruzzese A, Beninati S (2000) Theophylline administration markedly reduces hepatic and pulmonary implantation of B16-F10 melanoma cells in mice. Melanoma Res 10(5):435–443.

Lotan R, Lotan D (1980) Stimulation of melanogenesis in a human melanoma cell by retinoids. Cancer Res 40:3345–3350.

Mitsias DI, Vovolis V (2011) Anaphylaxis to dimenhydrinate caused by the theophylline component. J Investig Allergol Clin Immunol 21(4):317–318.

Moschou PN, Roubelakis-Angelakis KA (2014) Polyamines and programmed cell death. J Exp Bot 65(5):1285–1296. https://doi.org/10.1093/jxb/ert373.

Piacentini M, Martinet N, Beninati S, Folk JE (1988) Free and protein-conjugated polyamines in mouse epidermal cells. Effect of high calcium and retinoic acid. J Biol Chem 263(8):3790–3794.

Qi L, Ding Y (2018) Screening of Differentiation-Specific Molecular Biomarkers for Colon Cancer. Cell Physiol Biochem 46(6):2543–2550. https://doi.org/10.1159/000489660.

Raza M, Al-Shabanah OA (2010) Simultaneous determination of different polyamines and their mono-acetylated derivatives in gastric tissue by HPLC with post-column derivatization. Sci Pharm 78(2):249–258. https://doi.org/10.3797/scipharm.1001-02.

Russell DH (1983) Clinical relevance of polyamines. Crit Rev Clin Lab Sci 18(3):261–311.

Russell DH, Durie BG, Salmon SE (1975) Polyamines as predictors of success and failure in cancer chemotherapy. Lancet 2(7939):797–799. https://doi.org/10.1016/S0140-6736(75)80009-2.

Shah U, Kavad M, Raval M (2014) Development and Validation of Stability-indicating RP-HPLC Method for Estimation of Pamabrom in Tablets. Indian J Pharm Sci 76(3):198–202.

Sharma S (2009) Tumor markers in clinical practice: General principles and guidelines. Indian J Med Paediatr Oncol 30(1): 1–8. Doi: 10.4103/0971-5851.56328.

Simons SS Jr, Johnson DF (1978) Reaction of o-phthalaldehyde and thiols with primary amines: fluorescence properties of 1-alkyl(and aryl)thio-2-alkylisoindoles. Anal Biochem 90(2):705–725. https://doi.org/10.1021/jo00408a030.

Tabolacci C, De Martino A, Mischiati C, Feriotto G, Beninati S (2019) The Role of Tissue Transglutaminase in Cancer Cell Initiation, Survival and Progression. Med Sci (Basel) 7(2): E19. https://doi.org/10.3390/medsci7020019.

Tabolacci C, Lentini A, Mattioli P, Provenzano B, Oliverio S, Carlomosti F, Beninati S (2010) Antitumor properties of aloe-emodin and induction of transglutaminase 2 activity in B16-F10 melanoma cells. Life Sci 87(9-10):316–324. https://doi.org/10.1016/j.lfs.2010.07.003.

Tabolacci C, Oliverio S, Lentini A, Rossi S, Galbiati A, Montesano C, Mattioli P, Provenzano B, Facchiano F, Beninati S (2011) Aloe-emodin as antiproliferative and differentiating agent on human U937 monoblastic leukemia cells. Life Sci 89(21-22):812–820. https://doi.org/10.1016/j.lfs.2011.09.008.

Thiele CJ, Gore S, Collins S, Waxman S, Miller W (2000) Differentiate or die: the view from Montreal. Cell Death Differ 7(10):1014–1017. https://doi.org/10.1038/sj.cdd.4400723.

Thomas T, Thomas TJ (2001) Polyamines in cell growth and cell death: molecular mechanisms and therapeutic applications. Cell Mol Life Sci 58(2):244–258. https://doi.org/10.1007/PL00000852.

Venza M, Visalli M, Cicciu D, Teti D (2001) Determination of polyamines in human saliva by high-performance liquid chromatography with fluorescence detection. J Chromatogr B Biomed Sci Appl 757(1):111–117.

