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Original Research Article

Proteomic analysis of 17-DMAG effects on colon cancer HT-29 cells

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17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) is an inhibitor of heat shock protein 90 (HSP 90) that stabilizes a variety of proteins required for survival of cancer cells. 17-DMAG was developed as chemotherapeutic agents, having an increasing importance in cancer treatment. However, their effect on colon cancer and on the regulated proteins remains largely unknown. In this study, 17-DMAG was used and its effects on colon cancer cell line HT-29 were examined. 17-DMAG can induce apoptosis and inhibit the proliferation of HT-29 cells in a dose-and time-dependent manner. Through two-dimensional electrophoresis (2DE), we detected 19 differentially expressed protein spots in the HT-29 cells after 17-DMAG (1 μM) treating for 24 hours (h) compared with the un-treated. Of which, 2 up- and 14 down-regulated non-redundant proteins were identified by liquid chromatography-tandem mass spectrometry (LC-MS). Furthermore real-time RT-PCR showed that 4 differentially expressed proteins were down-regulated in mRNA level with consistent regulation in protein level. Two of them (cortactin (SRC8) and Heat shock 70 kDa protein 1A/1B (HSP71) were verified by western blotting to have consistent change with 2DE based proteomic study. In conclusion, we demonstrated that 17-DMAG inhibits proliferation and induces apoptosis. Moreover, 17-DMAG altered the expression profile of 16 proteins including cortactin and HSP71. This data suggest that SRC8 or HSP71 may be potential HSP90 client proteins and new targets for anti-cancer drug development.

Key words: 17-DMAG, proteomics, colon cancer, HT-29, cortactin, HSP71

Abbreviations: HSP90: heat shock protein 90; 17-DMAG: 17-dimethylaminoethylamino-17-demethoxygeldanamycin; 2DE: two-dimensional electrophoresis; LC-MS: liquid chromatography-tandem mass spectrometry; HSP71: Heat shock 70 kDa protein 1A/1B; SRC8: cortactin

INTRODUCTION

In cancer-related death, colon cancer is the second leading cause (Katicic et al., 2012). Recently, with the development in understanding of the molecular biology in colon cancer, targeted therapy with low toxicity and few side effects has drawn the attention of more and more people (Nelson and Benson, 2013). In the search for new colon cancer therapeutics, it is very important to discover better molecular targets which are crucial to tumorigenesis. Heat shock proteins (HSPs) have been considered as especially useful anti-cancer drug targets for their location at the crossroads of multiple signal pathways related to tumor cell proliferation, differentiation and apoptosis in colon cancer (Stravopodis et al., 2007).

Among the several HSPs, HSP 90 has the most important functions. Clinical trials of HSP90 inhibitors, such as 17-DMAG, have been performed and observed promising results in some malignant diseases (Banerji, 2009; Schwock et al., 2008 ; Stravopodis et al., 2007 ; Whitesell and Lin,

2012; Whitesell et al., 2012).

However, due to the important function of HSP90 in several signaling pathways, the inhibition of HSP90 in healthy cells can lead to severe side effects. To optimize chemotherapeutic treatment and to minimize side effects, it is important to identify regulated proteins and understand the molecular mechanism of inhibiting HSP90-dependent pathways in cancer cells. 17-DMAG was found to enhance growth inhibition of rhabdomyosarcoma (RMS) cells via autophagy (Peron et al., 2012), and it can also ameliorate the anti-tumor efficacy of $CD8^+$ T effector cells (Kawabe et al., 2009). However, the global effects of 17-DMAG on the proteome remains largely unknown.

Recently, comparative proteomic analysis has been widely used for identifying proteins which could potentially be associated to cancer etiology, progression, therapy and prognosis (de Wit et al., 2013; Luo et al., 2013). A proteomic study was performed to identify the alteration of proteins by 17-DMAG to Hela cells, and found that 17-DMAG preferentially targets tyrosine kinases and the DNA damage response (Sharma et al., 2011). However, few proteomic studies have been conducted on 17-DMAG effects on colon cancer. Therefore, in this study, we investigated the proliferation and apoptotic effects of 17 -DMAG on HT-29 cells *in vitro*. Then, a proteomic study based on two-dimensional electrophoresis (2DE) combined with tandem mass spectrometry was performed to elucidate the mechanism of the effect of 17-DMAG on HT-29 cells.

MATERIALS AND METHODS

Reagents

17-DMAG from Sigma Company (St. Louis, New Jersey, USA); Annexin-V-FITC and CCK-8 from Shanghai Beyotime Institute of Biotechnology (Nantong, Jiangsu, China); β-actin antibody from Cell Signaling Technology (CST) Company (Boston, Massachusetts, USA); Electrophoresis kits from General Electric Healthcare Company (GE) (Schenectady, New York, USA); Other materials were chemically pure and obtained from Sinopharm Chemical Reagent Company, Ltd (Shanghai, China).

Cell culture

HT-29 cells were cultured in McCoy's 5A medium with 10 % fetal bovine serum containing 100 μg/mL streptomycin, 100 IU/mL penicillin and were maintained in a 37 °C humidified chamber containing 5 % CO_2. HT-29 cell line was free of Mycoplasma contamination. Exponentially growing cells were used for the following studies.

