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Quantitative Proteomic Analysis of Bromotetrandrine and Tetrandrine in K562 Cell Line Using ^{18}O-labeling Method

TAN Ying[1], GE Zhi-qiang[1*], LIU Chang-xiao[2]

1. School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, China
2. Tianjin National Key Laboratory of Pharmacokinetics and Pharmacodynamics, Tianjin Institute of Pharmaceutical Research, Tianjin 300193, China

Abstract: **Objective** To compare quantitative proteomic analysis of bromotetrandrine (W198) which was a Class I new antitumor drug in China and tetrandrine (Tet) in K562 cell line using ^{18}O-labeling method. **Methods** To illustrate its mechanism, a shotgun quantitative proteomic strategy employing 2D LC-MS-MS and trypsin catalyzed ^{18}O-labeling quantification was carried out in this study. Compared to normal chronic leukemia cell line K562 and K562 induced by Tet, the proteomic changes of K562 induced by W198 were investigated. In order to validate the quantitation by the ^{18}O-labeling, the analysis was done on an equivalent sample composed of the same amount of labeled and unlabeled proteins from normally cultured cells to act as a reference to the comparative sample. **Results** A threshold of ± 2-fold change for deciding whether a protein concentration was changed was settled for the following experiments. Comparing the 105 identified soluble proteins' expression levels of the apoptosis starting up K562 cells after W198 induction with the normally cultured cells, 16 proteins were found with significantly altered expression levels after W198 treatment. Eight proteins were up-expressed including HMGB2, peroxiredoxin-2, and eIF4A-I, etc. Eight proteins were down-expressed including TCP-1, GRP94, GST-π, and SFGHs, etc. Compared to K562 induced by Tet, eight proteins of K562 were found with significantly altered expression levels after W198 treatment. Five proteins were up-expressed including HSP 90-β and 40S ribosomal protein S15a, etc. Three proteins were down-expressed including phosphoglycerate kinase 1, isoform 5 of interleukin enhancer-binding factor 3, etc. **Conclusion** The ^{18}O-labeling MS-MS-based method is ideal as a discovery tool, but it is not suitable for validation using a large number of samples. Other more effective methods, such as Western blotting should be used for further validation of candidate cancer proteins discovered from ^{18}O-labeling samples. In total, 105 soluble proteins were discovered, and 16 proteins were found with significantly altered expression levels after W198 treatment. These repressed or activated proteins are the potential drug targets of W198, which may provide novel targets for future development of biomarkers for cancer therapy.

Key words: bromotetrandrine; K562; ^{18}O-labeling; quantitative proteomics; tetrandrine
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Introduction

Bromotetrandrine, 5-bromotetrandrine (W198, Fig. 1), is a 5-bromo-compound of tetrandrine (Tet) which is a bibenzylisoquinoline alkaloid. Large preclinical studies of W198 were carried out in China (Jin, Wang, and Wei, 2005; Xiao *et al*, 2004a; 2004b; 2004c; 2005; Song *et al*, 2009). W198 is a Class I new drug for reverse multidrug resistance and the clinical trails are going on in China. But the mechanism of the action is not yet clear. Multidrug resistance (MDR) is a major factor in the failure of many forms of chemotherapy. Several cellular pathways are believed to have a role in

* Correspondence author: Ge ZQ Address: School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, China
E-mail: gezhiq@tju.edu.cn
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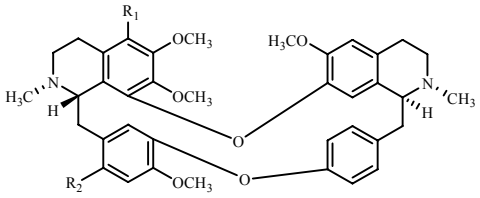
MDR. The cells could reduce the concentration of the drug by increasing efflux to eject the compound, or by decreasing influx. The cells might also activate the detoxifying systems and DNA repairing proteins. Finally the cells might alter proteins in the apoptosis pathways in order to avoid cell death (Opiteck *et al*, 1997).

Undoubtedly these pathways are associated with many proteins. In order to explain the mechanism of MDR and anticancer MDR, it is necessary to have a strategy for observation and quantitation of the changes of multiple proteins. Quantitative analysis of global protein levels, termed "quantitative proteomics", is important for the system-based understanding of the molecular function of each protein component and is expected to provide insights into molecular mechanisms of various biological processes and systems (Opiteck *et al*, 1997). Currently, several approaches have been developed for quantitative proteomics, including two-dimensional gel electrophoresis (2DGE) followed by MS analysis, stable isotope labelling-based quantitation, MS signal intensity-based quantitation, and protein array-based quantitation.



