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A chemo-resistant protein expression pattern of glioblastoma cells (A172) to perillyl alcohol

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Abstract

Glioblastoma multiform (GBM) is by far the most malignant glioma. We have introduced a new treatment for GBMs that comprises the inhalation of a naturally occurring terpene with chemotherapeutic properties known as perillyl alcohol (POH). Clinical trial results on recurrent GBM patients showed that POH extends the average life by more than eight months, temporarily slows tumor growth, and in some cases even decreases tumor size. After approximately seven months the tumor continues to grow and leads to a dismal prognosis. To investigate how these tumors become resistant to POH we generated an A172 human glioblastoma cell culture tolerant to 0.06 mM of POH (A172r). We used Multidimensional Protein Identification Technology (MudPIT) to compare the protein expression profile of A172r cells to the established glioblastoma A172 cell line. Our results include a list of identified proteins unique to either the resistant or the non-resistant cell line. These proteins are related to cellular growth, negative apoptosis regulation, Ras pathway, and other key cellular functions that could be connected to the underlying mechanisms of resistance.

Keywords

Glioblastoma; MudPIT; perillyl alcohol; A172 cells; resistant; protein profile

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Introduction

Glioblastoma multiform (GBM) is by far the most common and malignant glioma. During the last 25 years, no significant advances in the treatment of glioblastoma multiform (GBM) have been developed. Currently, patients with surgical resection who are treated by radiation or chemotherapy have an approximate survival time of 12 months.¹ In such a scenario, the quest for more effective chemotherapeutic agents is of great importance. Perillyl alcohol (POH), a naturally occurring terpene, is a new and promising chemotherapeutic agent. It has cytostatic and cytotoxic effects² and induces apoptosis in lung,³ leukocyte,⁴ prostate,⁵ and breast cancer cell lines.⁶ *In vivo*, POH has anti-metastatic effects and is a potent inhibitor of angiogenesis.⁷⁻⁸ Its chemotherapeutic effects are under evaluation in several clinical trials, including patients with colorectal, breast, or ovarian cancer.⁹⁻¹¹

In particular, we previously reported an ongoing clinical trial (phase I) comprising GBM patients treated by a new method of administration through intranasal delivery that has statistically shown to increase survival time.¹ The side effects of treatment with POH are almost none, even in patients that survived for more than a year. Our results showed POH slows tumor growth and in some cases even decreases tumor size as shown in Figure 1. However, after approximately seven months the tumor becomes resistant to POH, continues to grow, and leads to an adverse prognosis.¹

In a previous report, we used Differential Gel Electrophoresis (DIGE) to compare the plasma protein profile of these patients before and after 4 months of treatment with POH. Our protocol included depleting the six most abundant plasma proteins, excising spots of interest and analyzing them by MALDI-TOF-TOF.¹² We identified candidate prognostic proteins (e.g., anti-thrombin) that correlated with the progression of the disease; however, they were common plasma proteins that were not specific to the disease. In this regard, the results shed little light on the effects of the POH treatment at a molecular level. Moreover, few proteins were identified because of the nature of the technique employed.¹²

To continue evolving the use of POH as a treatment we need to identify the molecular changes that lead to tumor resistance to POH. Identifying pathways activated by treatment with POH could provide insights on how to combine chemotherapeutic agents to synergistically treat the disease or in developing new treatments. To increase the number of identified proteins we employed the state-of-the-art proteomic methodology known as Multidimensional Protein Identification Technology (MudPIT). The later uses two-dimensional liquid chromatography (LC/LC) online with tandem mass spectrometry and is capable of identifying thousands of proteins in complex mixtures. To overcome the problem of finding specific tumor proteins, we focused our experiments on human glioblastoma cells instead of plasma.

To tackle the problem of investigating tumor resistance to POH, we generated a new glioblastoma cell culture, referred heretofore as A172r, which can tolerate up to 0.06 mM of POH; in contrast, the commercial A172 cell line dies when exposed to this concentration. The A172r protein profile was compared with the established human glioblastoma multiform cell line A172. Relative protein quantitation was performed using spectral counting¹³⁻¹⁴ and the profile comparisons were carried out using the PatternLab for proteomics suite.¹⁵

Experimental section

1. A172 cell culture

A172 cells were obtained from the American Type Culture Collection and grown as monolayers in 25 cm² tissue culture flasks in Dulbecco's modified Eagle medium (D-MEM) supplemented with 0.2 mM nonessential amino acids, 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (fungizone, 2.5 mg/ml). For sub-cultivations, confluent monolayers were gently washed with phosphate-buffered saline (PBS 1×) pH 7.2, and after a short period of trypsin treatment, the cells were suspended in the previous culture medium for the control cells.

