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Metabolic perturbation of an Hsp90 C-domain inhibitor in a lung cancer cell line, A549 studied by NMR-based chemometric analysis

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Abstract Hsp90 is a good drug target molecule that is involved in regulating various signaling pathway in normal cell and the role of Hsp90 is highly emphasized especially in cancer cells. Thus, much efforts for discovery and development of Hsp90 inhibitor have been continued and a few Hsp90 inhibitors targeting the N-terminal ATP binding site are being tested in the clinical trials. There are no metabolic signature molecules that can be used to evaluate the effect of Hsp90 inhibition. We previously found a potential C-domain binder named PPC1 that is a synthetic small molecule. Here we report the metabolomics study to find signature metabolites upon treatment of PPC1 compound in lung cancer cell line, A549 and discuss the potentiality of metabolomic approach for evaluation of hit compounds.

Keywords Hsp90, C-domain inhibitor, biomarker, metabolomics

Introduction

The ~90 kDa heat shock protein Hsp90 is a highly conserved molecular chaperone found in great abundance in eukaryotic cells (~1% of cytosolic protein).[1] It is involved in a large number of cellular processes, mainly related to the conformational stabilization and regulation of a specific set of client proteins, including steroid hormone receptors, kinases and polymerases.[2]

Hsp90 is an emerging target for anticancer drugs. Because many client proteins are related to tumorigenesis, inhibiting Hsp90 function should be result in disruption of tumorigenic signals in cancer cell.[3-6] In addition, the cellular expression level of Hsp90 is highly elevated in many tumor cells, which provides a clinical relevance of effect of Hsp90 inhibition.[7]

The main target site for ligands is the N-terminal ATP binding site of Hsp90. Actually, many developmental studies are on-going currently and several inhibitors of the N-terminal domain of Hsp90 are being clinically tested. Recently, we found a novel compound (PPC1 hereafter) that specifically binds the C-domain of Hsp90 and its potency in various cancer cell lines is promising (unpublished data).

The effectiveness of the N-domain blockers are evaluated by monitoring the expression level of clients proteins such as RAF-1, CDK4, AKT, ErbB2, BRAF, BCR-ABL, EGRF, EML4-ALK, HSF-1 (transcription negative regulator of Hsps), and so on. Currently, the clinical effectiveness or potency of the N-domain blockers is evaluated in the expression level of Hsp70 in the peripheral tissues.[8] However, the effectiveness of the C-domain blocker is not well studied like that of the N-domain. Only the limited reports are shown some changes of several client proteins expression upon treatment of C-domain blockers. Moreover, the metabolic signature

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molecules for evaluation of Hsp90 inhibition are never studied.

Here we first report metabolic perturbation and the changed metabolites identified NMR spectra upon treatment of the C-domain blocker, PPC1 in a lung cancer cell line, A549.

Experimental Methods

Cell treatments- Human lung adenocarcinoma A549 cell was acquired from Korean Cell Line Bank. The cells were cultured in RPMI-1640 (Welgene, Korea), 10% fetal bovine serum (Welgene), 1% penicillin/streptomycin (Welgene) and maintained in humidified 5% CO_2 atmosphere at 37°C. To compare metabolites of PPC1- treated A549 cell and untreated cell, 0.25 ㎛ of PPC1 was treated. The vehicle of PPC1 was 0.1% DMSO in which concentration A549 is rarely affected. After 48 hrs, 1×10^7 cells were harvested with trypsin treatment and washed with PBS×2.

Sample preparation of soluble fraction and lipophilic fraction- The 1x 10^7 cell was re-suspended with 400 μL of methanol and 200 μL of chloroform. The samples were sonication for 15 min. Then, 200 μL of chloroform and 200 μL of distilled water were added to the disrupted cell suspension, and the lysates were clarified by centrifugation at 15,000g for 20 min at 4°C. After centrifugation, the aqueous and lipid layers were carefully transferred into a new 1.5 mL microcentrifuge tube. The samples were dried using a centrifugal concentrator. The aqueous residue was dissolved in 600 μL of NMR buffer (2 mM Na_2HPO_4, 5 mM NaH_2PO_4, 0.025% TSP in D_2O) and lipid residue was dissolved in 600 μL of $CDCl_3$.