Tet R_1=H; R_2=H W198 R_1=Br; R_2=H

Fig. 1 Chemical structures of W198 and Tet

Although two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)-based method has been a historically important and currently widely-used approach for quantification of proteins, 2DGEs are cumbersome to run, have a poor dynamic range, and are biased toward abundant and soluble proteins (Opiteck *et al*, 1997). Label free strategy has shown a promising future for further development, but at present, it often includes experimental variation and signal noise which could affect the quantitative value and accuracy (Yan and Chen, 2005).

Current approaches of quantitative proteomics are mainly based on incorporation of stable isotope tags into proteins/peptides. Stable isotope labeling experiments require the incorporation of a stable isotope such as ^{2}H, ^{13}C, ^{15}N or ^{18}O. These stable isotope labeling techniques could be classified into two major classes: *in vivo* labeling and *in vitro* labeling. In general, stable isotope labeling *in vivo* has been proved to be an effective method of quantitative proteomic analysis. A disadvantage, however, is that this method is not practical for analyzing biological samples that cannot grow in culture, such as tissues or body fluids. In addition, it requires a relatively long labeling incubation time in cell culture to satisfactorily incorporate the isotopic labels. One of the stable isotope labelings *in vitro*, named isotope coded affinity tags (ICAT), occurs most frequently in literature (Opiteck *et al*, 1997). However, the sulfhydryl-group-directed ICAT approach is limited to cysteine- containing peptides and proteins. Alternative labeling methods which are independent of the amino acid composition are necessary.

Up to today, many methods of quantitative proteins were published (Ong, Foster, and Mann, 2003; Keller *et al*, 2003; Li *et al*, 2004; Oh *et al*, 2004; Wiener *et al*, 2004; Old *et al*, 2005; Chen *et al*, 2006). Among the MS-based *in vitro* stable isotope-labeling methods, the proteolytic ^{18}O-labeling method is particularly attractive. ^{18}O-labeling quantitative method is one of the relatively practical and feasible methods, which has been widely used in recent years (Yao *et al*, 2001; Yan and Chen, 2005). During trypsin proteolysis, ^{16}O or ^{18}O isotopes could be incorporated into the C-termini of peptides in the presence of ^{16}O or ^{18}O water. The relative quantity of proteins is determined by the ratio of peak intensities or areas of ^{16}O- to ^{18}O-labelled peptides measured by MS.

For establishment of quantitative proteome, we carried out a study on ^{18}O-labeling quantitative method (Tan, Ge, and Liu, 2007). In this study we analyzed the whole cell protein extraction of the K562 cell using MudPIT, and identified the 155 soluble proteins. For the first time, the expression levels of proteins associated with W198 and Tet were discussed. These pathways make the mechanisms of MDR as a complex network. Therefore, it is important in the need for a more effective approach to study the mechanism of its antitumor activity.

Materials and methods

Chemicals and reagents

W198 and Tet were synthesized by Prof. WANG

Feng-peng in Pharmaceutical College of Sichuan University, stored as a 100 mmol/L solution in absolute ethanol at −20 ℃, and diluted with the medium prior to use.

Ammonium bicarbonate, sequence grade modification trypsin, iodacetyl amine, urea, and ammonium chloride were from Sigma (St. Louis, MO). HPLC grade formic acid was from Acros (Loughborough, UK). HPLC grade acetonitrile (ACN) was from Merck (Darmstadt, Germany). 1,4-Dithiothreitol (DTT) was from Roche (Mannheim, Germany). PMSF were from Amresco (Solon, OH). Centricon filters (1×10^4) were from Millipore (Bedford, MA). $H_2{}^{18}O$ (95%) was from ISOTEC^{TM}. Water was purified using a Milli-Q system (Millipore, Bedford, MA). Bio-basic strong cation exchange HPLC column (100 mm × 0.32 mm, 5 μm) and the reverse phase HPLC column (100 mm × 0.18 mm, 5 μm) were from ThermoHypersil (Hemel Hempstead, UK). All other chemicals used were of analytical grade and obtained commercially.

