Proteomic analysis of cervical cancer cells treated with suberonylanilide hydroxamic acid

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Suberonylanilide hydroxamic acid (SAHA) is an orally administered histone deacetylase inhibitor (HDACI) that has shown significant antitumour activity in a variety of tumour cells. To identify proteins involved in its antitumour activity, we utilized a proteomic approach to reveal protein expression changes in the human cervical cancer cell line HeLa following SAHA treatment. Protein expression profiles were analysed by 2-dimensional polyacrylamide gel electrophoresis (2-DE) and protein identification was performed on a MALDI-Q-TOF MS/MS instrument. As a result, a total of nine differentially expressed proteins were visualized by 2-DE and Coomassie brilliant blue (CBB) staining. Further, all the changed proteins were positively identified via mass spectrometry (MS)/MS analysis. Of these, PGAM1 was significantly downregulated in HeLa cells after treatment with SAHA. Moreover, PGAM1 has been proven to be downregulated in another cervical cancer cell line (CaSki) by western blot analysis. Together, using proteomic tools, we identified several differentially expressed proteins that underwent SAHA-induced apoptosis. These changed proteins may provide some clues to a better understanding of the molecular mechanisms underlying SAHA-induced apoptosis in cervical cancer.

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1. Introduction

Cervical cancer is one of the most common cancers and a leading cause of death among women worldwide. It is characterized by a well-defined premalignant phase that can be suspected on cytological examination of exfoliated cervical cells and confirmed on histological examination of cervical material. However, this is procedurally inefficient (Woodman *et al* 2007). Suberonylanilide hydroxamic acid (SAHA), which has been reported to induce cell growth inhibition and/or apoptosis in a variety of tumour cells, is a targeted inhibitor of histone deacetylases (Gray *et al* 2004). In clinical trials, SAHA has shown significant anticancer activity against both haematological and solid tumours at doses well tolerated by patients (Marks *et al* 2007; Richon *et al* 1998). Proteomics is the study of protein expression in a tissue or biological fluid. Comparison of protein patterns in biological fluids between healthy individuals and patients

Keywords. Cervical cancer; 2-DE; proteomics; suberonylanilide hydroxamic acid

Abbreviations used: ACN, acetonitrile; AKR, aldoketoreductase; AR, aldose reductase; CBB, Coomassie brilliant blue; CHCA, cyano-4-hydroxy-cinnamic acid; 2-DE, 2-dimensional polyacrylamide gel electrophoresis; DMEM, Dulbecco modified Eagle medium; DMSO, dimethyl sulphoxide; HDACI, histone deacetylase inhibitor; HSP27, heat shock protein 27; IEF, isoelectric focusing; IPG, immobilized pH gradient; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; PBS, phosphate buffered saline; PVDF, polyvinylidene difluoride; Q-TOF, quadrupole time-of-flight; RNAi, RNA interference; SAHA, suberonylanilide hydroxamic acid; SOD, superoxide dismutase; TBST, Tris-buffered saline Tween-20; TFA, trifluoroacetic acid; TNF, tumour necrosis factor

with disease is increasingly being used to both discover biological markers of disease and identify biochemical processes important in disease pathogenesis (Barratt and Topham 2007). Proteomics has been revolutionized in the past decade by the application of techniques such as protein arrays, 2-dimensional polyacrylamide gel electrophoresis (2-DE) and mass spectrometry (MS), which have tremendous potential for biomarker development, target validation, diagnosis, prognosis and optimization of treatment in medical care, especially in the field of clinical oncology.

As SAHA has shown good results in human cancer therapy, we used HeLa cells as a model to identify whether SAHA would be effective in cervical cancer. We took the proteomics approach, particularly 2-DE and MS, to identify the altered proteins in HeLa cells before and after SAHA treatment. With proteomics, our analysis revealed nine differentially expressed proteins, which functioned in biological processes as diverse as protein metabolism, molecular chaperone, transcription, carbohydrate metabolism, telomere maintenance and biosynthesis, as well as redox regulation. Furthermore, all the proteins were correlated with apoptosis or antiproliferation, which supported the theory that SAHA could be effective in the therapy of cervical cancer.