2. A172 resistant cell culture

The A172r were generated by gradually increasing the concentration of POH in the medium, starting with 0.003% (v/v) during three passages, followed by 0.006% (v/v) during four passages, 0.0075% (v/v) throughout 5 passages, then 0.0085% (v/v) through 5 passages and finally 0.01% (0.06 mM) during 8 passages. We then tested the effects of 0.06 mM on normal A172 cells. One passage of A172 cells directly to a medium containing 0.06 mM of POH kills these cells in 36 hours.

3. Protein solubilization and trypsin digestion

Each culture medium (A172 & A172r) was discarded and the pellets were obtained by centrifugation at 700 RCF. After washing three times with PBS, the pellets were incubated on ice for 45 minutes with 100 µL of 10 mM Tris (pH 7.5), 1 mM EDTA for enrichment of the membrane fraction. Cells were lysed in a 1cc U-100 insulin syringe filled with cell suspension by pushing and compressing the plunger several times. The soluble fraction was separated from the membrane fraction by ultra-centrifugation (18,000 RCF). The proteins pellets were then re-suspended with RapiGest and trypsinization was carried out as described by Fischer *et al.*¹⁶

4. Protein identification by MudPIT

Seventy micrograms of the digested peptide mixture were loaded onto a biphasic (strong cation exchange / reversed phase) capillary column and washed with a buffer containing 5% acetonitrile, 0.1% formic acid diluted in HPLC grade water. The two-dimensional liquid chromatography separation and tandem mass spectrometry conditions were as described by Washburn *et al.*¹⁷ Mass spectrometry data were collected using a Finnigan LTQ-XL (Thermo, San Jose, CA) set to the data-dependent acquisition mode with dynamic exclusion turned on. One MS survey scan was followed by nine MS/MS scans and 12 salt steps were performed. The Xcalibur data system (Thermo, San Jose, CA) was used to control the mass spectrometer scan functions and HPLC solvent gradients. The analyses were performed in triplicates.

Tandem mass spectra were extracted from the raw files using RawExtract1_9_8¹⁸ and were searched against the *Homo sapiens* sequences plus common contaminant proteins; all sequences were downloaded as FASTA-formatted from the EBI-IPI protein database (version 3.23, released on November 2, 2006).¹⁹ The confidence levels and false positive rates were calculated using a decoy database that contained the reverse sequences of the original dataset appended to the target database was used²⁰, and the best matching sequences from the combined database were indicated by SEQUEST. The peptide mass search tolerance was set to 3 Da. No differential modifications were considered. The search space included all candidate peptides whose theoretical mass fell within the 3 Da mass tolerance window, regardless of their tryptic status; no enzymatic cleavages were imposed.

The validity of peptide / spectrum matches was assessed in DTASelect 2.0.31 21 according to the SEQUEST 22 cross-correlation score (XCorr) and the SEQUEST normalized difference in cross-correlation score (DeltaCN). The search results were grouped by charge state (+1, +2, and +3) and tryptic status (fully tryptic, half-tryptic, and non-tryptic), resulting in nine distinct subgroups. For each subgroup, the distribution of XCorr and DeltaCN values for the direct and decoy database hits was obtained, and the two subsets were separated by quadratic discriminant analysis. Outlier points in the two distributions were discarded. The full separation of the direct and decoy subsets is not generally possible, so the discriminant score was set such that a false discovery rate of 1% was determined based on the number of accepted decoy database peptides. This procedure was independently performed on each data subset, resulting in a false-positive rate that is independent of tryptic status or charge state. Additionally, a minimum sequence length of seven amino acid residues was required, and each protein on the list was supported by at least two peptide identifications. The last filtering step results in an estimated false discovery rate lower than 1%.