Wallace HM, Fraser AV, Hughes A (2003) A perspective of polyamine metabolism. Biochem J 376(Pt 1):1–14. DOI: 10.1042/bj20031327.

Legends to figures

Figure 1. Chemical structures of naturally occurring polyamines and diaminooctane (internal standard).

Figure 2. Representative chromatograms of (A) OPA derivative standards (separation was performed five times injecting into the column 500 pmoles of each polyamine standards), and polyamines in MEK cells (B), B16-F10 melanoma cells (C) and in SKMEL-110 melanoma cells (D). Peaks: putrescine (3), cadaverine (4), spermidine (2), spermine (1) and diaminooctane (5).

Figure 3. Levels of (A) di- and (B) polyamines in lysates of MEK cells (white bars), murine B16-F10 melanoma cells (grey bars) and human SK-MEL110 melanoma cells (black bars). Each value represents the mean ± SD of five different determinations.

Figure 4. Theophylline derivatives as differentiation inducers. (A) Chemical structure of theophylline (Theo), 8-chlorotheophylline (8-Cl-Theo), and 8-bromotheophylline (8-Br-Theo). (B) Proliferation curve of murine B16-F10 melanoma cells treated with 1 mM methylxantines after 24, 48, and 72 h. (C) Reduction of intracellular polyamine levels (SPD, spermidine; SPM, spermine) in murine B16–F10 melanoma cells after 72 h of treatments. (D) Theo, 8-Cl-Theo and 8-Br-Theo increase transglutaminase 2 (TG2) activity in melanoma cells and produces an intracellular accumulation of melanin after 72 h of treatments. Each point represent the mean of three different determinations ± SD (*$p<0.01$, compared to the control).

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Istituto Superiore di Sanità, Rome, Italy

Rome, 12 August 2019

Cover Letter

Dear Editor,

Please find the manuscript: "**Evaluation of polyamines as marker of melanoma cell proliferation and differentiation by an improved High Performance Liquid Chromatographic method**" by B. Provenzano, A. Lentini, R. Tatti, A. De Martino, I. Borromeo, C. Mischiati, G. Feriotto, C. Forni, S. Beninati and myself, submitted as full article for publication on **Amino Acids**.

The liquid chromatographic separation of basic compounds and thus of diamines and polyamines has always been difficult. This was mainly due to their interaction with residuing silanol groups of the packing material, resulting in longer elution and broad tailing peaks. The use of OPA-label introduces another problem. It is well known that OPA-polyamine derivatives degrade quickly after their formation and the polyamines spermidine and spermine, containing unreacted secondary amino groups, show a very low fluorescence response, with most common reversed-phase packing materials.

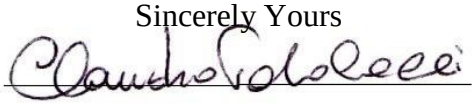
From these considerations, we suspected that the low fluorescence response, especially for spermidine and spermine, was caused by on-column degradation and therefore we attempted to decrease the permanence of these two polyamines into the HPLC column. The introduction of ultra-pure silica, has enabled the production of reversed-phase packing materials (Inertsil, Supelcosyl etc...), which allow the application of much higher ionic strength solvents, with minimal silanol interactions. These two properties make possible spermine to be eluted first followed by spermidine, putrescine and cadaverine, with a large increase of the fluorescence response and a better reproducibility.

The applicability of the resulting method was investigated, using three different types of samples, two melanoma cell lines (B16-F10 and SKMEL-110) and a normal one (MEK) as reference. Moreover, In addition, this technique has been applied in determining the potential role of 8-Cl-Theo and 8-Br-Theo in the differentiation of melanoma B16-F10 cells, evidenced by decreased intracellular levels of polyamines.

In conclusion, the present method enables an easy and sensitive determination of intracellular polyamines in normal and neoplastic cell lines.

We hope that this manuscript could appropriately fit with the aim of Amino Acids journal.