NMR measurements- All 1D NOESY NMR spectra were recorded at 298K on a Bruker AVANCE III 600 spectrometer equipped with a cryoprobe. Residual water signal of 1D NMR spectra was suppressed by using a pre-saturation water suppression pulse sequence and these data were processed with the program Topspin 3.1. The mixing time in the NOESY sequence has a value of 100 ms.

Statistical analysis and metabolite identification- The statistical analysis was performed using the Bruker Biospin's AMIX program. Briefly, a principal component analysis (PCA) was first performed to detect any group separation based on NMR signal variability and to identify any abnormalities within the data. This method also enabled detection of any excluded outliers, defined as observations situated outside the 95% confidence region of the model. The identification of each peak and quantitative analysis was achieved using Chenomx NMR Suite 7.1 (Chenomx, Alberta, Canada), following manufacturer's guide. TMS was used for the ^1H reference. All NMR spectra were phased and baseline-corrected with the program Chenomx NMR Suite 7.1. For chemometric analysis, PLS-DA was used in a web-based program MetaboAnalyst (www.metaboanalyst.ca) as a supervised method that uses multiple linear regression technique to find the direction of maximum covariance between a data set (X) and the class membership (Y).

Results & Discussion

PCA analysis of the water soluble fraction- To address the metabolic changes occur by PPC1 treatment, metabolites of A549 were extracted from cell pellets. Combined extraction of polar and lipophilic metabolites from cells using methanol/chloroform/water was used.

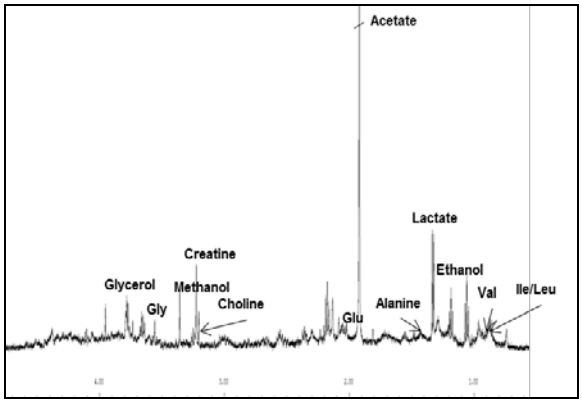

Figure 1. 1D NOESY spectrum of A549 control sample.

Several metabolites identified are depicted on the spectrum. For the water soluble metabolites, 1D NOESY spectra were obtained. Figure 1 showed that one of

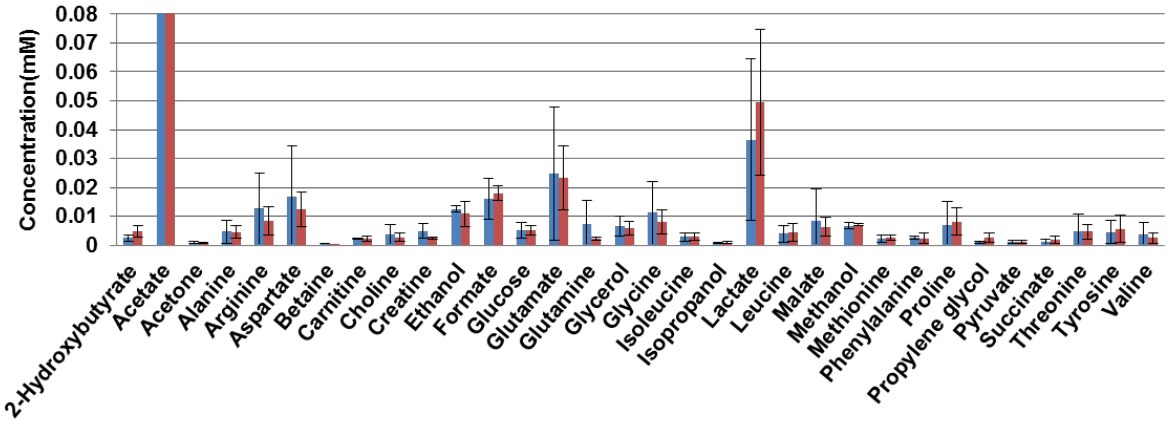

Figure 4. The concentrations of metabolites identified in the NMR spectra of soluble fractions. Around 32 metabolites were quantitated using Chenomx NMR Suite 7.1. Blue bars represent the control group and brown bars represent the PPC1-treated group.