5. PatternLab for Proteomics data parsing

The DTASelect-filtered files (which contain the spectral counting information) were converted to PatternLab's native data format (the index and sparse matrix files) by using PatternLab's DTASelect to Sparse Matrix parser. The PatternLab output includes an index and a sparse matrix file. The index file lists all identified proteins within all the project's assays and assigns to each a unique Protein IDentification (PID) integer. The sparse matrix file contains rows where each one corresponds to an assay and follows the schema: $\langle \text{class label} \rangle \langle \text{PID}_1 \rangle : \langle \text{value}_1 \rangle \dots \langle \text{PID}_n \rangle : \langle \text{value}_n \rangle$, where n is the number of identified proteins for that assay. Here, $\langle \text{class label} \rangle$ was assigned as 1 for the A172r cells and -1 for non treated A172 cells. Accordingly, $\langle \text{PID}_i \rangle$ and $\langle \text{value}_i \rangle$ correspond, respectively, to the i th protein's identification integer and its spectral count for the respective assay. The resulting sparse matrix has 6 rows obtained from each condition in triplicates. The index and sparse matrix are available in Supplemental Data I.

6. Selection of differentially expressed proteins according to the TFold methodology

The PatternLab TFold module was used to pinpoint differentially expressed proteins between the A172 and the A172r cells. Proteins having an absolute fold change greater than 2.5 and a t-test p -value of 0.01 were considered as differentially expressed. The fold change of 2.5 was obtained through the TFold procedure as to maximize the number of proteins that satisfy the fold change, t-test and the Benjamini-Hochberg theoretical false discovery rate estimator (BH-FDR)(q -value of 0.05). 23 Additionally, only proteins present in at least five of all six MudPIT assays were considered for this analysis.

7. Selection of unique proteins

The proteins that were only identified in either the A172 or the A172r were assessed using PatternLab's Approximately Area Proportional Venn Diagram module. A stringent selection criterion was imposed to only consider proteins that were identified in all three assays of a condition and found in no assays of the other condition.

8. Gene Ontology Explorer analysis

To help interpret the set of selected proteins we used PatternLab's Gene Ontology Explorer (GOEx) module.²⁴ Our data analysis used the gene ontology database (OBO v1.2, downloaded June 2nd, 2010) and the human annotation file (GOA, downloaded on June 2nd, 2010) from <http://geneontology.org> and <http://www.ebi.ac.uk>, respectively. The Gene Ontology Explorer specialist mode was used to search proteins in the Venn diagram and

TFold results that mapped to key words such as negative apoptosis regulation and cellular growth.

9. Validation of differential expression by Western Blot

Western blot for HSP 70 was performed according to Fischer *et al* 16 using fifteen micrograms of the membrane-enriched fraction lysate.

Results

Selection of differentially expressed proteins according to the TFold methodology

A report listing the proteins considered for the TFold analysis (present in at least 5 out of the six assays) with their fold change and p-value for differential expression is presented on Supplemental Data II. Table 1 list fifty seven proteins that were considered differentially expressed according to the TFold methodology. Figure 2 shows the graphical representation of the TFold analysis; it maps proteins according to their p-value for differential expression and their fold change.

The results were further verified by confirming the differential expression of a chosen protein, in this case HSP70, with western blot (Figure 3). As shown in Figure 3 the expression of HSP70 in A172r is greater than in the A172 and is in accordance with the results obtained by spectral counting.

Selection of Unique Proteins

An approximately area proportional Venn diagram (AAPVD) analysis was generated using PatternLab to pinpoint proteins that were uniquely identified in the A172 (Table II) or in A172r (Table III). The selection criterion was stringent because it considered only proteins found in all three replicates of each state and not found in any of the other state. We identified 19 and 72 unique proteins related to the A172 and A172r cells, respectively. The Venn diagram is presented in Figure 4. A less stringent analysis considering only proteins found in at least two of the three replicates is found in Supplemental Data III; this analysis yielded 133 and 257 uniquely identified proteins for A172 and A172r, respectively.

Discussion and Conclusions

Prior pharmacological studies have generated several clues on the mechanism of action of POH. It has been demonstrated that POH acts by inhibiting the isoprenylation of the small GTPase Ras proteins by blocking their tethering in the cytoplasmic membrane; this leads to inhibiting Ras signal transduction. 25, 26 Moreover, POH was shown to induce apoptosis, 4,27,28 causes G0/G1 arrest in several types of cancers, 29,30 instigates transitory G2/M arrest, and induce enhanced Fas-mediated apoptosis. 31 The ability of POH to disrupt protein anchorage to cell membrane suggests it may interfere with other signaling processes.