2. Materials and methods

2.1 *Cell culture and treatment*

HeLa and CaSki cell lines were purchased from ATCC (Rockville, MD, USA). HeLa cells were maintained in Dulbecco modified Eagle medium (DMEM, Gibco, USA) containing 10% foetal calf serum (Hyclone, USA), penicillin (10^7 U/l) and streptomycin (10 mg/l). CaSki cells were cultured in RPMI-1640 (Gibco, USA) containing 10% foetal calf serum (Hyclone, USA), penicillin (10^7 U/l) and streptomycin (10 mg/l) at 37°C in an atmosphere containing 5% CO_2. SAHA (Alexis, San Diego, CA, USA) was dissolved in dimethyl sulphoxide (DMSO). When the cells reached 50%–80% confluency, the medium was replaced by a fresh culture medium containing SAHA. Control cells were cultured in a medium containing an equal amount of DMSO instead of SAHA. For 2-DE analysis, HeLa cells were treated with 2.5 μM SAHA for 48 h. Cells were washed twice by centrifugation in phosphate buffered saline (PBS) and transferred to sterile plastic tubes for storage at –80°C until use.

2.2 *2-Dimensional polyacrylamide gel electrophoresis and image analysis*

2-DE was performed as described previously (Tong *et al* 2007) with minor modification. Approximately 4×10^7 cells were lysed in 1 ml lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT, 0.2% pH 3–10 ampholyte; BioRad, USA) containing protease inhibitor cocktail 8340 (Sigma, St Louis, MO, USA). Samples were then kept on ice and sonicated in six cycles of 10 s, each consisting of 5 s sonication followed by a 10 s break. After centrifugation at 14 000 rpm for 1 h at 4°C, the supernatant was collected and the protein concentrations were determined using the DC protein assay kit (BioRad). Protein samples (2 mg) were applied to an immobilized pH gradient (IPG) strip (17 cm, pH 3–10 NL, BioRad) using a passive rehydration method. After 12–16 h of rehydration, the strips were transferred to an isoelectric focusing (IEF) cell (BioRad) and focused for a total of 60 000 VH. The second dimension was performed using 12% SDS-PAGE at 30 mA constant current per gel after equilibration. The gels were stained using CBB R-250 (Merck, Germany) and scanned with a BioRad GS-800 scanner. At least four independent runs were made for each cell line to ensure the accuracy of analyses. The maps were analysed by the PDQuest software version 6.1 (BioRad). The quantity of each spot in a gel was normalized as a percentage of the total quantity of all spots in that gel and evaluated in terms of optical density (OD). The paired *t*-test was used for statistical analysis of the data. Only spots that showed consistent and significant differences (± over 2-fold, $P<0.05$) were selected for analysis with MS.

2.3 *In-gel digestion*

In-gel digestion of proteins was carried out using MS-grade Trypsin Gold (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, spots were cut out of the gel (1–2 mm diameter) using a razor blade, and destained twice with 100 mM NH_4HCO_3/50% acetonitrile (ACN) at 37°C for 45 min in each treatment. After dehydration and drying, the gels were pre-incubated in 10–20 μl trypsin solution for 1 h. Then samples were added in adequate digestion buffer (40 mM NH_4HCO_3/10% ACN) to cover the gels and incubated overnight at 37°C. Tryptic digests were extracted using MiliQ water initially, followed by extraction twice with 50% ACN/5% trifluoroacetic acid (TFA) for 1 h each time. The combined extracts were dried in a vacuum concentrator at room temperature. The samples were then subjected to MS analysis.

2.4 *MALDI-Q-TOF analysis and protein identification*

Mass spectra were acquired using a quadrupole time-of-flight (Q-TOF) mass spectrometer (Micromass, Manchester, UK) with a matrix-assisted laser desorption ionization (MALDI) source (Micromass). Tryptic digests were dissolved in 5 μl of 70% ACN/0.1% TFA, and then 1 μl of the digests was

mixed with 1 μl saturated alpha-cyano-4-hydroxy-cinnamic acid (CHCA) in 50% ACN/0.5% TFA and spotted onto a 96-well target plate. MS/MS was performed in a data-dependent mode in which the top ten most abundant ions for each MS scan were selected for MS/MS analysis. The MS/MS data were acquired and processed using the MassLynx software (Micromass) and MASCOT was used to search the database. Database searches were carried out using the following parameters: database, Swiss-Prot; taxonomy, *Homo sapien*; enzyme, trypsin; and allowance of one missed cleavage. Carbamidomethylation was selected as a fixed modification and oxidation of methionine was allowed to be variable. The peptide and fragment mass tolerance were set at 1 and 0.2 Da, respectively. The data format selected was Micromass PKL and the instrument selected was MALDI-Q-TOF. Proteins with probability-based MOWSE scores exceeding their threshold (P<0.05) were considered to be positively identified.