Cell line, cell culture, and drug treatment

K562 cell line was obtained from the Chinese Academy of Medical Sciences. The cells were grown in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin. Cultures were maintained in a humidified atmosphere of 5% CO_2 at 37 ℃. Exponentially growing cells were used for all experiments. Control and test cell (10 mL) suspensions were separately seeded at a density of 5×10^5/mL to 9 cm Petri dishes, after which the culture medium of the test cells was added with W198 to a final concentration of 10^{-8} mol/L. After 18 h which is a typical time point of the apoptosis starting up process, the proteins in cells were respectively extracted and used as control and test samples.

Preparation of cell extracts

Proteins from control and test cells were respectively extracted as follows: cells were washed with ice cold PBS for three times, then 100 μL lysis buffer (6 mol/L urea, 100 mmol/L NH_4HCO_3, 1% DTT, and 1 mmol/L PMSF) was added to per 10^6 cells to dissolve the proteins. The lysis lasted for 30 min at 4 ℃. After that the extracts were centrifuged at 18 000 × *g* for 30 min at 4 ℃ using the Sigma laboratory centrifuge to remove the debris and the supernatants were collected. Protein concentration was determined by the Bradford assay and adjusted to 1 mg/mL with the lysis buffer. The proteins with concentration of 1 mg/mL were reduced with 5 mmol/L DTT at 37 ℃ for 1 h to disconnect the disulfide bonds, followed by carboxyamido-methylated with 10 mmol/L idoacetamide in the dark at room temperature for 30 min to avoid the robonding of the disulfide bonds. Then the proteins were precipitated overnight at −25 ℃ by adding 14-fold cold organic solvent (ethanol-acetone-acetic acid is 50:50:0.1). After being centrifuged and lyophilized to dryness, the protein pellets were stored at −25 ℃ until use.

Trypsin catalyzed digestion and co-digestion ^{16}O-/^{18}O- labeling of proteins

Control and test protein samples were respectively dissolved in digestion buffer (1 mol/L urea and 100 mmol/L NH_4HCO_3), made in ^{16}O or ^{18}O water at the concentration of 1 μg/μL (protein/digestion buffer), and digested with sequencing grade trypsin at protein mass-trypsin mass (50:1) at 37 ℃ for 24 h. After that, additional trypsin was added to the final ratio of 20:1 and the incubation was at 37 ℃ for another 18 h to improve the labeling efficiency. Digestions were terminated by adding formic acid to the final volume concentration of 5%.

Two same control protein samples were also digested respectively in the presence of ^{16}O or ^{18}O water using the above method.

The equivalent sample was made by mixing same amount of ^{16}O- and ^{18}O-labeled control peptide samples just before loading onto the column to avoid back exchange.

Two replicate comparative samples were both made by mixing the same amount of ^{16}O-labeled control peptide sample and ^{18}O-labeled test peptide sample just before loading onto the column. All the samples were centrifuged using the 1×10^4 filter membrane before injected into the LC column.

2D LC-MS/MS analysis of peptides

The analysis was performed on 2D LC-ESI-MS-MS (LCQ DecaXP MAX, Thermo Finnigan, Palo Alto, CA) and the whole process was controlled by the Xcalibur Data System (Thermo Finnigan, Palo Alto, CA). Sample (100 μg) was injected into the strong cation exchanger column followed by 20 min-washing

without salts. Then the peptides were separated by 11-step-elution from the strong cation exchanger column followed by a gradient elution from the reversed-phase chromatography. The salt steps used were 10, 25, 40, 50, 75, 100, 150, 200, 300, 500, and 1000 mmol/L ammonium chloride, respectively. The reverse phase elution gradient procedure was 1-min 100% buffer A (5% acetonitrile and 0.1% formic acid in water), 70-min gradient to 65% buffer B (0.1% formic acid in acetonitrile), 20-min gradient to 80% buffer B, 5-min 80% buffer B, 1-min gradient to 100% buffer A, and 12-min re-equilibration with 100% buffer A.

The Ion Trap Mass Spectrometer was operated in the automatic gain control mode with three data-dependent MS-MS scans after each full MS scan (*m/z* 300－1500). A precursor ion was excluded from further MS-MS analysis for 3 min if it was analyzed once in the previous 0.5 min. Exclusion mass width was set as 3.0, and reject mass width was set as 1.0. The normalized collision energy was set at 35%, and the precursor ion mass range was set at 2. The temperature and voltage for the capillary and the ion source were maintained at 160 ℃ and 3.2 kV, respectively.