A more detailed understanding of the effects of POH on A172 could help in developing new synergic and more efficient treatments for this disease. Some key differentially expressed proteins from Table I that could be linked to the POH treatment or the underlying mechanisms of POH resistance are pinpointed below:

- Cofilin (IPI00012011.6) is related to cell locomotion. This process is the one by which tumor cells spread to surrounding tissues by active invasion. *In vivo*, cells that have a more rapid locomotion are found to be more invasive and metastatic. 32 *In vitro*, cell motility has been shown to be an key part of the invasive potential of glioma tumors; moreover, the locomotory habits of particular glioma cell lines derived from tumors has been shown to be stable, and unaffected by passage

number.³³ Cofilin is known to be overexpressed in glioma tumor cells, ³⁴ and in several other tumor cell types.³⁵⁻³⁶ We found a decrease in the expression of cofilin in the A172r cells as shown in Table I. This suggests that POH reduces the chances of tumor metastasis and invasiveness.

- Several heat shock proteins (HSPs) were found to be up-regulated in A172r (e.g., HSP90AA1, HSP90AB1, HSPA1B, HSPA1A). HSPs, also known as stress proteins are present in cells of all life forms and appear to confer protection against a wide range of cellular and tissue injuries. ³⁷ The HSPs superfamily includes several different molecular weight class families of which the HSP70 family is the most highly conserved. HSP70 is a prominent cytoprotective factor: its downregulation can kill tumor cells and facilitate the induction of apoptosis.³⁸⁻³⁹ In contrast, its upregulation, as a consequence of either cellular stress or transfection, is linked with inhibiting apoptosis.⁴⁰⁻⁴¹ For example, expression levels of HSP70 were shown to correlate negatively with response to chemotherapy in breast cancer. ⁴² Our results showed HSP70 to be over-expressed in the A172r cells suggesting a contribution to the resistance of POH.
- The 4F2 cell-surface antigen heavy chain (IPI00027493.1) was found to be upregulated in A172r and is annotated in the GO to be related to cell growth. Its over-expression was previously linked with the resistance to mitoxantrone in MCF-7 cells (human breast cancer cell line). ⁴³
- ARHGAP1 Rho GTPase-activating protein 1 (IPI00020567.1), NPC1 Niemann-Pick C1 protein precursor (IPI00005107.2), ITGB4 Isoform Beta-4C of Integrin beta-4 p (IPI00027422.1) mediates the transfer of a signal from the outside to the inside of a cell by means other than the introduction of the signal molecule itself into the cell. They were found downregulated in A172r; however, ITGA2 Integrin alpha-2 precursor (IPI00013744.1), that serves the same purpose, was found to be overexpressed suggesting major changes in signal transduction. The expression of the latter has been noted to be correlated with resistance in malignant mesotheliomas. ⁴⁴

The PatternLab Venn diagram analysis pinpointed proteins that were uniquely identified in either the A172 or A172r. It should be noted that “uniquely identified” does not necessarily mean that the protein is non-existent in a given condition; however, it was below the detection limits of our technique. Among these proteins we pinpoint a few found in the A172r that have been linked with tumor resistance or cell growth:

- NOTCH2 Neurogenic locus notch homolog protein 2 precursor (IPI00297655.4) is linked with a negative regulation of apoptosis and programmed cell death. Recently, Wang *et al.* linked this protein with promoting resistance of glioma stem cells to radiotherapy.⁴⁵ Moreover, the authors showed that inhibition of Notch pathway with gamma-secretase inhibitors (GSIs) renders the glioma stem cells more sensitive to radiation at clinically relevant doses.
- G3BP2 Isoform A of Ras GTPase-activating protein-binding protein 2 (IPI00009057.2) is linked with signal transduction. Auger *et al.*, has also linked the over-expression of this protein with resistance to temozolomide in glioma cell lines.⁴⁶
- HSPA6 Heat shock 70 kDa protein 6 (Hsp70-6) was identified among the unique proteins. The human cytosolic Hsp70s are composed of six canonical members, named Hsp70-1a, Hsp70-1b, Hsp70-1t, Hsp70-2, Hsp70-6, and Hsc70. In particular, HSP 70-6 is, most likely, the least studied of this family. It usually shows almost no basal expression and is only expressed in cases of extreme

stresses.⁴⁷ Such appearance suggests a link with the glioblastoma cell line and resistance to POH.