17-DMAG was dissolved in dimethyl sulfoxide (DMSO) and used as stock solution. Before use, the stock solution was diluted with McCoy's 5A medium. The purity of 17-DMAG was assessed to be > 96 % based on high-performance liquid chromatography analysis according to the product manual.

Proliferation assay

CCK-8 assay was performed to measure the anti-proliferation effects of 17-DMAG on HT-29 cells according to the company's protocol. Briefly, the cell suspension were dispersed to 96-well flat-bottomed microplates containing 100 μL of the various 17-DMAG dilutions with final concentrations at 0 (0.1 % DMSO or not), 0.25, 0.5, 1.0, 2.5 and 5.0 μmol/L in 0.1 % DMSO in three replicate wells. The cells were incubated for 24, 48 and 72 h, respectively. Each well then received 10 μL CCK-8 solutions according to the kit's user guidelines explanations. After 1 h incubation, the plate was agitated on a shaker before reading absorbance at 450 nm using a microplate reader (1500-UV, Thermo, USA). The experiments were repeated for three times.

Apoptosis assay

The cells were stained with Propidium iodide (PI) and Annexin-V according to the kit instruction. Briefly, cells were washed with PBS twice and stained with Annexin-V FITC and PI staining for 10 min at room temperature. Then the stained cells were detected by FACS Aria II flow cytometer (BD Biocsiences, San Jose, California, USA).

Protein sample preparation

Exponentially growing HT-29 cells (3×10^5) were cultured in 96-well flat-bottomed microplates for 24 h. After being washed by PBS twice, the cells were treated with 1.0 μmol/L 17-DMAG or 1‰ DMSO for 24 h. The cells were washed three times with PBS and lysed in a buffer containing 8 M urea, 2 M thiourea, 0.5 mM PMSF, 4 % CHAPS, 0.5 % IPG buffer 3–10, 1 % NP-40, 65 mM DTT, DNA I (1 μg/mL) and RNase A (1 μg/mL), and centrifuged at 4 °C, 12,000 rpm for 10 min and the supernatant was collected and stored at -80 °C at the volume of 50 μg (for western blot) and 1000 μg (for two-dimensional electrophoresis).

Two-dimensional gel electrophoresis (2DE) and Image Analysis

The 2DE and Image Analysis were performed according to the previously published (Zhang et al., 2006). Briefly, the first dimension of 2DE was carried out on an IPGphor iso-electronic focusing (IEF) system (GE Company, Schenectady, New York, USA). Solubilized proteins (1 mg) were mixed with IEF sample buffer containing 8 M urea, 2 M thiourea, 65 mM DTT, 20 mM Tris-base, 4 % CHAPS, 0.5 % IPG buffer, pH 3–10 NL to a total volume of 350 μL with a trace of bromophenol blue, and applied to IPG DryStrips (pH 3–10 NL; 180×3×0.5 mm). IEF was performed at 20 °C under the conditions: 50 V for 14 h, 500 V for 1 h, 1000 V for 1 h, 8000 V gradient for 2250 Vhr, 8000 V for 6 h up to 51.7 kVh. After IEF, the gel strips were equilibrated twice in equilibration buffer containing 6 M urea, 50 mM Tris-HCl, 30 % glycerol, 2 % SDS, pH 8.8 for 15 min in which 0.2 %

Table 1. The Effects of 17-DMAG on abundance of RNAs encoding 6 differentially expressed proteins identified by LC-MS

spot	Protein name(_HUMAN)	primers(5'→3')	fold change[a]
1	AMD	F: TTCGTGATTGACTTCAAGCCTC R: CTCGCCCAGGCATACAGAAT	0.11
2	LMNB1	F: GATCGAGCTGGGCAAGTG R: TTCTCGAAGCTTGATCTGGG	0.21
4	SRC8	F: AGCACGAGTCACAGAGAGATT R: AAAGCCAACGGCGCTCTTA	0.24
10	HSP71	F: TTTGAGGGCATCGACTTCTACA R: CCAGGACCAGGTCGTGAATC	0.06
11	K2C8	F: TCCTCAGGCAGCTATATGAAGAG R: GGTTGGCAATATCCTCGTACTGT	0.03
19	TCTP	F: GAAAGCACAGTAATCACTGGTGT R: GCAGCCCCTGTCATAAAAGGT	0.12

a: Compared with the control (1‰ DMSO), the changed rate of genes in the 17-DMAG treated group. The experiments were repeated for three times, $P<0.05$.

DTT was added into the first step equilibration buffer and 3 % iodoacetamide was added into the second. The second dimensional run was carried out on a SDS-PAGE vertical slab, 12 % separation gel with Bio-Rad Protein II electrophoresis apparatus (Bio-Rad Company, Hercules, California, USA). Mr calibration was added via a molecular weight marker. Separate gels were run at constant current of 30 mA/gel. After completion of the 2DE, gels were stained using Coomassie brilliant blue G-250. The 2DE gels were scanned in transmission mode via Imagescaner machine (GE Company, Schenectady, New York, USA).