Protein identification

The raw MS-MS data were searched against IPI human protein database (version 3.18, released on June 13, 2006 by EBI, number of entries is 60090) using the SEQUEST algorithm in Bioworks version 3.1 software (Thermo Finnigan). DTA files were generated with the parameters of MS-MS threshold 10^5, peptide mass tolerance 1.5, fragment mass tolerance 0.8 and minimum ion count of 35%. Search parameters were as follows: The tryptic enzyme was used restricted with the maximum of two internal missed cleavages sites; Differential modification was set as 4 at C terminus to include both the unlabeled and labeled peptides incorporated with two ^{18}O; Static modification was set as 57 for cysteine to account for its carboxamido-methylation. The matched peptides were filtered based on the acceptable sensitivity and error rates. Statistical assessment of sensitivity and error rates for the identified proteins was performed using INTERACT™, PeptideProphet™, and ProteinProphet™. These software tools utilize expectation-maximization rules to model predictable sensitivity and false positive identification rates. The algorithm penalizes identifications based on single hits and weighs additional factors such as the number of unique peptides per protein and the presence of tryptic ends. The accepted error rate was controlled below 10% in peptide and protein filter in this study.

Quantification of labeled and unlabeled peptide ratios

The labeled and unlabeled ratios were quantified by calculating the relative abundances ($^{18}O/^{16}O$) of peptide pairs based on the area of their extracted ion chromatograms and reconstructed manually from the full MS scans. The extracted ion chromatogram XIC for a peptide was generated by summing the intensities within a narrow *m/z* range from the full scan mass spectra for each scan cycle, using a lower limit of [(average mass of the first two isotopic variants of peptides – 1) + charge]/charge and an upper limit of [(average mass of the first two isotopic variants of peptides + 1) + charge]/charge. Information sciences and interaction sciences (ICIS) algorithm was selected to generate peaks and the 15 smoothing points' Gaussian method was used to smooth the peaks. Peak areas were measured and selected only when the MS-MS scans of the identified ion were contained within the peak boundaries, in order to eliminate ions within the corresponding *m/z* range but eluting in other regions of the HPLC run.

The following equation, slightly modified by Yao *et al* (2001), was used to calculate the labeled and unlabeled peptide pair ratios.

$$\text{Ratio}\left(\frac{^{18}O}{^{16}O}\right) = \frac{I_4 - \frac{M_4}{M_0}I_0 + I_2\left(1-\frac{M_2}{M_0}\right) - \left(1-\frac{M_2}{M_0}\right)\frac{M_2}{M_0}I_0}{I_0}$$

where I_0 = measured relative intensities for the first two isotopic variants of unlabeled peptides; I_2 = measured relative intensities for the two peaks with masses 2 higher; I_4 = measured relative intensities for the two peaks with masses 4 higher; M_0 = sum of theoretical relative intensities for the first two isotopic variants of unlabeled peptides; M_2 = sum of theoretical relative intensities for the two monoisotopic peaks with masses 2 higher; and M_4 = sum of theoretical relative intensities for the two monoisotopic peaks with masses 4 higher. The theoretical natural isotopic distribution was calculated based on the peptide sequence using the MS-isotope program available on Protein Prospector Website (http://prospector.ucsf.edu/ucsfhtml4.0/msiso.htm).

Differential expressions and modifications of protein

To cover more proteins from the cells, peptides identified in the equivalent sample or either one of the comparative samples were all quantified in the three samples in the respective salt concentration file and the adjacent retention time (t_R), based on the theory that the same peptides would elute in the same salt concentration step in the strong cation exchange column and have similar t_R in the reverse phase column under identical LC-LC-MS-MS conditions.

Proteins' expression levels were considered to be changed when the calculating ratios in the two replicate comparative samples were altered in the same direction compared to the ratio calculated in the equivalent sample. And the ratios of peptides in the comparative samples were normalized to the corresponding peptide ratios in the equivalent sample. If several identified peptides belonging to the same protein have different change directions, these peptides would be carefully examined to find whether different modifications of these peptides existed under different conditions.

Bioinformatics analysis of the differentially expressed proteins

Proteins with expression levels determined to be up-regulated or down-regulated after docetaxel administration was searched against protein database (Swissprot and EBI) and PubMed (NCBI) to obtain their functions and their relationships with apoptosis.

