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Discovery of Licochalcone A as a Natural Product Inhibitor of Hsp90 and Its Effect on Gefitinib Resistance in Non-Small Cell Lung Cancer (NSCLC)

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The epidermal growth factor receptor (EGFR), or ERBB family regulates essential cellular processes, including proliferation, survival, migration, and differentiation, and appear to play an important role in the etiology and progression of tumors.[1] Among its members, EGFR/ERBB1 and Her2/ERBB2 seem to be successfully targeted by therapeutic agents of gefitinib (Iressa, Astra Zeneca) and trastuzumab (Herceptin, Genentech), respectively. Over the past two decades, several cancer drugs targeting a biologically relevant single protein have been discovered to eradicate tumors in more specific ways and reduce the harmful nonspecific side effects of chemotherapeutics.[2] However, despite the robust efficacy of the targeted cancer drugs, the development of drug-resistance remains a major obstacle to the successful treatment of cancer. Although mechanisms of resistance might vary, one essential problem is the extremely complex nature of tumors. Tumors can survive against targeted therapy by mutating targeted proteins, down-regulating death signals, or up-regulating survival pathways. Therefore, the notion of targeting a single protein is not intrinsically a silver bullet to battle against cancers.

Numerous oncogenic proteins represent important biological targets for cancer therapy. Among them, Hsp90 has received considerable attention and emerged as an attractive drug target that is responsible for the stabilization and maturation of a wide range of oncogenic proteins, including EGFR, Her2, Met, Akt, Raf, HIF-1α and MMP2.[3,4] Hsp90 is, so called "nodal" protein that orchestrates protein-folding quality control in multiple signaling pathways. Due to its nodal properties, Hsp90 has been extensively pursued as a target for overcoming drug resistance.[5] Disruption of Hsp90 chaperone activity induces client proteins degradation via the ubiquitin-proteasome pathway, which can ultimately lead to cell death. Moreover, Hsp90 is constitutively expressed at 2-10 fold higher levels in tumor cells compared to their normal counterparts.[6] Hence, the discovery of a small molecule, disrupting Hsp90 chaperone machinery is believed to be a promising strategy for cancer treatment. Besides, accumulating evidence in recent studies has demonstrated that Hsp90 is a potential therapeutic target for neurodegenerative diseases, including Alzheimer's, Parkinson's, Prion and Hodgkin's diseases.[7-9] The potential therapeutic benefits associated with Hsp90 modulation emphasize the importance of identifying novel Hsp90 inhibitors.

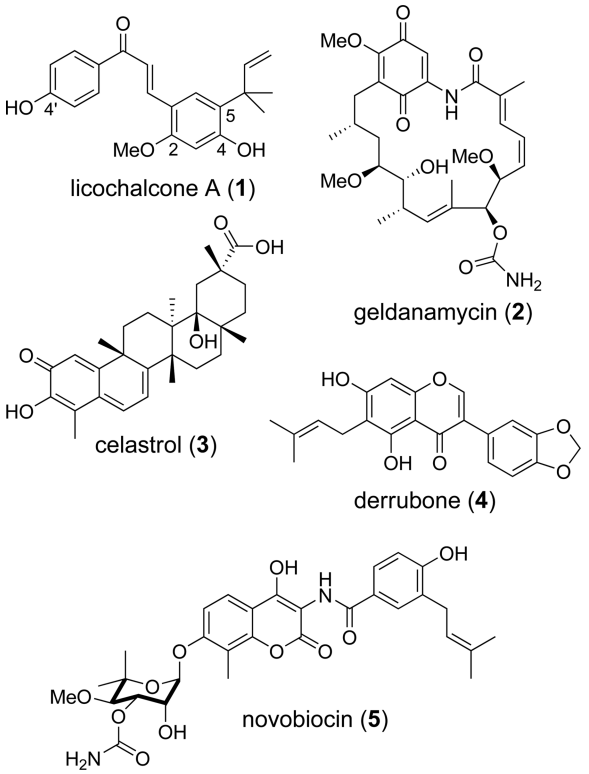

Figure 1. Structure of licochalcone A and known natural product inhibitors of Hsp90.

The discovery of new drugs from natural products has proved to be the single most successful strategy and natural products still provide ample sources of chemical diversity in drug discovery. Natural products targeting Hsp90 are investigated for the treatment of cancerous diseases. Several natural products, including geldanamycin[10] (GA, **2**), celastrol[11] (**3**), derrubone[12] (**4**), and novobiocin[13] (**5**) have been reported to target Hsp90 (Figure 1).