- TPT1 Tumor protein, translationally-controlled 1 (IPI00009943.2). The expression of this protein is up-regulated by growth stimuli and repressed when growth arrest is induced. This protein has been shown to be correlated with glioma growth in previous studies.⁴⁸
- PXN Isoform Alpha of Paxillin (IPI00220030.1) is a target of a number of oncogenes involved in key signal transduction and important in cell motility and migration. The up-regulation of this protein was linked with an increase in tumor growth and cell proliferation in lung cancer.⁴⁹
- RIC8A resistance to inhibitors of cholinesterase 8 homolog A (IPI00100106.1). This protein is a candidate marker for tumor aggressiveness or tamoxifen resistance in estrogen receptor -positive breast cancers. ⁵⁰

It is too much to expect that Ras therapies alone can be successful against high-grade malignant gliomas in the clinic. Our results mark a step forward in understanding the resistance mechanisms and candidates that could be target for knock-down experimentation. The results include lists of candidate protein markers that are representative of either the resistant or the non-resistant state of the A172 in response to POH, thus providing a good starting point for future investigations. Even though the linkage of several proteins have been made to the context of cancer disease states current knowledge only allows us to at best link the presence of these proteins to a phenotype of a disease. Therefore, these proteins are not necessarily causative of the disease and could just as likely be proteins associated to a cellular state occurring during the progression of the tumor without special consequences to the actual process of the disease. Among the challenges and limitations of this work, we should point out a few: a) the great complexity and dynamic range of proteins in the biological sample analyzed limits our analysis only to the most abundant proteins; b) The study did not target differential expression of post-translational modifications; c) The results obtained by using a human glioblastoma cell line do not necessarily reflect the same changes that occur in patients' tumors, but our expectation is that there should be many common markers. We note that disease biology encompasses the interplay of interacting proteins within the entire cellular proteome. Tailoring experiments to better understand how these multiple gene products interact and how they are linked to each level of the disease development could provide further molecular insights on how to treat such pathologies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Magnetic resonance imaging (MRI) follow-up on a patient with recurrent primary glioblastoma multiforme (GBM) in the lobar location under treatment with perillyl alcohol (POH) by intranasal delivery. A: MRI before starting the treatment with POH. B: MRI after 5 months of treatment with POH. C: MRI after 7 months of treatment with POH.

Figure 2.

A TFold comparative plot between the A172r and the A172 cells. Only proteins found in all 6 MudPIT assays were considered for this analysis. Each protein (represented as a dot) is mapped according to its $\log_2$ (fold change) on the ordinate axis and its $-\log_2$ (t-test **p**-value) on the abscissa axis. The latter indicates how likely the observed fold change is a result of chance. Fold changes refer to the ratios of the average relative quantitation values obtained for each state. Accordingly, blue-dot proteins have p -value < 0.01 and an absolute fold change greater than 2.5, the established fold-change cutoff. Orange-dot proteins did not meet the fold-change cutoff but were indicated as statistically different. Green-dot proteins met the fold-change cutoff but cannot be claimed to be statistically different. Red dots did not satisfy the fold-change or the statistical cutoffs. The BH theoretical false-positive estimator ($q < 0.05$) indicates that all proteins selected as differentially expressed are likely to be truly differentially expressed. There are 57, 60, 72, and 1284 blue, orange, green, and red dots, respectively.

Figure 3.

Western blot of HSP70: Slots 1 and 2 correspond to A172 and A172r, respectively. The western blot shows HSP70 to be over-expressed in A172r. Beta-tubulin, a protein not indicated as differentially expressed by the TFold, was used as an internal standard.

Figure 4.

Approximately proportional Venn diagram of the identified proteins presents in all three replicates of each state: The dark green represents the proteins unique of the A172 cells; the yellow represents the proteins unique of the resistant cells (A172r). The number in parentheses represents the unique identified proteins in each condition. Only proteins present in all three replicates of a state were considered.

Table I

Differentially expressed proteins selected by the TFold Analysis. All proteins were found to be statistically differentially expressed ($p < 0.01$) and have an absolute fold change greater than 2.5. The set of proteins also satisfies the BH-FDR of 0.05 criterion. A positive fold indicates a greater expression on the A172r.