Validation

Validation was assessed either through external analysis of gene products previously reported in the cancer literature, or through Western blotting. We developed a comprehensive pancreatic cancer database using an electronic review of the known pancreatic cancer database, including DNA and RNA arrays, SAGE analysis, and differential display (Chevallet *et al*, 2003; Rabilloud *et al*, 2002). Hundreds of genes were organized into a compendium, which is electronically searchable, to compare the cancer proteins that were isolated from our experiments to externally validate our proteins and to determine their potential use as biomarkers. In addition, we searched PubMed for any non-pancreatic cancers that might be associated with the proteins discovered.

Data analysis

MS-MS spectra were searched against the human sequence database in National Cancer Institute or International Protein Index using SEQUEST (Chevallet *et al*, 2003). The database search results were validated using the PeptideProphet program (Chevallet *et al*, 2003). PeptideProphet uses various SEQUEST scores and a number of other parameters to calculate a probability score for each identified peptide. The identified peptides were then assigned to a protein identification using the ProteinProphet software (Rabilloud *et al*, 2002). ProteinProphet allows filtering of large-scale datasets with predictable sensitivity and false-positive identification error rates, and then generates statistically validated protein identification from the identified peptides. In this study, we used a ProteinProphet probability score 0.5 as a cut-off value for protein identification.

This would ensure that the false-positive rate (error rate) for protein identification was controlled below 10%. Quantification of the ratio of each protein (isotopically heavy versus light) was calculated using the ASAP Ratio program (Chevallet *et al*, 2003). Information on the software could be found on line at http://www.systemsbiology.org/Default.aspx?pagename=FullList. The identified proteins were classified based on GeneOntology (GO) consortium.

Results

Identification of proteins

Quantitative proteomic analysis of K562

We used ^{18}O-labeling, chromatographic fractionation and purification, and MS-MS to identify and quantify soluble proteins in K562. In this study, two separate comparisons were performed: (1) normal K562 *vs* normal K562 (equivalent sample), and (2) normal K562 *vs* K562 induced by W198 (control/W198 sample). The first experiment was performed to validate the quantitation by the ^{18}O-labeling. The analysis was done on an equivalent sample composed of the same amount of labeled and unlabeled proteins from normally cultured cells to act as a reference to the comparative sample. Once the protein variability had been established in equivalent sample, we sought to compare the findings from the first experiment with the second experiment (control/W198 quantification).

For each of the two separate ^{18}O-labeling experiments, we labeled the comparator and target samples protein with the isotopically heavy or light ICAT reagents, respectively. The labeled proteins from the comparator and target proteomes were then combined, digested, fractionated, and purified by multi-dimensional chromatography, and analyzed by MS-MS. The protein identification and quantification were accomplished using a suite of software tools (Chevallet *et al*, 2003; Rabilloud *et al*, 2002).

In the equivalent experiment, we identified and quantified 61 proteins, with a false-positive rate of less than 0.9%. In the control/W198 experiment, we identified and quantified 78 proteins, also with a false-positive rate of less than 0.9%. These two experiments identified a total of 105 proteins in K562, of which 34 proteins were identified in both experiments, 27 proteins were identified only in the equivalent experiment, and 44 proteins were identified only in control/W198 experiment. For the 105 unique proteins identified in this study, 45 proteins were previously reported to be pancreatic juice constituents, while the remaining 60 proteins were not reported in previous studies (Zhu, Bilgin, and Snyder, 2003). In the previous study (Rao *et al*, 2005), pancreatic juice obtained from pancreatic cancer patients was first fractionated by 1-DE and subsequently analyzed by LC-MS-MS for protein identification. In this study, we used cation exchange chromatography for sample fractionation which increased the total number of proteins identified. Moreover, we employed ICAT labeling, which enabled us to do quantitative proteomic analysis of the proteins in pancreatic juice. Thus, the study presented here identified additional pancreatic juice proteins that have not been reported before, and employed a quantitative proteomic method to quantify the protein ratios in cancer sample *vs* normal.

Proteins identified in K562

The 105 proteins identified in K562 were examined by molecular function and cellular component, and classified to GO nomenclature. Proteins in the cells were divided in accordance with the positioning of the protein into cytoplasmic protein, nuclear protein, and membrane protein. The proteins and other cell types were specifically shown in Fig. 2. The majority of the identified proteins was in the extracellular region (62%) or bound to the plasma membrane (14%). This is consistent with the fact that proteins from pancreatic juice are primarily secreted. In addition, 12% of the proteins were from the intracellular space: cytoplasm 5%, endoplasmic reticulum 3%, mitochondrion 1%, and nucleus 3%. Since pancreatic juice is mostly cell free, the presence of these cellular proteins supports the assumption that cellular proteins might be shed into pancreatic juice by cell turnover or secretion.