2.5 *Western blot*

Collected cells were lysed in RIPA buffer (50 mM Tris-base, 1.0 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% TritonX-100, 1% sodium deoxycholate, 1 mM PMSF) to extract all the proteins and quantified by the DC protein assay kit (BioRad). Samples were separated by 12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences). The membranes were blocked overnight with PBS containing 0.1% Tween 20 in 5% skimmed milk at 4°C, and subsequently probed by the primary antibodies: goat-anti-PGAM1 (diluted 1:500, Abcam, UK). Blots were incubated with the primary antibodies for 2 h at room temperature and washed 3 times in TBST. The blots were then incubated with secondary antibody conjugated to horseradish peroxidase for 2 h at room temperature. Target proteins were detected by enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, USA), and β-actin was used as an internal control.

3. Results

3.1 *2-DE profiling of the differentially expressed proteins between HeLa and SAHA-treated HeLa cells*

The protein expression profile of HeLa cells and HeLa cells treated with SAHA were examined by 2-DE. The experiments were repeated at least three times with reproducible results. A pair of representative 2-DE maps is shown in figure 1. The spot with a remarkable expression change is shown in figure 2 in an enlarged form.

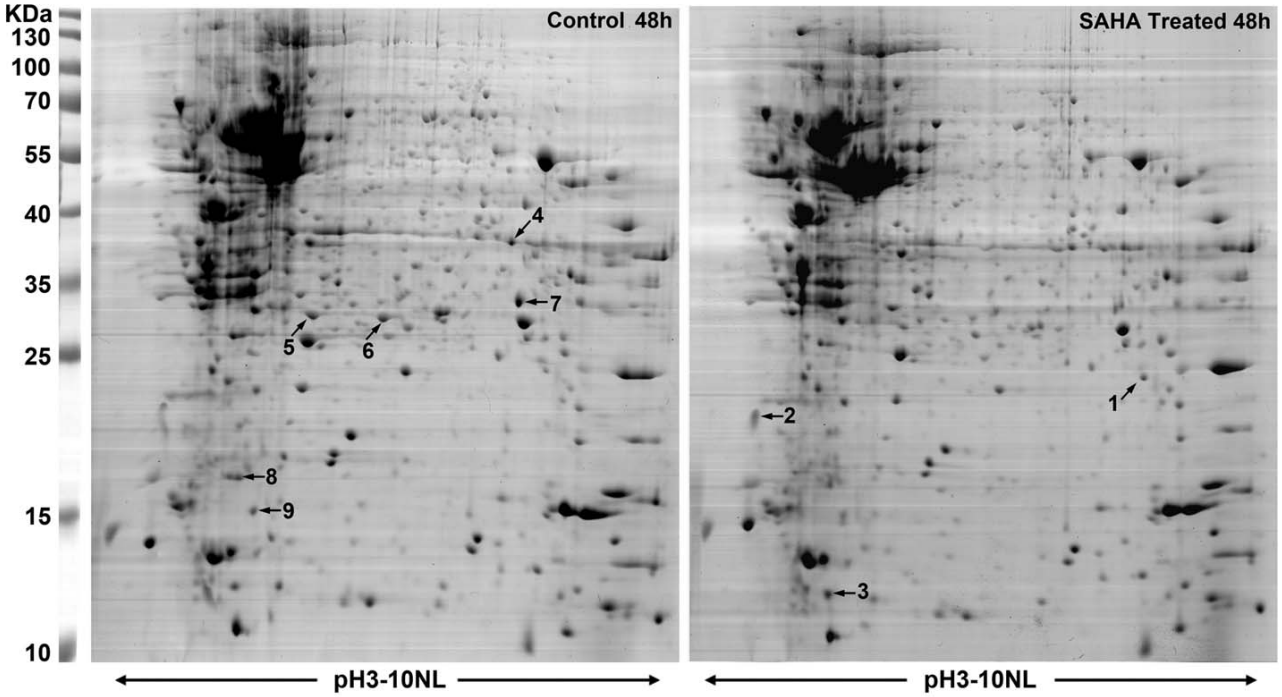


Figure 1. Comparison of the protein expression patterns between control and suberonylanilide hydroxamic acid (SAHA)-treated HeLa cells. The arrows indicate the nine differentially expressed proteins.