IPI	Fold Change	Description
IPI00027493.1	5.36	SLC3A2;LOC442497 4F2 cell-surface antigen heavy chain
IPI00382470.3	4.69	HSP90AA1 Heat shock protein HSP 90-alpha 2
IPI00013744.1	4.47	ITGA2 Integrin alpha-2 precursor
IPI00384280.5	3.98	PCYOX1 Prenylcysteine oxidase precursor
IPI00334775.6	3.72	HSP90AB1 85 kDa protein
IPI00033494.3	3.71	MRLC2 Myosin regulatory light chain
IPI00298237.7	3.67	TPP1 Isoform 1 of Tripeptidyl-peptidase 1 precursor
IPI00332106.2	3.56	PBXIP1 Pre-B-cell leukemia transcription factor-interacting protein 1
IPI00065486.2	3.55	ABCB6 Isoform 4 of Mitochondrial ATP-binding cassette subfamily B member 6
IPI00031357.1	3.43	PPOX Protoporphyrinogen oxidase
IPI00304925.5	3.13	HSPA1B;HSPA1A Heat shock 70 kDa protein 1
IPI00029133.4	2.90	ATP5F1 ATP synthase B chain, mitochondrial precursor
IPI00099463.2	2.86	SGPL1 Sphingosine-1-phosphate lyase 1
IPI00003527.5	-2.50	SLC9A3R1 Ezrin-radixin-moesin-binding phosphoprotein 50
IPI00456940.5	-2.50	RPL7L1 Ribosomal protein L7-like 1
IPI00106847.3	-2.50	MSH6 Isoform GTBP-alt of DNA mismatch repair protein MSH6
IPI00186290.6	-2.51	EEF2 Elongation factor 2
IPI00000816.1	-2.52	YWHAE 14-3-3 protein epsilon
IPI00012773.1	-2.57	MTA1 Isoform Long of Metastasis-associated protein MTA1
IPI00029111.2	-2.58	DPYSL3 DPYSL3 protein
IPI00007207.2	-2.60	LIPA Isoform 1 of Lysosomal acid lipase/cholesteryl ester hydrolase precursor
IPI00005107.2	-2.64	NPC1 Niemann-Pick C1 protein precursor
IPI00029019.5	-2.75	UBAP2L Isoform 2 of Ubiquitin-associated protein 2-like
IPI00169383.3	-2.77	PGK1 Phosphoglycerate kinase 1
IPI00183786.1	-2.89	FADS2 fatty acid desaturase 2
IPI00008868.3	-2.97	MAP1B Microtubule-associated protein 1B
IPI00218918.5	-3.00	ANXA1 Annexin A1
IPI00000875.6	-3.07	EEF1G Elongation factor 1-gamma
IPI00376798.3	-3.10	RPL11 Isoform 1 of 60S ribosomal protein L11
IPI00221093.7	-3.13	RPS17 40S ribosomal protein S17
IPI00020567.1	-3.25	ARHGAP1 Rho GTPase-activating protein 1
IPI00299086.3	-3.33	SDCBP Syntenin-1
IPI00479018.2	-3.33	SDCBP Syntenin isoform 3
IPI00297261.3	-3.50	PTPN1 Tyrosine-protein phosphatase non-receptor type 1
IPI00000070.1	-3.53	LDLR Low-density lipoprotein receptor precursor

IPI	Fold Change	Description
IPI00739099.2	-3.64	COL5A2 Collagen alpha-2(V) chain precursor
IPI00418471.6	-3.80	VIM Vimentin
IPI00296141.3	-3.82	DPP7 Dipeptidyl-peptidase 2 precursor
IPI00008894.2	-3.92	CPA4 Carboxypeptidase A4 precursor
IPI00479186.5	-3.98	PKM2 Isoform M2 of Pyruvate kinase isozymes M1/M2
IPI00013068.1	-4.00	EIF3S6 Eukaryotic translation initiation factor 3 subunit 6
IPI00072377.1	-4.24	SET Isoform 1 of Protein SET
IPI00289758.6	-4.25	CAPN2 Calpain-2 catalytic subunit precursor
IPI00025491.1	-4.27	EIF4A1 Eukaryotic initiation factor 4A-I
IPI00328298.6	-4.29	SMC4 Isoform 2 of Structural maintenance of chromosomes protein 4
IPI00007812.1	-4.56	ATP6V1B2 Vacuolar ATP synthase subunit B, brain isoform
IPI00043598.2	-4.63	IKIP CDNA FLJ31051 fis, clone HSYRA2000605, weakly similar to MYOSIN HEAVY CHAIN, CLONE 203
IPI00027422.1	-4.69	ITGB4 Isoform Beta-4C of Integrin beta-4 precursor
IPI00396171.3	-4.88	MAP4 Isoform 1 of Microtubule-associated protein 4
IPI00184330.5	-5.78	MCM2 DNA replication licensing factor MCM2
IPI00465248.5	-6.05	ENO1 Isoform alpha-enolase of Alpha-enolase
IPI00021033.2	-6.71	COL3A1 Isoform 1 of Collagen alpha-1(III) chain precursor
IPI00027626.3	-6.90	CCT6A T-complex protein 1 subunit zeta
IPI00784347.2	-8.92	KRT18 Keratin, type I cytoskeletal 18
IPI00026781.2	-10.00	FASN Fatty acid synthase
IPI00554648.3	-11.31	KRT8 Keratin, type II cytoskeletal 8
IPI00012011.6	-15.64	CFL1 Cofilin-1