NMR spectra of soluble metabolites. To explore the difference of metabolism, PCA (Principle components analysis) was performed. In Figure 2, the black dot represents the control (untreated) cell and the red dot represents the PPC1-treated cell. PCA analysis for the soluble metabolite did not showed clear difference between the tested groups. Interestingly, slight difference between groups could be identified shown in Figure 2, which may suggest that clear grouping could be achieved by well-designed experiments such as controlling drug treatment concentrations.

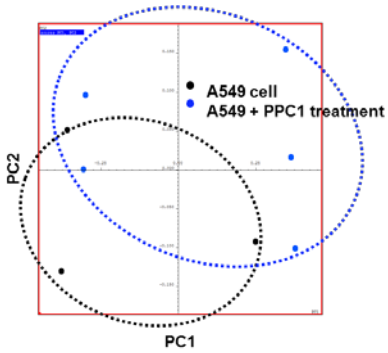

Figure 2. PCA analysis using AMIX. Black dots represent the control group and Blue dot represent the PPC1-treated group.

PCA analysis of the lipid soluble fraction- For the water soluble metabolites, 1D NOESY spectra were obtained. Figure 3 showed that one of NMR spectra of soluble metabolites. Actually, the lipid metabolites are hardly analyzed by NMR spectrum since the peak overlapping is significant in the spectrum.

In Figure 3, the black dot represents the control (untreated) cell and the red dot represents the PPC1-treated cell. PCA analysis for the lipid metabolite did not showed clear difference between the tested groups like soluble fraction. However, as shown in Figure 3, the grouping is relatively improved compared to that of soluble fractions.

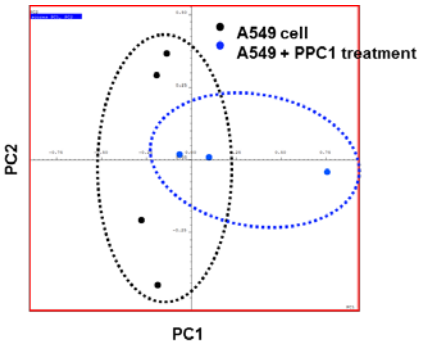

Figure 3. PCA analysis of lipid fractions. Black dots represent the control group and Blue dot represent the

PPC1-treated group.

Quantitative difference between groups- The soluble metabolites were assigned and identified in the NMR spectra. The quantitation of identified metabolites is summarized in Figure 4. As shown in Figure 4, we identified 32 metabolites in the NMR spectra among the soluble metabolites.

We first performed univariate analysis to obtain an overview of potentially important features before applying more sophisticated methods for the soluble metabolites. This analysis, that is t-tests, examines each variable separately and does not consider the effect of multiple comparisons. Figure 5 shows the t-tests result between two groups. Among the identified metabolites, methanol and formate significantly increased with the p-value 0.023484 and 0.038114, respectively.

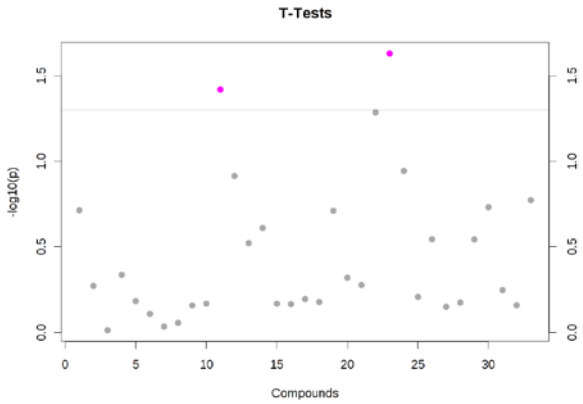

Figure 5. T-tests result between PPC1-treated and PPC1–untreated groups. Two metabolites, methanol and formate were identified as the significants (Pink dots).

Formic acid is involved in glyoxylate and dicarboxylate metabolism as well as methane metabolism. Methanol is mainly produced in methane metabolism, in which formaldehyde is processed into methanol and formic acid by the enzyme, N-dimethyl-4-nitrosoaniline oxidoreductase. It is not clear yet whether activation of N-dimethyl-4-nitrosoaniline oxidoreductase is induced by Hsp90 inhibition or not, for which more studies should be required.

