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Application of Proteomic Profiling Based on 2D-DIGE for Classification of Compounds According to the Mechanism of Action

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SUMMARY

The development of new anticancer agents derived from natural resources requires a rapid identification of their molecular mechanism of action. To make this step short, we have initiated the proteomic profiling of HeLa cells treated with anticancer drugs representing a wide spectrum of mechanisms of action using two-dimensional difference gel electrophoresis (2D-DIGE). Unique proteome patterns were observed in HeLa cells treated with the HSP90 inhibitor geldanamycin, and were similar to the patterns induced by radicicol, a structurally different HSP90 inhibitor. On the other hand, etoposide and ICRF-193, compounds claimed to be topoisomerase II inhibitors, showed different proteomic profiles, which reflect their different biological activities as revealed by cell-cycle analysis. Thus far, combined data from 19 compounds have allowed their successful classification by cluster analysis according to the mechanism of action.

INTRODUCTION

Cell-based assays are widely used in drug discovery because the assessment of molecular interaction occurs within the context of a living cellular environment (Baker et al., 2007). Many bioactive compounds inhibiting the growth of cancer cells have been isolated using a cell-based screen (Kakeya et al., 2002; Kawada et al., 2009). In most instances, the molecular target for newly isolated compounds remains unknown. The identification of a plausible target is sometimes possible based on the results of cell-based assays; however, the exact target must be proven by enzymatic assays, analyses of binding proteins, or genetic methods employing an siRNA (Kazami et al., 2006; Sato et al., 2007; Teruya et al., 2005). The confirmation of molecular targets, however, is usually a difficult and time-consuming process.

Multidimensional phenotype profiling approaches have a capacity to generate a testable hypothesis related to the mechanism of action and eventual off-target effects of new compounds. The differential sensitivity of the panel of cancer cell lines to the compounds has been used to identify their molecular target(s). The most commonly used assay, the NCI 60 antitumor screen, allowed the identification of benzolactone enamide as an inhibitor of V-ATPase (Boyd et al., 2001). Another panel consisting of 39 different cancer cell lines identified the compound encoded as ZSTK474 to be an inhibitor of phosphatidylinositol 3-kinase (Yaguchi et al., 2006). Recent advances in the field of molecular biology have provided a wide spectrum of methods suitable for target identification. The application of the Connectivity Map, developed by Golub and coworkers, which uses gene expression signature for profiling (Lamb et al., 2006), led to the identification of a class of HSP90 pathway modulators (gedunin and celastrol) (Hieronymus et al., 2006). Cell morphology-based profiling (Abassi et al., 2009; MacDonald et al., 2006) and activity-based proteomic profiling (Leung et al., 2003) are also used for molecular target identification.

Compared with gene expression profiling, which can simultaneously measure the expression of more than 20,000 genes, proteome analysis provides us only with the opportunity to trace at most 1,000 protein spots. However, any change of molecular weight and isoelectric point of proteins after posttranslational modification is often detectable as a mobility shift of protein spots in two-dimensional gel electrophoresis (2DE) analyses. Because biologically active compounds affect cellular processes and induce changes in both expression level and modification of proteins, proteome profiling is an informative approach for investigating the effects of a compound. Indeed, several research groups have shown that a biologically active compound alters the proteome (Cecconi et al., 2007; Towbin et al., 2003). Recent advances in two-dimensional difference gel electrophoresis (2D-DIGE) have allowed the measurement of the abundance of each protein spot between different gels with high accuracy due to introduction of an internal standard (Van den Bergh and Arckens, 2004). With 2D-DIGE, the abundant proteomic expression data obtained from different treatments can be collected and the expression patterns can be compared. In this study, we have used 2D-DIGE to perform a comprehensive proteome analysis of protein expression changes caused by the treatment of cancer cells with anticancer drugs claimed to possess the exact mechanisms of action.

It is well known that the anticancer drugs of known and similar mechanisms of action such as doxorubicin and daunorubicin, both classified as anthracyclines, are clinically active against different types of cancers. Doxorubicin is mainly used in the treatment of solid tumors, whereas daunorubicin shows activity in hematologic malignancies. Another case is cisplatin and oxaliplatin, the former active against lung and ovarian cancers and the latter active against colon cancer. Keeping in mind a subtle difference in clinical activity of the compounds of similar exact mechanism of action, we have made an attempt to establish differential protein profiles in cancer cells treated with anticancer agents representing several main mechanisms of action, including also several compounds possessing the same well-established mechanism of action. The proteomic profiling of mechanism of action may play an essential role in the planning of individualized chemotherapy of cancer patients once the correlation between drug sensitivity and the drug-induced proteomic profile is found.

Here we report the procedure and results of the proteome analysis using 2D-DIGE that revealed significant similarities in protein expression changes induced by the compounds belonging to the same class. Furthermore, we were also able to distinguish subtle differences among compounds attacking the same molecular target, though in a different way.