Table II

Uniquely identified proteins in the A172 cell culture

IPI00384938.1	IGHG1 Hypothetical protein DKFZp686N02209
IPI00011454.1	GANAB Isoform 2 of Neutral alpha-glucosidase AB precursor
IPI00008603.1	ACTA2 Actin, aortic smooth muscle
IPI00015737.1	DCAKD CDNA: FLJ22955 fis, clone KAT09907
IPI00386258.1	MTCH1 Isoform 1 of Mitochondrial carrier homolog 1
IPI00003926.3	CLN8 Protein CLN8
IPI00027776.6	FECH Ferrochelataase, mitochondrial precursor
IPI00016915.1	IGFBP7 Insulin-like growth factor-binding protein 7 precursor
IPI00177940.2	RDH14;NT5C1B Retinol dehydrogenase 14
IPI00328383.2	SLC25A46 48 kDa protein
IPI00396329.1	BXDC1 Brix domain-containing protein 1
IPI00171445.1	ATAD1 ATPase family AAA domain-containing protein 1
IPI00294380.5	PCK2 Phosphoenolpyruvate carboxykinase [GTP], mitochondrial precursor
IPI00030380.1	GTF2H1 TFIIF basal transcription factor complex p62 subunit
IPI00295598.4	CBARA1 calcium binding atopy-related autoantigen 1
IPI00433279.3	SLFN5 Isoform 1 of Schlafen family member 5
IPI00304331.2	B3GAT3 Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase 3
IPI00302966.5	TTC17 Tetratricopeptide repeat protein 17
IPI00027443.5	CARS cysteinyl-tRNA synthetase isoform c

Table III

Uniquely identified proteins in the A172r cell culture

IPI00216952.1	LMNA Isoform C of Lamin-A/C
IPI00383581.3	GANAB Isoform 1 of Neutral alpha-glucosidase AB precursor
IPI00021428.1	ACTA1 Actin, alpha skeletal muscle
IPI00026302.3	RPL31 60S ribosomal protein L31
IPI00219910.1	BLVRB 23 kDa protein
IPI00007063.5	HDGFRP3 Hepatoma-derived growth factor-related protein 3
IPI00328616.3	MRPL52 39S ribosomal protein L52, mitochondrial precursor
IPI00219446.5	PEBP1 Phosphatidylethanolamine-binding protein 1
IPI00010201.4	- 40 kDa protein
IPI00221091.9	RPS15A 40S ribosomal protein S15a
IPI00022442.2	NDUFAB1 Acyl carrier protein, mitochondrial precursor
IPI00291093.3	POLR2E DNA-directed RNA polymerases I, II, and III subunit RPABC1
IPI00215790.6	RPL38 60S ribosomal protein L38
IPI00003949.1	UBE2N Ubiquitin-conjugating enzyme E2 N
IPI00419979.3	PAK2 Serine/threonine-protein kinase PAK 2
IPI00218830.1	NMT1 Isoform Short of Glycylpeptide N-tetradecanoyltransferase 1
IPI00152658.1	NEK7 Serine/threonine-protein kinase Nek7
IPI00100106.1	RIC8A resistance to inhibitors of cholinesterase 8 homolog A
IPI00741973.1	LOC131691 similar to peptidylprolyl isomerase A isoform 1
IPI00031650.3	NUDT16L1 Isoform 1 of Protein syndesmos
IPI00018272.3	PNPO Pyridoxine-5'-phosphate oxidase
IPI00247063.3	MME Neprilysin
IPI00295851.4	COPB1 Coatamer subunit beta
IPI00017763.4	NAP1L4 Nucleosome assembly protein 1-like 4
IPI00037283.3	DNM1L Isoform 5 of Dynamin-1-like protein
IPI00101532.5	CEP55 Isoform 1 of Centrosomal protein of 55 kDa
IPI00007402.2	IPO7 120 kDa protein
IPI00009982.1	TDRKH Isoform 1 of Tudor and KH domain-containing protein
IPI00019901.1	ADD1 Isoform 1 of Alpha-adducin
IPI00006029.2	FOXK2 Isoform 1 of Forkhead box protein K2
IPI00032957.1	UBE2I SUMO-conjugating enzyme UBC9
IPI00456635.1	UNC13D Isoform 1 of Unc-13 homolog D
IPI00024364.2	TNPO1 transportin 1 isoform 1
IPI00034159.1	ATP6V0D1 Vacuolar ATP synthase subunit d 1
IPI00012726.4	PABPC4 Isoform 1 of Polyadenylate-binding protein 4
IPI00220030.1	PXN Isoform Alpha of Paxillin
IPI00009943.2	TPT1 Tumor protein, translationally-controlled 1
IPI00004962.2	GOLPH4 Golgi phosphoprotein 4