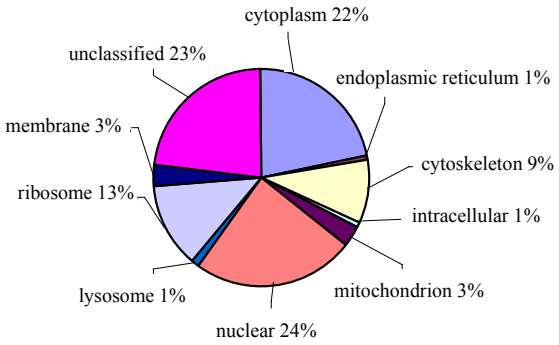


Fig. 2 Cellular component distribution based upon their cellular location of the identified proteins annotated by GO convention

As expected, many of the proteins identified in pancreatic juice were enzymes (catalytic activity, 38%). Other GO molecular function categories identified included binding function 19%, enzyme regulation 5%, obsolete molecular function 7%, signal transduction 2%, structural molecules 2%, transcription regulation 1%, and transporter activity 8%. Molecular functions for 18% of the proteins are still unknown at present.

Identification of proteins involved in accordance with the classification of biological processes was shown in Fig. 3. Proteins were involved in the biological processes including cell communication of cell-to-cell, cell cycle, cell motility, cell growth or maintenance, apoptosis, physiological process, stress response, transportation, protein metabolism, nucleic acid metabolism nucleotide nucleotide (DNA/RNA processing), and so on. One protein involved in protein metabolism has the largest proportion (29%), followed by nucleic acid metabolism nucleotide (17%). Protein metabolism included protein biosynthesis, alienation, folding, and complex assembly, such as aggregation and modification. “Other metabolism” is mainly

involved in glycolysis, TCA cycle, gluconeogenesis, and the metabolism of other small molecules which are involved in apoptosis (Oh *et al*, 2004).

In accordance with the protein molecules, the protein annotation is divided into the following categories (Fig. 4): enzyme, enzyme-regulater protein, transporter, vector or ligand binding protein (ligand binding or carrier), the signal transduction protein (signal transducer), nucleic acid binding protein, transcription regulatory proteins (transcription regulater), and structural protein. Enzymes account for the largest proportion of protein identified in 21.0%, mainly including synthetase, reductase, transferase, esterase, dehydrogenase, connect enzyme (ligase), oxidoreductase, superoxide dismutase (SOD), lyase, and so on. Ligand binding proteins, and the proportion of which is 13%, include protein binding, macro-molecular binding, GTP binding, metal ion binding, nucleic acid binding proteins, etc. In addition to the binding proteins outside, transcription regulatory proteins have the smallest percentage of about 3%.

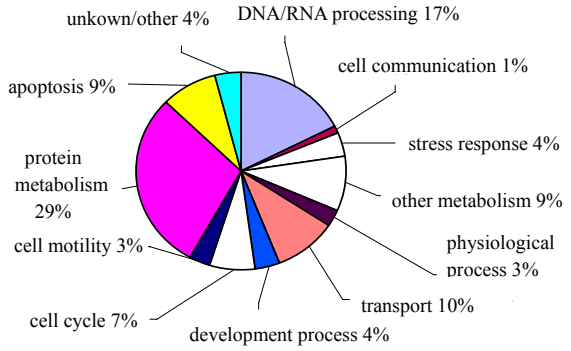


Fig. 3 Biological process distribution based upon the processes they participated in of the identified proteins annotated by GO convention

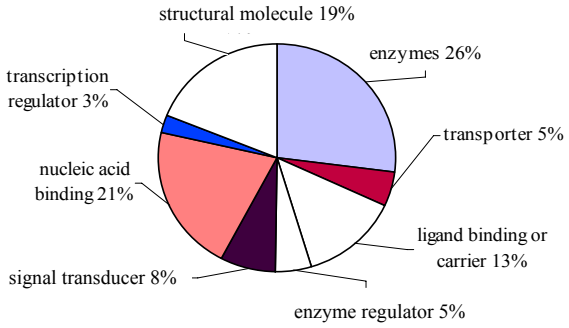


Fig. 4 Molecular function distribution based upon their biological roles of the identified proteins annotated by GO convention

Analysis of differential proteins

The quantification of each protein is presented as a protein ratio between two samples at two tests of Control/W198 and Tet/W198 using ^{18}O-labeling technology. Comparing the 105 identified soluble proteins' expression levels of the apoptosis starting up K562 cells after W198 induction with the normally cultured cells, 16 proteins were found with significantly altered expression levels after W198 treatment. Eight up-expressed proteins and eight down-expressed proteins were found. Compared to K562 induced by Tet, eight proteins of K562 were found with significantly altered expression levels, five up-expressed proteins and three down-expressed proteins of the proteins, after W198 treatment.