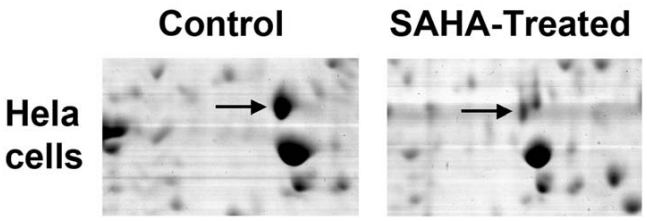


Figure 2. Enlargement of PGAM1 spot before and after suberonylanilide hydroxamic acid (SAHA) treatment of HeLa cells in 2-dimensional polyacrylamide gel electrophoresis (2-DE) gel.

3.2 *Identification of differentially expressed proteins*

Of the differentially expressed spots, spot #7 was positively identified with high confidence (table 1). The average values of the MOWSE score and the number of unique peptides identified by MS/MS sequencing were 244 and 4, respectively. A representative MS map of spot #7 is shown in figure 3.

3.3 *Protein validation by western blot*

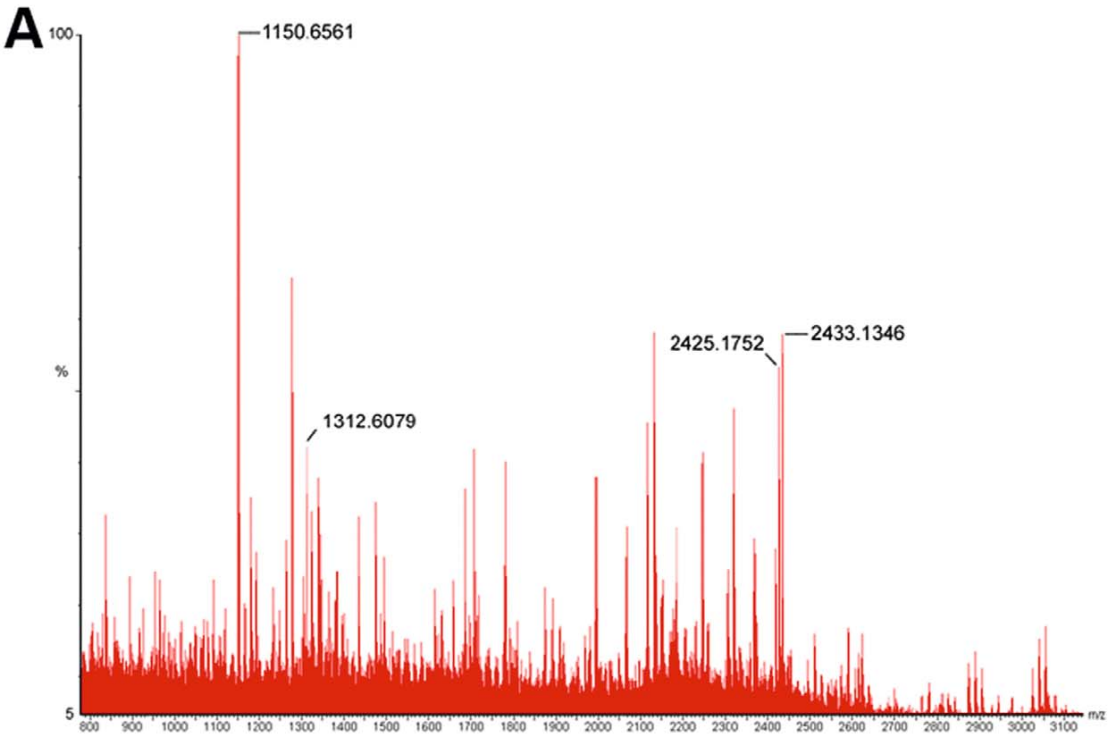
PGAM1 was further validated by western blot. Consistent with the observations in 2-DE analysis, PGAM1 was downregulated particularly in the SAHA-treated HeLa cells compared with the parental HeLa cells (figure 4). A similar change was detected in CaSki cells treated with SAHA (figure 4).