The image analysis was performed using ImageMaster software (GE Company, Schenectady, New York, USA). A match set consisting of six images, including three from the control group and three from the experiment group. The differential protein spots were discovered by comparing the relative volume of each spot. The differential protein spots were defined by >2-fold difference in the average volume of a spot.

Tandem mass spectrometric identification of proteins

The differential protein spots were cut out and in-gel digested as previously published (Zhang et al., 2006). The digested peptides were analyzed via nanoLC-ESI-MS/MS (esquire HCT, Bruker Company, Germany). The digested peptide mixtures were injected onto a C18 μ-precolumn (300 μm id × 5 mm, 5 μm, PepMap™) (Dionex, USA) with a flow rate of 20 μL/min in an Ultimate 3000 High Performance Liquid Chromatography (Dionex, USA). After being desalted by precolumn, the peptides were eluted to a C18 reversed-phase nano-column (75 μm id × 15 cm length, 3 μm, PepMap™) (Dionex, USA). The flow rate was 300 nL/min with continuous gradient consisting of 3-48 % and 0.1 % FA. The eluted peptides from the reversed-phase nanocolumn were on line injected to HCT mass spectrometer through a PicoTip emitter nanospray needle (New Objective, Woburn, MA, USA), and used for real-time ionization and peptide fragmentation. The MS/MS data was input in MASCOT 2.0 program (MatrixScience, Boston, MA, USA) to search the SWISS-PROT database. Search parameters were set as follows: trypsin was used as enzyme with one missed cleavage; mass and MS/MS tolerance were 1.2 Da and 0.6 respectively; carbamoylmethylation and oxidation (M) were selected as fixed and variable modification respectively; for species, Homo sapiens was selected. Proteins were identified based on the threshold ($p < 0.05$) set in the mascot software followed by manual check. When the proteins were identified by more than 4 peptides, no manual check was done. Those identified by less than 3 peptides were manually inspected. Of which, there must be at least one peptide with four or more continuous y-or b-series ions (e.g., y2, y3, y4, y5).

RNA extraction and RT-PCR

To isolate the total RNA, 1×10^6 HT-29 cells were collected in micro-centrifuge tubes containing 1 mL TRIzol reagent (Sigma, USA). The RNA was extracted according to the manufacturer's protocol and treated with 5 U/μL DNase I (Promega, USA) to remove contaminating genomic DNA. The purity and concentration of the RNA were determined with a NanoDrop (NanoDrop Technologies, USA). Subsequently, cDNA was synthesized through using the Superscript II kit (Life Technologies, USA). Real-time RT-PCR was performed on a 25 μL reaction mixture containing 700 nM forward and reverse primers, 80nM template and 1x Sybr Green reaction mix (Applied Biosystems, CA). The primer sequences used are listed in Table 1. GAPDH was used for normalization. Fold change was calculated according to the $2^{-\Delta\Delta CT}$ (cycle threshold) method.

Western blot detection

The protein samples treated with 17-DMAG (1.0aμmol/L) or 1 ‰ DMSO for 24 h and or 48ah were used for WB experiment. The extracted 50 μg protein was separated by

Table 2. The inhibiting rate of 17-DMAG to HT-29 at different concentration and treating time

Concentration (μmol/L)	24h	48h	72h
0	0	0	0
0.1	14.36±0.95#	20.8±1.17#△	29.62±2.27#△
0.25	22.17±1.15#	27.55±0.65#△	39.19±1.74#△
0.5	28.45±1.16#	33.33±1.23#△	44.29±2.00#△
1	35.04±1.58#	46.20±4.76#△	50.66±2.17#△
2.5	46.85±2.44#	55.45±4.47#△	58.84±3.18#△
5	57.19±2.06#	61.75±2.72#△	70.74±2.65#△

#, P<0.05, compared to the concentration of 0aμmol/L, significant differences were detected at other concentrations, with the same treating time.
△, P<0.05, comparison between different treating time using 24ah as control.

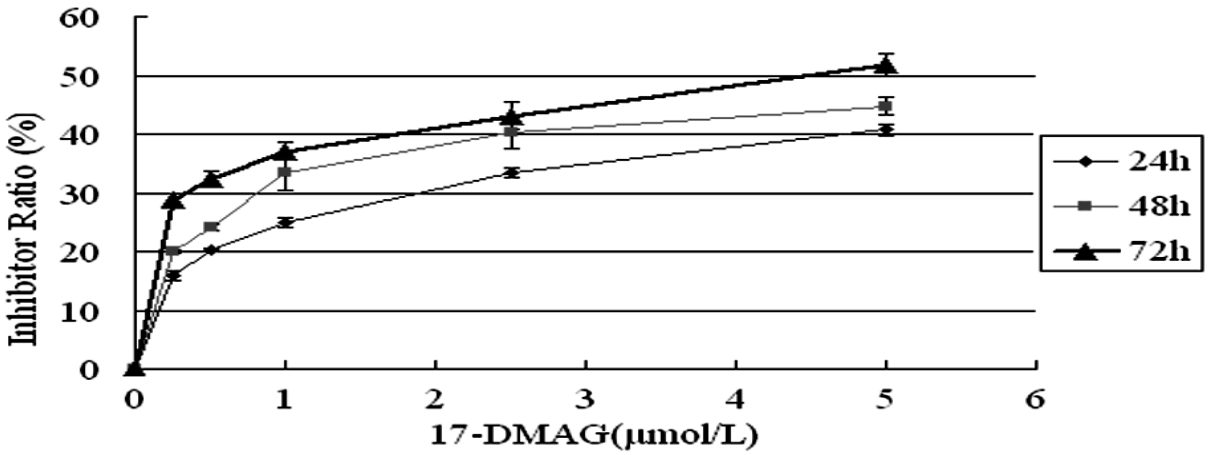

Figure 1: The cell proliferation inhibiting rate by 17-DMAG
Cells were treated with 17-DMAG at indicated dose and time point. The proliferation was determined by CCK-8 staining. Each spot represents the mean of the data from three independent experiments, p<0.05.