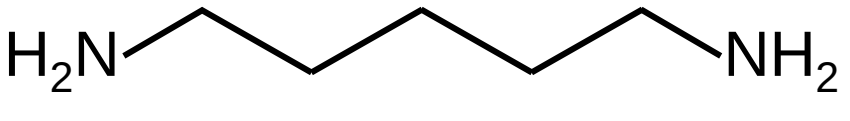
Sincerely Yours



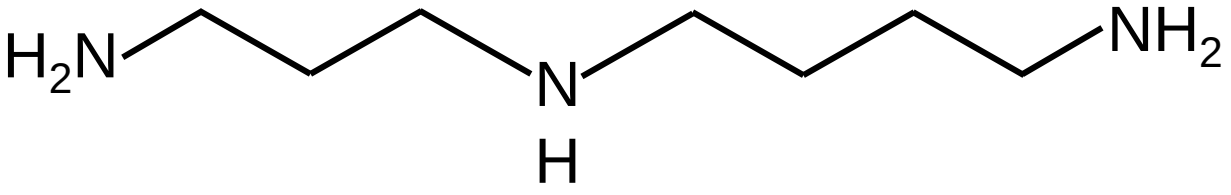
C. Tabolacci, Ph.D.

Figure 1

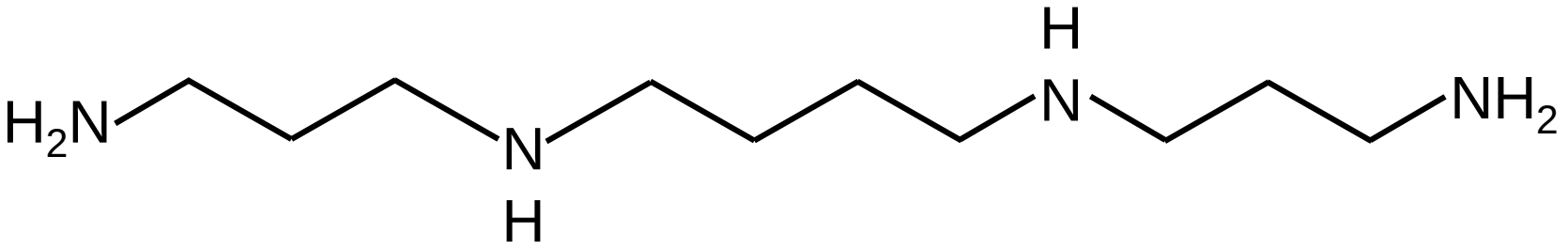
Putrescine (PUT)



Cadaverine (CAD)



Spermidine (SPD)



Spermine (SPM)

Diaminoctane (DAO)