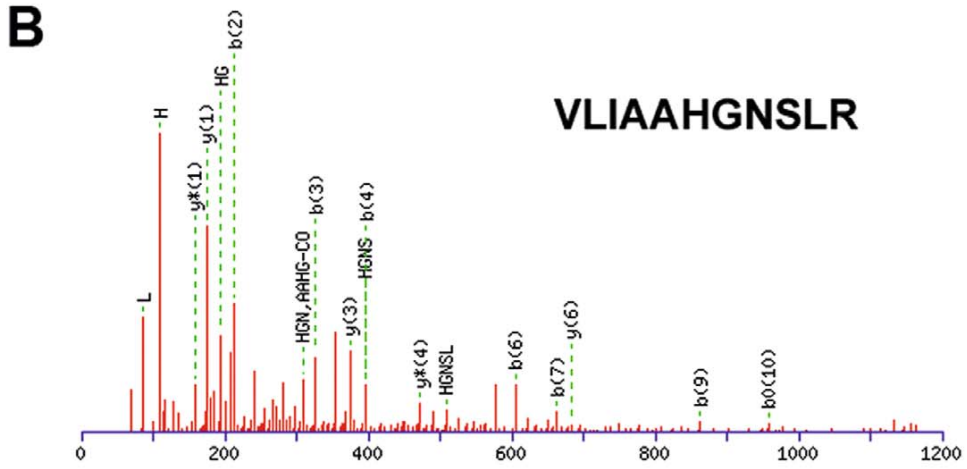
4. Discussion

In this study, human cervical cancer cells (HeLa) were used as a model, and a 2-DE-based proteomics approach was undertaken to annotate the altered proteins in the HeLa cells before and after treatment with SAHA. Our proteomic analysis revealed a total of nine differentially expressed proteins, which functioned in diverse biological processes including protein metabolism, molecular chaperone, transcription, carbohydrate metabolism, telomere maintenance and biosynthesis, as well as redox regulation. Among these, five proteins were found to be downregulated by SAHA – PGAM1, TBCA, HSPB1, ALDR and IF5A1. Furthermore, significant downregulation of PGAM1 after treatment with SAHA was confirmed in two cervical cancer cell lines (HeLa and CaSki) by western blot analysis.

PGAM1 is a key enzyme in glycolysis, which is upregulated during the process of carcinogenesis, both in primary and metastatic cancers, and finally leads to increased glucose consumption, which can be observed with clinical tumour imaging (Gatenby and Gillies 2004). Recent findings support an important role of PGAM1 in cell proliferation. For example, overexpression of PGAM1 leads to immortalization and indefinite proliferation (Evans *et al* 2005). Conversely, inhibition of PGAM1 promotes growth arrest in breast carcinoma cell lines (Engel *et al* 2004), and RNA interference (RNAi) against PGAM1 produces a senescent phenotype in some cancer cells (Kondoh *et al* 2005). Aldose reductase (AR) belongs to the NADPH-dependent aldoketoreductase (AKR) superfamily. However, recent studies suggest that AR is involved in key signalling events associated with cell growth and proliferation (Hamaoka *et al* 2004; Murata *et al* 2001). The upregulation of AR can promote colon cancer (Gokmen-Polar *et al* 2001) and inhibition of AR prevents phosphorylation of various kinases that regulate transcription factors required for cell survival and death (Ramana *et al* 2005). Heat shock protein 27 (HSP27) is one of the members of the small HSP family that functions as a molecular chaperone in protein translocation, folding and overlapping, and thus plays an essential role in cancer cell growth, both by promoting autonomous cell proliferation and by inhibiting death pathways such as the activation of caspase (Kamradt *et al* 2002) and NF-kappa B (Kammanadiminti and Chadee 2006). Upregulation of HSP27 is commonly found in breast (Oesterreich *et al* 1993) and ovarian cancer (Langdon *et al* 1995). TBCA and IF5A1 are also correlated with antiproliferation or apoptosis induction (Nolasco *et al* 2005; Cracchiolo *et al* 2004). In our studies, three proteins – SOD2, TEBP, MTPN – were markedly upregulated in SAHA-treated HeLa cells. Superoxide dismutase [Mn] (SOD2) is essential for eliminating superoxide radicals (O^{2-}) and thus protects cells from the damage induced by free radicals with low activity in tumour cells (Oberley and Buettner 1979). SOD2 is a tumour necrosis factor (TNF)-inducible gene product and is suggested to be a tumour suppressor gene in a variety of cancer cells (Weydert *et al* 2006; Weydert *et al* 2003; Church *et al* 1993; Venkataraman *et al* 2005). In addition, TEBP and MTPN also have an effect on apoptosis induction (Meadows *et al* 2004; Gupta *et al* 2002).