SDS-PAGE using 12.5 % separating gel. Then the proteins were transferred to 0.45aμm PVDF membrane. The membrane was blocked with 10% nonfat dry milk overnight and washed with TBST for three times and detected with antibodies raised against HSP90, HSP71, SRC8 (cortactin) or β-actin. Primary antibodies were used at a suitable dilution (1:1000 for HSP90, 1:2000 for HSP71 and SRC8, and 1:5000 for β-actin) in blocking buffer. After incubation with primary antibodies for 2 h at room temperature, the membrane was washed with TBST and incubated for 1 h at room temperature with secondary antibodies. Finally, the membrane was washed with TBST for three times and the immune complexes were revealed by enhanced chemiluminescence and detected by X-ray films.

Statistical analysis

Values are expressed as mean ± standard deviation (SD). One-way ANOVA was used for assessing the statistical significance of the effect of 17-DMAG concentrations. P values less than 0.05 were considered statistically significant.

RESULTS

17-DMAG inhibits HT-29 cells proliferation

As HSP90 has very important functions in cell proliferation, in this study, we checked the proliferation rate of 17-DMAG on HT-29 to select a suitable 17-DMAG concentration for follow-up studies. As shown in Table 2, after treatment with 17-DMAG at concentrations of 0, 0.25, 0.5, 1.0, 2.5 and 5.0 μmol/L, the proliferation inhibition rate (%) was significantly increased. For example, after treatment for 24 h, the inhibition rate (%) of HT-29 cells was increased to 14.36±0.95, 28.45±1.16, 35.04±1.58, 46.85±2.44 and 57.19±2.06 compared to the control respectively; at 48 h, the inhibitor rate was 20.80±1.17, 33.33±1.23, 46.20±4.76, 55.45±4.47 and 61.75±2.72 respectively. Furthermore, at the same concentration, the proliferation inhibiting rate increased with the treating time delay from 24 h, 48h to 72h. These data demonstrate that the proliferation of HT-29 cells are suppressed by 17-DMAG in dose and time dependent manner (Figure 1).

17-DMAG induces apoptosis in HT-29 cells

HSP90 families are the molecular chaperones of ubiquitous, involved in folding, activation and maturation of many proteins. The inhibition of HSP90 can induce cell apoptosis. Therefore in this work, the cell apoptosis induced by 17-DMAG were analyzed by flow cytometry technology. As shown in Figure 2A and 2B, after treatment with 17-DMAG for 24 h, both the early and total apoptotic rates were markedly increased with increase in concentration, from 0, 0.25, 0.5, 1.0 to 2.5 μmol/L ($P<0.05$). Moreover, following treatment with 1.0 μM 17-DMAG the apoptotic ratio was also significantly increased with the treating time extending from 6, 12, 24 to 48 h (Figure 2C).

17-DMAG inhibitor the expression of HSP90

Since 17-DMAG is an inhibitor of HSP90, the expression of HSP90 after treated by 17-DMAG (1.0 μM) for 24h and 48h was detected by western blot. As shown in Figure 3 A and 3B, HSP90 was decreased to 0.79- and 0.64- fold compared with its control ($p<0.05$).

17-DMAG regulates proteome expression

To better discover the complexity of HSP90-dependent cellular signaling we analyzed the effect of 17-DMAG on protein abundance at 24 h. 17-DMAG-treated HT-29 cells (1.0 μM) were compared to its control (1‰ DMSO) by spot expression patterns of 2-DE. 661±10 and 672±18 protein spots were detected in 2DE-gels of the experimental group and the control groups respectively (Figure 4). Using Image Master software analysis, 19 proteins spots were found to be differentially expressed with a threshold greater than 2-fold, including 2 with increased volume and 17 with decreased volume in the experiment group compared to the controls.

17-DMAG regulates the expression of 19 proteins

Following trypsin digestion, each spot was analyzed by ESI tandem mass spectrometry, 19 differentially expressed protein spots were successfully identified, including 2 up-regulated and 17 down-regulated proteins in 17-DMAG-treated groups compared to the control. The proteins with a decreased expression in the 17-DMAG-treated HT-29 cells were peptidyl-glycine alpha-amidating monooxygenase and L-lactate dehydrogenase B chain among others. The up-regulated ones were heat shock 70 kDa protein 1A/1B, and keratin and type II cytoskeletal 8 (Table 3).