With the normal K562 cells as reference, the expression of 16 soluble proteins changed, with up-regulation of expression in eight proteins and down-regulation of expression in eight proteins after 18 h of processing with W198 concentration of 1.5 mg/L K562 cells in Table 1. Comparing the 105 identified soluble proteins' expression levels of the apoptosis starting up K562 cells after W198 induction and the normally cultured cells, it was found that 16 proteins significantly altered expression levels after W198 treatment. Eight proteins were up-expressed including high mobility group protein B2 (HMG2), heat shock protein 90Bd, hypothetical protein LOC134147 peroxiredoxin (Prx)-2, eukaryotic initiation factor 4A-I, *L*-lactate dehydrogenase B chain, 60S ribosomal protein L30, and SLC9A1 protein. Eight proteins were down-expressed including T-complex protein 1 subunit α, endoplasmin precursor, glutathione S-transferase P, IF3I-HUMAN, isoform short of RNA-binding protein FUS, and ubiquitin-conjugating enzyme E2L3, esterase D and unknown protein similar to 60S ribosomal protein L32.

Compared to K562 induced by Tet (2 mg/L), eight proteins of K562 were found with significantly altered expression levels after W198 (1.5 mg/L) treatment (Table 2). Five proteins were up-expressed including Hsc70-interacting protein, HSP 90-β, 40S ribosomal protein S15a, eukaryotic translation initiation factor 3 subunit 6, and Prx-4. Three proteins were down-expressed including phosphoglycerate kinase 1, isoform 5 of interleukin enhancer-binding factor 3, and

Table 1 Differential proteins of control/W198 sample

Order	Swiss-Prot	Proteins	Protein ratio
1	P17987	T-complex protein 1 subunit α	0.274 267
2	P26583	high mobility group protein B2	2.802 49
3	(NULL)	heat shock protein 90Bd	4.153 888
4	P14625	endoplasmin precursor	0.386 305
5	P32119	Prx-2	3.589 058
6	P09211	glutathione S-transferase P	0.448 376
7	(NULL)	hypothetical protein LOC134147	2.734 037
8	P60842	eukaryotic initiation factor 4A-I	2.720 883
9	Q9Y262	IF3I_HUMAN	0.288 281
10	P07195	*L*-lactate dehydrogenase B chain	2.733 547
11	P35637-2	isoform Short of RNA-binding protein FUS	0.270 636
12	P68036	ubiquitin-conjugating enzyme E2 L3	0.422 68
13	P62888	60S ribosomal protein L30	2.901 986
14	(NULL)	predicted: similar to 60S ribosomal protein L32	0.316 369
15	P10768	esterase D	0.198 92
16	(NULL)	SLC9A1 protein	2.277 073

Table 2 Differential proteins of Tet/W198 sample

Order	Swiss-Prot	Proteins	Protein ratio
1	P50502	Hsc70-interacting protein	3.577 98
2	(NULL)	predicted: similar to Heat shock protein HSP 90-β	4.804 035
3	P60228	eukaryotic translation initiation factor 3 subunit 6	5.972 418
4	P00558	phosphoglycerate kinase 1	0.318 508
5	P62244	40S ribosomal protein S15a	3.356 531
6	Q13162	Prx-4	2.374 035
7	Q12906-5	isoform 5 of Interleukin enhancer-binding factor 3	0.466 722
8	Q9UBT2	phosphoglycerate kinase 1	0.425 31

phosphoglycerate kinase 1. These repressed or activated proteins were the potential drug targets of W198, which would offer the candidate proteins for tumor diagnosis and treatments.

Discussion

HMG2

HMG2 was found to be 2.8-fold more abundant in K562 cells induced by W198 compared with normal K562 cells in this study. Two other peptides, vlcfenr and cynelngctk, from this protein were also identified in the experiment (MS-MS spectra not shown). The identification of these three peptides gave an explicit identification of HMG2 in the samples. The quantification using Xpress (Nesvizhskii *et al*, 2003) revealed that this protein was nearly three times more abundant in the K562 induced by W198 (W198 to normal ratio = 2.802 49).