IPI00165357.4	MTA3 Isoform 1 of Metastasis-associated protein MTA3
IPI00052885.7	LOC729611;LOC731796 similar to 60S ribosomal protein L29
IPI00032137.1	ACTN3 Alpha-actinin-3
IPI00008380.1	PPP2CA Serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform
IPI00008339.5	C14orf173 hypothetical protein LOC64423 isoform 1
IPI00419847.3	SORBS3 Isoform Alpha of Vinexin
IPI00293073.4	MFN1 Mitochondrial transmembrane GTPase FZO-2
IPI00009822.1	SRP54 Signal recognition particle 54 kDa protein
IPI00009057.2	G3BP2 Isoform A of Ras GTPase-activating protein-binding protein 2
IPI00029723.1	FSTL1 Follistatin-related protein 1 precursor
IPI00185146.5	IPO9 Importin-9
IPI00005030.5	SH3BP4 Isoform 1 of SH3 domain-binding protein 4
IPI00061356.3	CCDC99 Isoform 1 of Coiled-coil domain-containing protein 99
IPI00291783.3	GEMIN5 Gem-associated protein 5
IPI00014456.4	STRN Isoform 1 of Striatin
IPI00030009.4	PAPSS2 Isoform A of Bifunctional 3'-phosphoadenosine 5'-phosphosulfate synthetase 2
IPI00375843.2	KRT80 Keratin-80
IPI00152701.7	PLCD3 phospholipase C delta 3
IPI00742944.2	FMN2 Formin-2
IPI00030781.1	STAT1 Isoform Alpha of Signal transducer and activator of transcription 1-alpha/beta
IPI00339269.1	HSPA6 Heat shock 70 kDa protein 6
IPI00003843.1	TJP2 Isoform A1 of Tight junction protein ZO-2
IPI00013871.1	RRM1 Ribonucleoside-diphosphate reductase large subunit
IPI00023339.2	CREBBP CREB-binding protein
IPI00014897.4	PLCB4 Isoform 2 of 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase beta 4
IPI00296388.4	BAZ2A Isoform 1 of Bromodomain adjacent to zinc finger domain protein 2A
IPI00107531.1	RAD50 Isoform 3 of DNA repair protein RAD50
IPI00479125.3	SRGAP2 SLIT-ROBO Rho GTPase-activating protein 2
IPI00034006.1	PTPN23 Tyrosine-protein phosphatase non-receptor type 23
IPI00409601.1	KIAA1219 Isoform 1 of Protein KIAA1219
IPI00166010.6	CNOT1 CCR4-NOT transcription complex, subunit 1 isoform a
IPI00297655.4	NOTCH2 Neurogenic locus notch homolog protein 2 precursor
IPI00159322.4	TCF20 Isoform 1 of Transcription factor 20
IPI00019447.5	FANCI Isoform 1 of Fanconi anemia group I protein