17-DMAG regulates the mRNA of 6 genes

To investigate the changes in gene expression at mRNA levels of the differential proteins found by 2-DE, we isolated total mRNA from 17-DMAG-treated HT29 cells and from the controls, and performed quantitative RT-PCR analysis of 6 candidate proteins which related to enzyme, chaperone and binding, respectively. As shown in Table 1, all the candidate genes were found to be inhibited at the mRNA levels after 17-DMAG treatment. Among these, ADM, LMNB1, SRC8 and TCTP have consistent regulation between mRNA and protein levels. However for two proteins, HSP71 and K2C8, their mRNA levels did not correspond with the protein levels. This is not surprising, because the final amount of a protein was an accumulation regulatory event at their transcriptional, post-transcriptional, translational and post-translational level. Therefore, the validation of the gene expression levels need further study.

Verification of HSP71 and SRC8 by western blotting

Protein expression of SRC8 and HSP71 was evaluated by western blotting densitometry (Figure 3C and 3D). In comparison to the controls (0.972±0.018 for SRC8 and 0.730±0.038 for HSP71), 17-DMAG treatment induced significant down-regulation of SRC8 expression (0.522±0.079, $P<0.05$), in contrast it promotes up-regulation of HSP71 expression (1.052±0.057, $P<0.05$).

DISCUSSION

Based on the importance of heat shock proteins (HSPs) in diseases such as cancer, Alzheimer's or malaria, inhibitors of these chaperons are needed and have emerged as promising anti-cancer drugs in both solid and hematologic malignancies (Usmani et al., 2010). There are many methods of selecting heat shock protein inhibitors including microarray-based screening (Schax et al., 2014; Taddei et al., 2014) and of studying the molecular mechanism of heat shock protein inhibitors (Ayrault et al., 2009; Caldas-Lopes et al., 2009). As reviewed by Sidera K, 13 HSP90 inhibitors representing multiple drug classes, with different modes of action, are, currently, undergoing clinical evaluation (Sidera and Patsavoudi, 2014). Of which, 17-DMAG has also been undergoing clinical trials, for its capacity as a potential drug (Jhaveri et al., 2012). However, our understanding of 17-DMAG on the proteome remains largely insufficient. In order to find the regulated protein induced by 17-DMAG, several proteomic studies were performed (Haupt et al., 2012; Sharma et al., 2011). In these studies, they focus on cervical, myeloma and breast cancer and the knowledge on colon cancer is very limited (Wang et al., 2010). Furthermore, these studies only performed proteomic analysis to identify proteins related to 17-DMAG stimulation. Limited data has been obtained relating to the systemic knowledge of 17-DMAG concentration, cell function and proteome.

In this study, we selected suitable concentration and stimulation time of 17-DMAG through the analysis of the effects of 17-DMAG on the proliferation and apoptosis in colon cell line HT-29. According to Figure 1, the cell proliferation inhibiting rate gradient is similar at the concentration of 1.0 μm compared with its previous concentration of 0.5 or next concentration of 2.5aμm after