Licochalcone A is a major phenolic constituent of the *Glycyrrhiza* plant, of which the root is commonly called licorice. Licorice is one of well-known traditional medicines in East Asia and has various pharmacological applications in detoxification, *anti*-inflammation, *anti*-cancer, *anti*-coagulation, and an *anti*-microbial effect.[14] Licochalcone A, one of the main effector components of licorice has been reported to have various biological activities, such as *anti*-inflam-

matory,[15] *anti*-oxidic,[16] *anti*-malarial,[17] and *anti*-leishmanial activity.[18]

A screening program of Hsp90 inhibitors from natural sources was recently launched, and licochalcone A was found to inhibit Hsp90 chaperoning functions. Herein we report *anti*-proliferative effect of lichochalcone A against gefitinib-resistant non-small cell lung cancer cells (H1975) and the study of Hsp90 inhibitory activity.

Non-small cell lung cancer (NSCLC) is the most common type of lung cancer and accounts for 80-85% of lung cancer patients.[19] Even with surgical resection at early diagnosis, approximately 50% of NSCLC patients face recurring cancers.[20] Moreover, 40-75% of NSCLC patients are unfortunately projected to die within 5 years even with surgery.[21] In the last decade, two EGFR-tyrosin kinase inhibitors, gefitinib and erlotinib have been extensively developed in NSCLC. However, tumor cells circumvent EGFR blockage by mutation of EGFR T790M, and activation and amplification of Met tyrosine kinase receptor as an alternative signaling pathway.[22,23] Considering both EGFR and Met are client proteins of Hsp90, to block Hsp90 protein folding machinary may be best suited to overcome the resistance from EGFR mutation and Met amplification.

To investigate the effect of licochalcone A on overcoming gefitinib-resistance in NSCLC, we measured *anti*-proliferative activity of *in vitro* model of gefitinib-resistant non-small cell lung cancer, H1975 (Figure 2). We treated H1975 cells with increasing concentration of licochalcone A and cell proliferation was measured at the time point of 24, 48, and 72 h using MTS colorimetric assay. The data indicated that 50 μM of licochalcone A significantly impaired growth of gefitinib-resistant H1975 cells.

To further determine whether the observed cytotoxicity was related to Hsp90 inhibition, H1975 cancer cells were incubated with various concentrations of licochalcone A and geldanamycin (GA, 1 μM) as positive control, and analyzed the expression levels of Hsp90's clients, EGFR, Her2, Met and Akt along with Hsp70 and Hsp90 (Figure 3). Licochal-

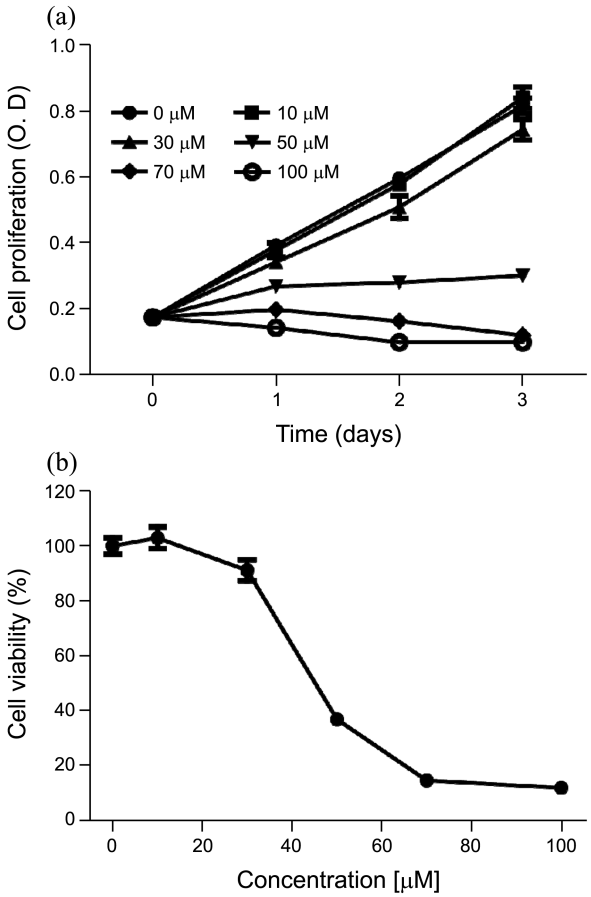

Figure 2. Effect of licochalcone A on cell proliferation of H1975 cells. (a) Cell growth was determined at 1, 2, and 3 days using MTS assay at various concentrations of licochalcone A (0, 10, 30, 50, 70, and 100 μM) (b) Cell death was induced by licochalcone A in H1975 cells. Cells were treated for 72 h at various concentrations of licochalcone A (0, 10, 30, 50, 70, and 100 μM) and cell viability was measured by MTS assay. Data are presented as mean ± SD (n = 4).