To analyze more, PLS-DA was employed as a supervised statistical method. We scaled the concentration data of each metabolite to completely eliminate global intensity differences by a procedure called autoscaling. Each variable is mean-centered and subsequently divided by its standard deviation in autoscaling. Figure 6 showed the 2D score plot of PLS-DA with autoscaled concentration data. This

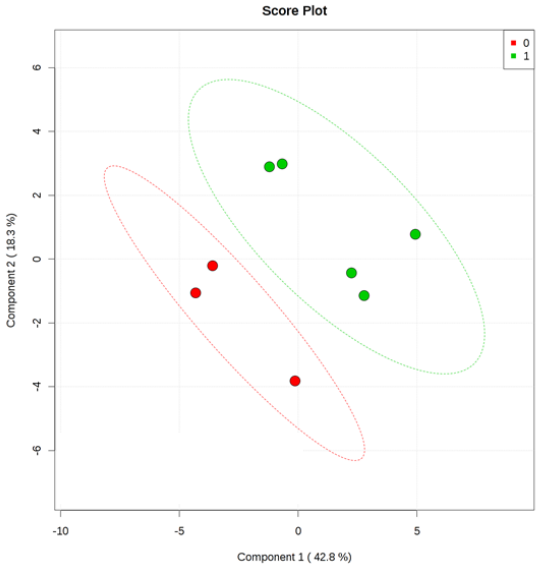

Figure 6. 2D Score plot of PLS-DA analysis. Red dots represent the control group and Green dot represent the PPC1-treated group.

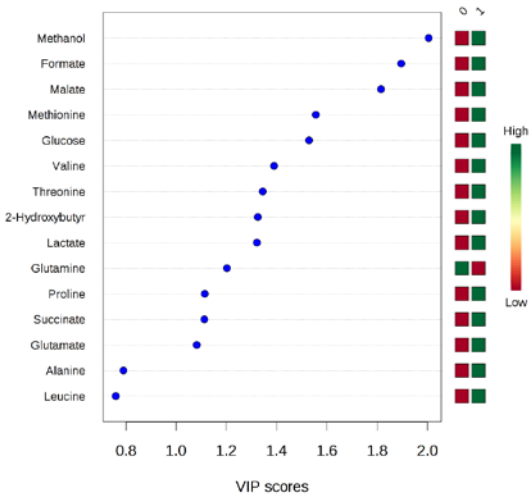

Figure 7. VIP score of PLS-DA analysis. VIP (Variable Importance in Projection) score is a weighted sum of squares of the PLS loadings. The weights are based on the amount of explained Y-variance in each dimension.

result showed improved grouping of two groups, which supports that PPC1 affects the metabolism of cancer cell, A549, compared to the untreated cells. The figure 7 is the VIP score plot of the PLS-DA analysis, which also indicates that methanol and formic acid are key metabolites in distinguishing groups.

Combined together, our results suggest that a specific C-domain inhibitor of Hsp90 affects the cancer metabolism of A549 and several metabolites including methanol and formic acid could be used as potential biomarkers to evaluate the effectiveness or potency of anti-cancer agents targeting Hsp90. This kind of study is the first approach in discovery of Hsp90 inhibitors, based on our knowledge and probably help for us to understand the mode of action of Hsp90 inhibition in cancer cells.

Acknowledgements

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References

1. Richter, K., Buchner, J. *J. Cell Physiol.* **188**, 281-290. (2001).
2. Terasawa, K., Minami, M., Minami, Y. *J Biochem. (Tokyo)* **137**, 443-447. (2005).
3. Vogelstein, B., Lane, D., Levine, A.J. *Nature* **408**, 307-310. (2000).
4. Workman, P., Burrows, F., Neckers, L., Rosen, L. *Ann. N.Y. Acad. Sci.* **1113**, 202–216. (2007).
5. Park, SJ., Borin, BN., Martinez-Yamout, MA., Dyson, HJ. *Nature Structural & Molecular Biology* **18(5)**, 537-41. (2011).
6. Park, SJ., Kostic, M., Dyson, HJ. *J Mol Biol.* **411(1)**, 158-73. (2011).
7. McDowell, C., Sutton, R., Obermann, W. *International Journal of Biological Macromolecules* **45**, 310–314. (2009).
8. Zhang, H., Chung, D., Yang, Y., *et al. Mol. Cancer Ther.* **5**, 1256-1264. (2006).
9. Yoon, D., Lee, M., Kim, S., Kim, S. *J. Korean Magn. Reson. Soc.* **17**, 1-10. (2011).
10. Kim, H., Choi, Y., Choi, K., Park, J., Kim, H., Jeon, J., Heu, M., Shin, D., Lee, J. *J. Korean Magn. Reson. Soc.* **16**, 91-102. (2011)