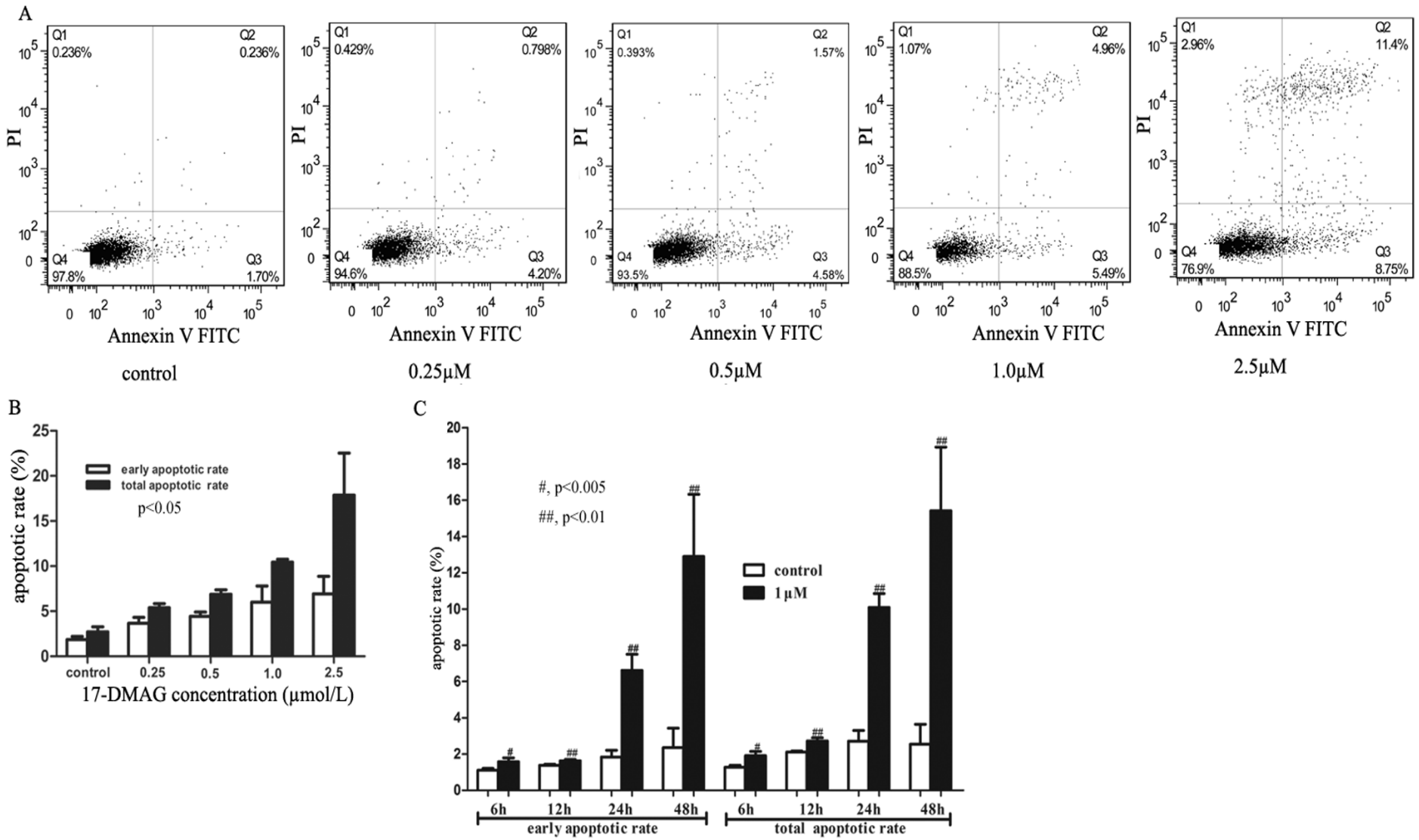

Figure 2: The apoptosis effect of 17-DMAG on HT-29 cells. The cells were treated with 17-DMAG at different concentration for 24 h, and then determined apoptotic cells by annexin-V & PI staining (A) and quantified (B)（p<0.05）; treated with 1.0 μM 17-DMAG at different time (C) . #p<0.05, ##p<0.01.

Q1: FITC－/PI+ Cells, necrotic cells, Q2: FITC+／PI+, late apoptotic cells; Q3: FITC+/PI－ cells, early apoptotic cells; Q4: FITC－/PI－, live cells. HT-29 cells were treated with 17-DMAG for 24h under different concentrations.

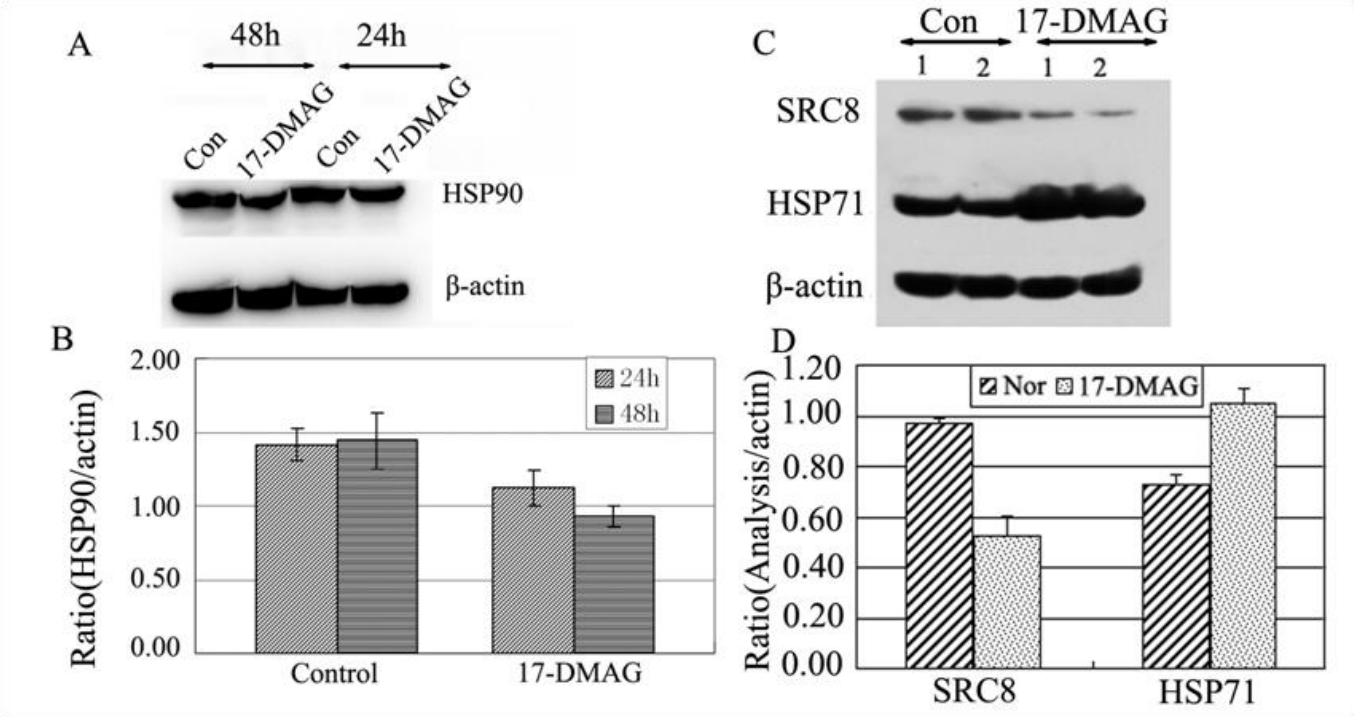

Figure 3: Western blotting analysis of HSP90, SRC8 and HSP71
(A) and (B) for HSP90, (C) and (D) for SRC8 and HSP71. (B) and (D) were from the statistical results of (A) and (C) respectively analyzed by Image J software.