Figure 2

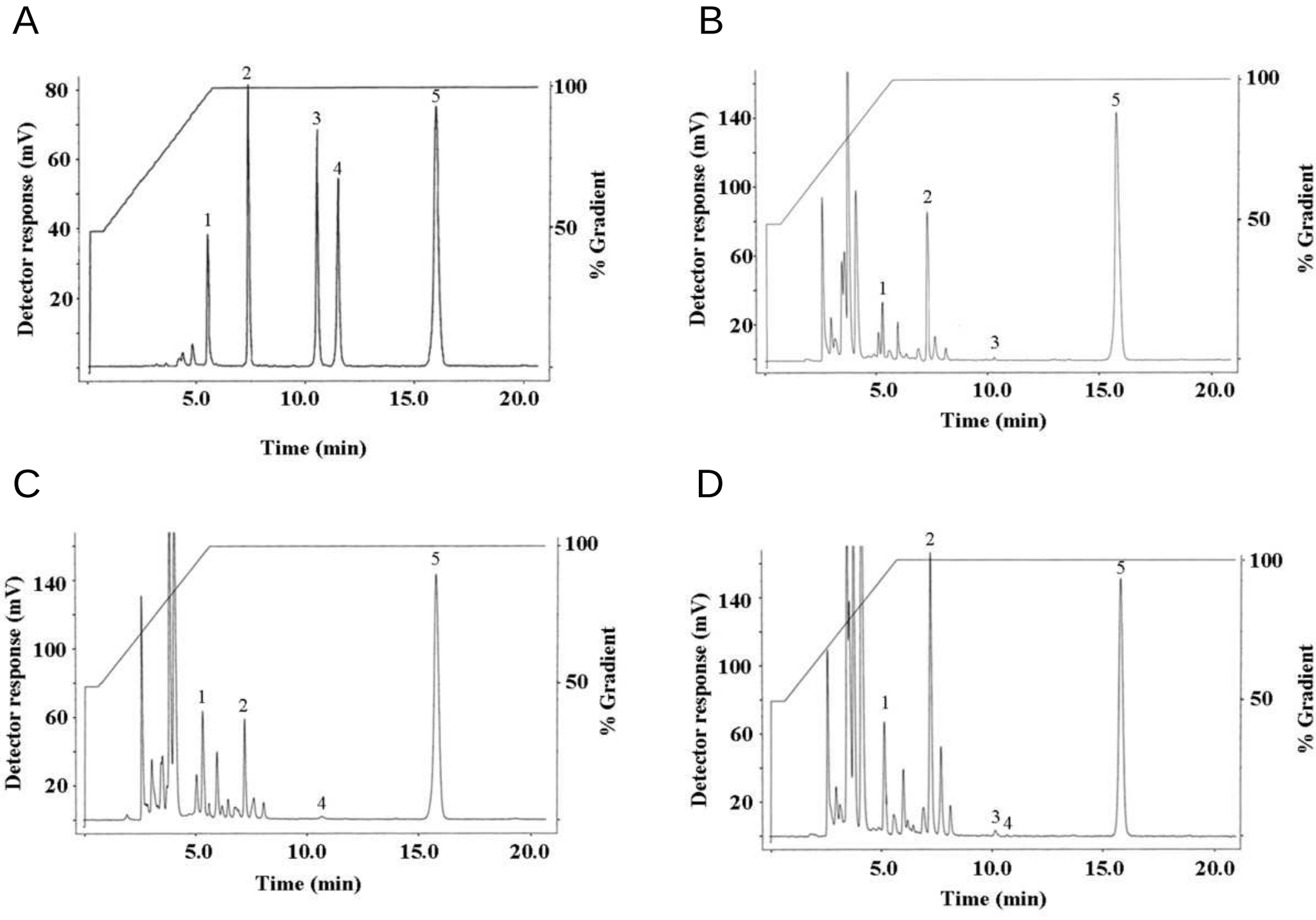


Figure 3

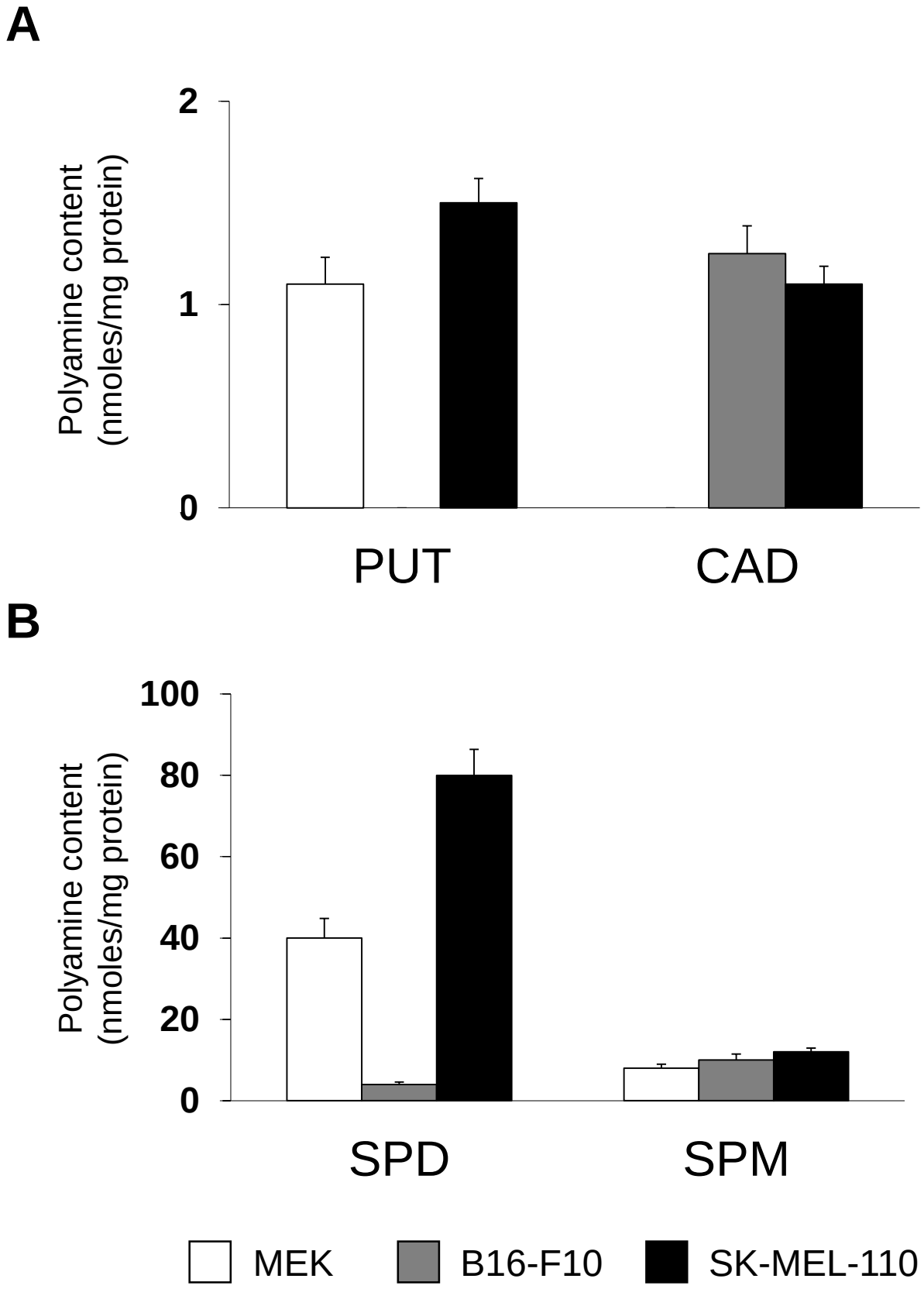

A
Polyamine content
(nmoles/mg protein)
2
1
0
PUT
CAD
B
Polyamine content
(nmoles/mg protein)
100
80
60
40
20
0
SPD
SPM
MEK
B16-F10
SK-MEL-110