In conclusion, using proteomics tools, we identified nine differentially expressed proteins following SAHA treatment of cervical cancer cells. The functions of the changed proteins were correlated to apoptosis and/or antiproliferation cellular processes. These results support the hypothesis that SAHA is a potential inhibitor of cervical cancer. However, many cellular regulatory proteins were not identified, possibly due to their low abundance. Therefore, more proteomics methods such as large-sized gel slabs, depletion of high-abundance proteins, enrichment of samples by prefractionation and using silver staining coupled with LC-MS/MS analysis would be helpful in identifying more antitumour proteins.





C

MAAYKLVLIR **HGESAWNLEN** **R**FSGWYDADL SPAGHEEAKR GGQALRDAGY
EFDICFTSVQ KRAIRTLWTV LDAIDQMWLP VVRTWRLNER HYGGLTGLNK
AETAAKHGEA QVKIWRR**SYD** **VPPPPMEPDH** **PFYSNISK**DR R**YADLTEDQL**
PSCESLKDTI **AR**ALPFWNEE IVPQIKEGKR **VLIAAHGNSL** **R**GIVKHLEGL
SEEAIMELNL PTGIPIVYEL DKNLKPIKPM QFLGDEETVR KAMEAVAAQG
KAKK

Figure 3. Identification of protein spot #7 seen in figure 1. (**A**) Mass spectrogram of tryptic peptides from spot #7. Four peptides with m/z values were subjected to mass spectrometry (MS)/MS analysis. (**B**) An example of an MS/MS spectrum of parent ion 1150.6561. (**C**) Protein sequence of PGAM1. The matched peptides are coloured in red.

Table 1. Proteins identified by MALDI-Q-TOF

Spot No.	Protein description	Abbreviations	Function	Accession No.	Ther Mr/pI[a]	No. of pep.[b]	Score[c]	Expr. level[d]
1	Superoxide dismutase [Mn]	SOD2	Redox regulation	P04179	24878/8.35	3	89	+
2	Prostaglandin E synthase 3	TEBP	Telomere maintenance and biosynthesis	Q15185	18971/4.35	2	102	+
3	Myotrophin	MTPN	Metabolism	P58546	12895/5.27	4	201	↑3.1 ± 0.9
4	Aldose reductase	ALDR	Carbohydrate metabolism	P15121	36099/6.56	4	142	–
5[e]	Heat-shock protein beta-1	HSPB1	Molecular chaperone	P04792	22826/5.98	3	122	–
6	Heat-shock protein beta-1	HSPB1	Molecular chaperone	P04792	22826/5.98	2	96	–
7	Phosphoglycerate mutase 1	PGAM1	Metabolism	P18669	28900/6.67	4	244	↓5.0 ± 1.6
8	Eukaryotic translation initiation factor 5A-1	IF5A1	Transcription	P63241	16918/5.08	3	171	–
9	Tubulin-specific chaperone A	TBCA	Molecular chaperone	O75347	12904/5.25	3	102	↓3.7± 1.2

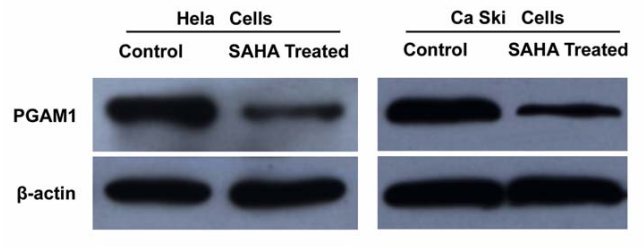


Figure 4. Western blot confirmation of PGAM1 expression change between control and suberonylanilide hydroxamic acid (SAHA)-treated HeLa and CaSki cells corresponding to spot #7 seen in figure 1. Protein extraction was performed at 48 h post-SAHA treatment of HeLa and CaSki cells. Proteins were probed with anti-PGAM1 antibodies; *β*-actin was used as an equal loading control.

Acknowledgments

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