HMG2 is a component of the SET complex. SET complex containing the nucleosome assembly protein SET, the tumor suppressor pp32, and the base excision repair enzyme APE could induce single-stranded DNA damage in isolated nuclei in a granzyme A-dependent manner. The normal functions of the SET complex are unknown, but the functions of its components suggest that it is involved in activating transcription and DNA repair. We now find that the SET complex contains DNA binding and bending activities mediated by the chromatin-associated protein HMG2. HMG2 facilitates assembly of nucleoprotein higher-order structures by bending and looping DNA or by stabilizing under-wound DNA. HMG2 is in the SET complex and coprecipitates with SET. By confocal microscopy, it is observed that cytoplasmic HMG2 colocalizes with SET in association with the endoplasmic reticulum, but most nuclear HMG2 is unassociated with SET. This physical association suggests that HMG2 may facilitate the nucleosome assembly, transcriptional activation, and DNA repair functions of SET and/or APE. HMG2, like SET and APE, is a physiologically relevant granzyme A substrate in targeted cells.

Granzyme A cleavage after Lys65 in the midst of

HMG box A destroys HMG2-mediated DNA binding and bending functions. Granzyme A cleavage and functional disruption of key nuclear substrates, including HMG2, SET, APE, lamins, and histones, are likely to cripple the cellular repair response to promote cell death in this novel caspase-independent death pathway (Opiteck *et al*, 1997; Fan *et al*, 2002).

Prx-2

In this study, we found that Prx-2 level was increased by 3.6-fold in K562 cells induced by W198. Prxs are redox enzymes using an activated cysteine as their active sites. This activated cysteine could be easily overoxidized to cysteine sulfinic acid or cysteine sulfonic acid, especially under oxidative stress conditions (Opitedk *et al*, 1997). Results obtained in a primary culture of Leydig cells challenged with tumor necrosis factor α suggested that this oxidized/native balance of Prx-2 might play an active role in resistance or susceptibility to tumor necrosis factor α-induced apoptosis (Yan and Chen, 2005).

Prxs are redox enzymes using an activated cysteine as their active site. The regeneration of Prxs after a short and intense oxidative stress was studied, using a proteomics approach. Important differences in regeneration speed were found, and Prx-2 was the fastest regenerated protein, followed by Prx-1, whereas Prx-3 and Prx-6 were regenerated very slowly. Further study of the mechanism of this regeneration by pulse-chase experiments using stable isotope labeling and cycloheximide demonstrated that the fast-regenerating Prxs are regenerated at least in part by a retroreduction mechanism. This demonstrates that the overoxidation could be reversible under certain conditions. The pathway of this retroreduction and the reasons explaining the various regeneration speeds of the Prxs remain to be elucidated.

Prxs are enzymes catalyzing the destruction of peroxides. In doing so, a reactive cysteine in the prx active site is weakly oxidized (disulfide or sulfenic acid) by the destroyed peroxides. Cellular thiols (e.g. thioredoxin) are used to regenerate the Prxs to their active state.

Conclusion

The aim of this study was to perform extensive proteomics analysis to discover any possible protein with differential expression in K562 cells induced by W198, using a limited number of samples. These potential biomarkers could then serve as a candidate pool for future validation in larger sample sets using other methods, such as label-free method for protein quantification, with the improvement of the accuracy. To the best of our knowledge, this is the first comprehensive proteome of K562 cells by quantitative global protein profiling using ^{18}O-labeling technology. The ^{18}O-labeling MS-MS based method is ideal as a discovered tool, but it is not suitable for validation using large numbers of samples. Other more effective methods, such as Western blotting should be used for further validation of candidate cancer proteins discovered from ^{18}O-labeling, using larger sample sizes.

Firstly, in order to validate the quantitation by ^{18}O-labeling, the analysis was done on an equivalent sample composed of the same amount of labeled and unlabeled proteins from normally cultured cells to act as a reference to the comparative sample. A threshold of ± 2-fold change for deciding whether a protein concentration was changed was settled for the following experiments.

In total, 105 soluble proteins were discovered, and 16 proteins were found with significantly altered expression levels after W198 treatment. Eight proteins were up-expressed including HMGB2, Prx-2, eIF4A-I, etc. Eight proteins were down-expressed including TCP-1, GRP94, GST-π, SFGHs, etc. These repressed or activated proteins are the potential drug targets of W198, which might provide novel targets for future development of biomarkers for early therapy.

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