RESULTS

Proteomic Patterns of Geldanamycin- and Radicicol-Treated HeLa Cells Are Similar

Geldanamycin (**1**) and radicicol (**2**) are well-known HSP90 inhibitors (Schulte et al., 1998; Whitesell et al., 1994). HSP90 is a target for cancer therapeutics, and 17-AAG is a derivative of geldanamycin undergoing clinical trials (Nowakowski et al., 2006). First, we determined the cell growth inhibitory effect of HSP90 inhibitors against HeLa cells using a WST-8 assay (Figure 1A). The 50% growth inhibitory concentration (IC_{50}) of geldanamycin against HeLa cells was approximately 0.05 μM in a 48 hr treatment. HeLa cell growth was not affected at concentrations lower than 0.01 μM, whereas complete growth inhibition was observed at concentrations greater than 0.1 μM. The IC_{50} of radicicol against HeLa cell growth was approximately 1 μM.

Next, we investigated the relationship between proteomic changes after exposing HeLa cells to effective concentrations of the compounds, 0.005, 0.05, 0.5, 5, and 10 μM for geldanamycin and 10 μM for radicicol; the results are shown in Figure 1B. In this analysis, 775 spots in 2DE gels were matched on all gel images and quantified by 2D-DIGE system software, resulting in 282 spots that were selected by ANOVA ($p < 0.01$) and Dunnett's test ($p < 0.01$) (see Table S1 available online). Then, hierarchical cluster analysis was performed. The results are displayed in the form of a heat map and a tree diagram (Figure 1B). In the heat map, the spots with increased expression are indicated in red, and the spots with decreased expression are indicated in green. As indicated in the tree diagram and the heat map, the patterns of protein expression were similar at geldanamycin concentrations greater than 0.5 μM.

To simplify the statistical evaluation of the 2D-DIGE experiments, the spots that were modified significantly between groups were typically selected using the ANOVA test and a volume ratio filter of no less than 2-fold for three biological replicates per group (Karp and Lilley, 2005). Using these parameters, 17 spots were selected and a similar result for the cluster analysis was obtained (data not shown).

HSP70 and HSP27 have been reported to be upregulated in HSP90 inhibitor-treated cells (Maloney et al., 2007; McCollum et al., 2006). To classify test compounds using proteomic profiling, the identity of each protein spot is not necessary; however, it is important to confirm whether a proteomic change of geldanamycin-treated cells matches that in previously reported results. Peptide mass fingerprinting identified 20 spots out of the total number of spots that had been significantly affected by the treatment with geldanamycin and other compounds (Tables S2 and S3). The application of the ANOVA test selected 15 out of 20 spots and the mean ratios between control and inhibitor-treated cells were tabulated (Table 1).

Spots 1114 and 1127, which were identified as heat shock 70 kDa protein 1 (HSP70, *HSPA1B*), were upregulated more than 7-fold when compared with control. At 0.05 μM geldanamycin, HSP70 upregulation was also detected, but the magnitude of increase was lower compared with higher concentrations. The expression level of spot 2382, identified as heat shock protein β-1 (HSP27, *HSPB1*), also reached a plateau, as did HSP70. By western blot, both HSP70 and HSP27 were upregulated to similar extents at concentrations greater than 0.05 μM (Figure 1C).

The upregulation of mitochondrial heat shock proteins (*HSP9B*, *HSPD1*) and protein disulfide isomerase and downregulation of eukaryotic elongation factor 2 (*EEF2*), fascin (*FSCN1*), adenylyl cyclase-associated protein 1 (*CAP1*), and aldo-keto reductase family 1 member C2 (*AKR1C2*) were observed in geldanamycin-treated HeLa cells. It is very important to note that the concentration corresponding to the IC_{50} in the WST-8 assay is insufficient to induce any obvious changes in the proteomic analysis. It is more reasonable to use concentrations at which cell growth is nearly completely inhibited.

When the cells were exposed to 0.5 μM geldanamycin, the amount of HSP70 increased in a time-dependent manner and reached a plateau after 18 hr (Figures 1D and 1E). Because long incubation with test compounds may be associated with secondary effects such as apoptosis, we purposely avoided long exposures. Instead, we performed a subsequent proteomic analysis of HeLa cells after 18 hr exposure to a test compound.

Radicicol (**2**) is another HSP90 inhibitor that structurally differs from geldanamycin (**1**). The expression patterns between radicicol- and geldanamycin-treated cells were compared by 2D-DIGE. Similar responses were observed between geldanamycin- and radicicol-treated cells (Figure 1B; Table 2). HSP70 (1114 and 1127), HSP27 (2372 and 2382), and 78 kDa glucose-regulated protein (GRP78, *HSPA5*; 972 and 983) increased during both treatments (Table 2). The spots representing eukaryotic elongation factor 2, fascin, and adenylyl cyclase-associated protein 1 were downregulated during both treatments. These results strongly suggest that compounds inhibiting the same molecular target generate similar proteomic profiles.

Proteomic Analysis of HeLa Cells Treated with Compounds of Known Mechanisms of Action

To compare proteomic patterns in HeLa cells treated with compounds whose targets are known, well-characterized