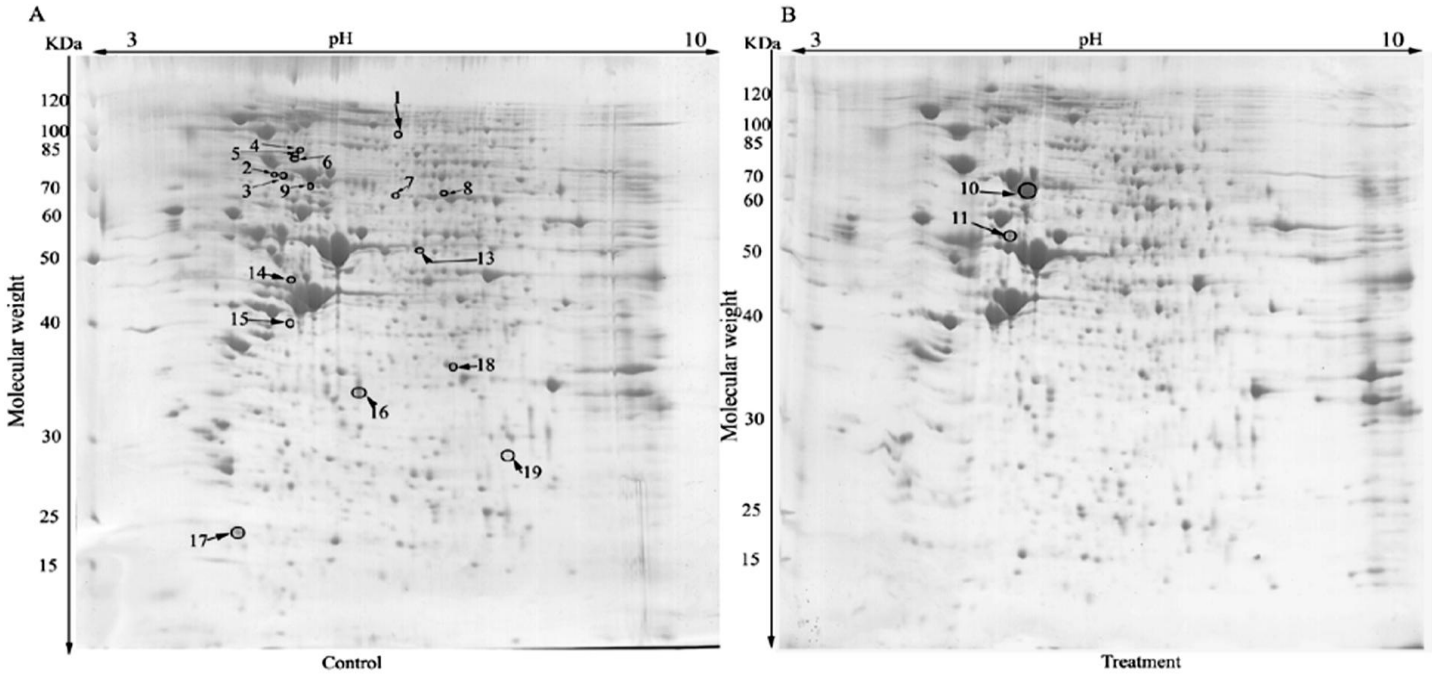

Figure 4: Separation and verification of differentially expressed protein spots from 17-DMAG-treated HT-29 cells and the control.
(A) and (B) represent G-250 stained 2-DE gels from the control group (A) and the treated group (B). Compared to the control, the up-regulated protein spots (spot 10 and 11) in the treated group were labeled in figure B, and down-regulated in figure A.

treated for 24 h or 48 h. So 1.0 μm was selected for proteomic study. Furthermore, according to Figure 2 C, a great change in cell apoptosis was detected after treated with 1.0aμm 17-DMAG for 24 h compared with that for 12 h and for 48ah. So 24 h is the best time. Then a proteomic study was carried out to find the proteins related to 17-DMAG stimulation at the concentration of 1.0 μm and treating time of 24 h. A total of 16 non-abundant proteins