Figure 4

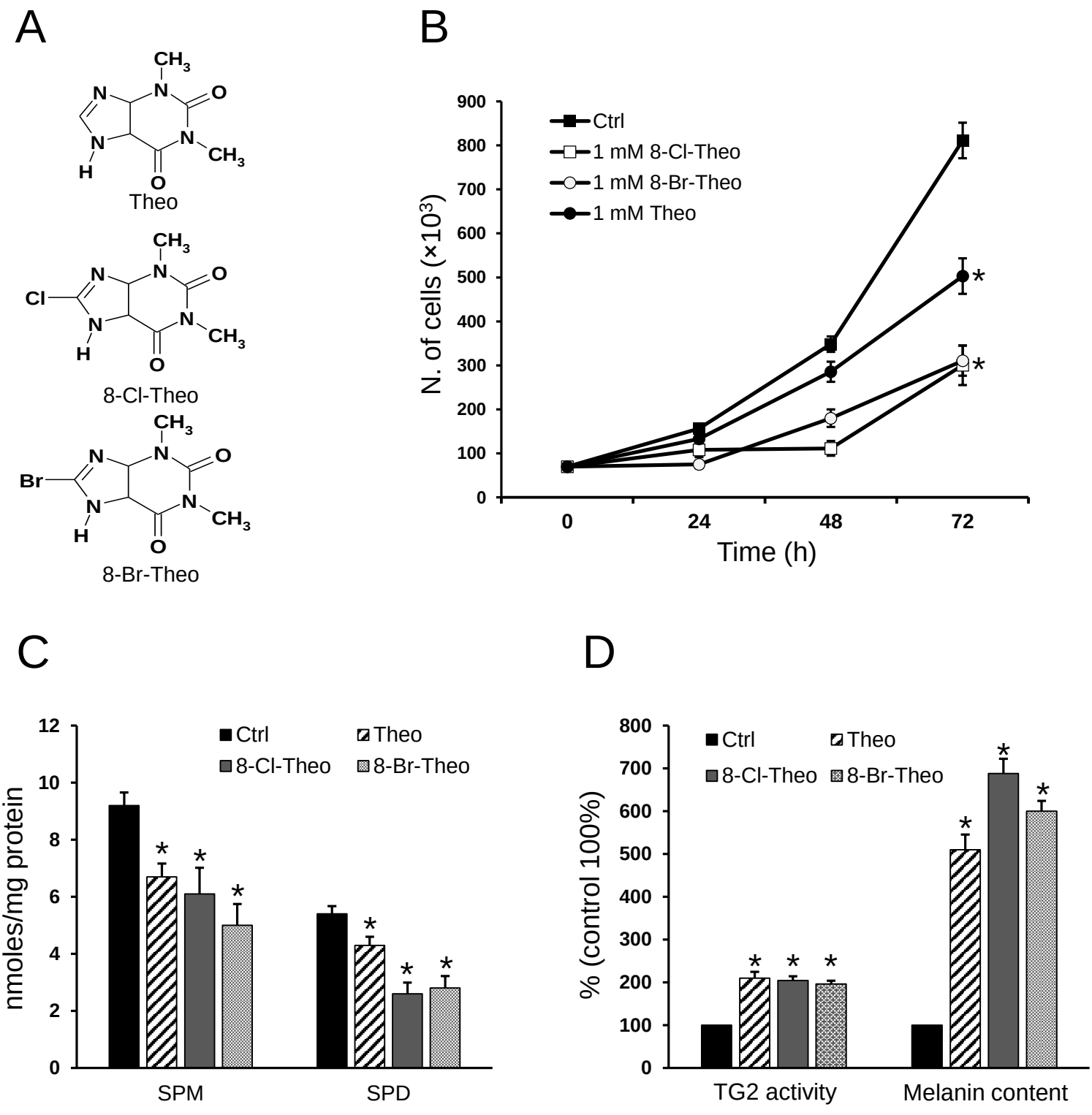

A
Theo
8-Cl-Theo
8-Br-Theo
B
Ctrl
1 mM 8-Cl-Theo
1 mM 8-Br-Theo
1 mM Theo
N. of cells (×10³)
Time (h)
C
Ctrl
Theo
8-Cl-Theo
8-Br-Theo
nmoles/mg protein
SPM
SPD
D
% (control 100%)
TG2 activity
Melanin content

Table 1

Peak areas of di- and polyamines at different times of incubation with OPA

Time (sec)	Peak Area (mm^2)				
	CAD	PUT	SPD	SPM	DAO
20	66 ± 2,8	72 ± 1,7	66 ± 1,4	28 ± 0,7	158 ± 5,8
30	78 ± 3,1	81 ± 3,1	76 ± 3,0	38 ± 0,9	174 ± 6,1
45	77 ± 2,6	84 ± 3,2	75 ± 2,6	35 ± 1,0	162 ± 5,9
60	75 ± 2,4	78 ± 2,5	68 ± 2,9	29 ± 0,8	159 ± 6,1
90	71 ± 1,8	77 ± 1,8	67 ± 1,6	28 ± 0,7	154 ± 5,7
120	65 ± 1,4	74 ± 2,0	64 ± 1,0	23 ± 0,5	146 ± 5,6

Sample containing 500 pmoles of di- and polyamines were derivatized with OPA with times of incubation ranging from 20 to 120 seconds. Data are expressed as the mean value of ten different determinations ± SD ($p<0.005$).