Table 3. The differentially expressed proteins identified in this study

Spot number[a]	accession number[b]	Protein name	Regulation (Treated/control)[c]	Score[d]	Mass[e]	PI[f]	Location[g]	Function[h]
1	P19021	Peptidyl-glycine alpha-amidating monooxygenase	-∞	56	109119	5.98	Membrane; Secreted	Enzyme
2	P20700	Lamin-B1	-∞	65	66653	5.11	Nucleus	structural
3	Q8WYA6	Beta-catenin-like protein 1	-∞	55	65588	4.96	Nucleus	Activator
4	Q14247	Src substrate cortactin	-∞	98	61720	5.24	Cytoplasm	structural
5、16	P35908	Keratin, type II cytoskeletal 2 epidermal	-∞	59	65678	8.07	Cytoplasm	structural
6	Q5BKZ1	Zinc finger protein 326	-∞	67	65955	5.08	Nucleus matrix	Activator
7、8	P02545	Prelamin-A/C	-∞	82	74380	6.57	Nucleus.	structural
9	P11142	Heat shock cognate 71 kDa protein	-∞	91	71082	5.37	Cytoplasmnucleolus	Chaperone
10	P08107	Heat shock 70 kDa protein 1A/1B	3.80±0.80	215	70294	5.48	Cytoplasm	Chaperone; Receptor
11	P05787	Keratin, type II cytoskeletal 8	5.91±1.04	151	53671	5.52	Cytoplasm;Nucleus	structural
12、14	P13645	Keratin, type I cytoskeletal 10	0.15±0.05	73	59020	5.13	Cytoplasm	structural
13	P31943	Heterogeneous nuclear ribonucleoprotein H	0.14±0.06	76	49484	5.89	nucleoplasm	binding
15	P05783	Keratin, type I cytoskeletal 18	0.12±0.09	135	48029	5.34	Cytoplasm; nucleolus	structural
17	P31942	Heterogeneous nuclear ribonucleoprotein H3	0.18±0.17	115	36960	6.37	Nucleus	binding
18	P07195	L-lactate dehydrogenase B chain	0.13±0.09	108	36900	5.71	Cytoplasm	Binding enzyme
19	P13693	Translationally-controlled tumor protein	0.09±0.01	56	19697	4.84	Cytoplasm	calcium ion binding

a. spot number shown in figure 3;
b, the protein accession number from SWISS-PROT database;
c, -∞represent only in the control
d, the score from mascot searching;
e, and f shows the calculated mass and PI;
g and h from UniProtKB/Swiss-Prot annotation.

(14 decreased and 2 increased expressions) were regulated by 17-DMAG treatment in HT-29 cells compare to the control. Furthermore, the differentially expressed proteins found in this work were not completely covered by the previously reported study (Sharma et al., 2011), which includes 10 covered and 6 newly discovered differently expressed proteins such as peptidyl-glycine, alpha-amidating, monooxygenase or Prelamin-A/C. Therefore, our work complements the previous studies.

Furthermore, in order to verify our findings, two differentially expressed proteins (HSP70 was consistent but SRC8 was not consistent with findings in a previously published paper (Sharma et al., 2011)) were verified to have consistent changes with both 2DE and WB. For SRC8, consistent results were found in three methods of 2DE, WB and PCR. So the difference between our work and the previously reported study (Sharma et al., 2011) may be due to the different cell lines or different concentration of 17-DMAG (1.0 μM in our work and 50 μM in the reported study. (Sharma et al., 2011))

Heat shock protein 90 family participates in several signal pathways, 17-DMAG can inhibit HSP90 (Mellatyar et al., 2014) and its signal proteins such as HSP70, NF-kB and STAT (Ge et al., 2006; Lazaro et al., 2015) to affect cell functions. In this work, we found that 17-DMAG causes 14 proteins to be down-regulated, including peptidyl-glycine alpha-amidating monooxygenase, Lamin-B1 and Src substrate SRC8. Analyzing the physicochemical properties of the regulated proteins may provide new clues to understand the interactions of HSP90-client protein. So, in this work, various protein annotation terms from uniprotkb including subcellular location, molecular function and biological processes were investigated. We found that 8 proteins (50%) were located in the nucleus. Our results further ascertaine

the previous knowledge that HSP 90 inhibition is involved in DNA damage (Ayrault et al., 2009; Sharma et al., 2011). 6 of the 16 differential proteins were structural proteins, 4 had binding activities, and others were enzymes or activator. In these differentially expressed proteins, there are molecular chaperones such as heat shock cognate 71 kDa protein, which is a repressor of transcriptional activation, and inhibits the transcriptional co-activator activity of CITED1 on Smad-mediated transcription (Yahata et al., 2000). There are also structural proteins involved in actin and Smad signal pathway, for example, SRC8, which contributes to the organization of the actin cytoskeleton and cell structure.

Furthermore, the regulation by 17-DMAG is complex and is considered to form a negative feedback loop, in which newly translated HSP90 accumulates gradually, and then activate some client proteins of HSP 90 and inactivate negative feedback proteins such as heat-shock factor 1 (HSF1) (Santagata et al., 2011). Indeed, we found that after 17-DMAG treatment, the abundance of both HSP71 (Heat shock 70 kDa protein 1A/1B) and K2C8 (Keratin, type II cytoskeletal 8) were increased. Of which, HSP71 can interact with HSF1 and HSP90 (http://string-db.org/newstring_cgi). HSP 71 has important functions in stabilizing pre-existent proteins against aggregation and mediating the folding of newly translated polypeptides (Perez-Vargas et al., 2006).

In conclusion, we have identified 16 proteins related to 17-DAMG treatment and ascertained SRC8 and HSP71 to be potential client proteins of HSP90. SRC8 and HSP71 can be new targets for anti-cancer therapeutic intervention. However, it is still necessary to examine these differentially expressed proteins in clinical samples.

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