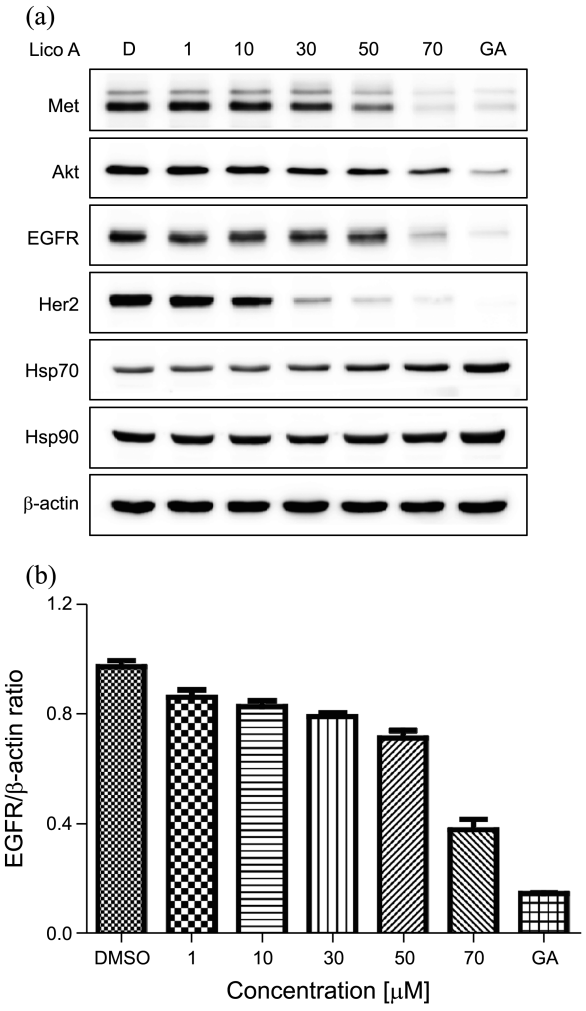

Figure 3. Licochalcone A induced degradation of Hsp90 client proteins. (a) Licochalcone A was evaluated for its ability to down-regulate several client proteins at various concentrations (μM). Geldanamycin (GA, 1 μM) and DMSO were employed, respectively as positive and negative controls. (b) Analysis of EGFR expression in H1975 cells treated with various concentrations of licochalcone A (0, 1, 10, 30, 50, and 70 μM). Results of densitometry analysis were reported as normalized to β-actin ratios.

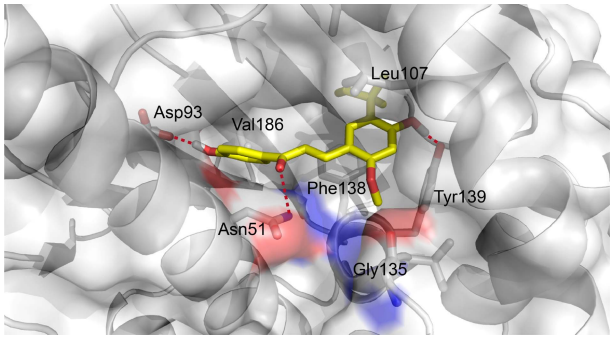

Figure 4. Docking pose of human Hsp90 with licochalcone A. The carbon, oxygen, and hydrogen atoms of licochalcone A are shown in yellow, red, and gray, respectively. The side chains of binding site are colored by atom types (carbon, gray; nitrogen, blue; oxygen, red) and labeled with their residue name. Hydrogen bonds are shown in dashed red lines.

cone A revealed a robust degradation of EGFR, Her2, Met and Akt and up-regulation of cochaperone Hsp70. The result strongly suggested that licochalcone A targeted the Hsp90 protein folding machinery, since molecular hallmarks of Hsp90 inhibition included marked proteosomal degradation of client proteins and transcriptional upregulation of Hsp70. It indicated that even 10 μM of licochalcone A induced the considerable degradation of Her2, whereas EGFR and Met was significantly depleted at 50-70 μM of licochalcone A.

To investigate the binding mode of licochalcone A in the Hsp90 active site, docking studies were performed using the human-Hsp90 crystal structure (PDB code: 1UYM). Licochalcone A was docked with the 3D coordinates of the Hsp90 N-terminal domain using AutoDock4.2 program. ATP N-terminal binding site of Hsp90 consists of hydrophilic region of Asp93, Asp54 and Thr184 residues and π-rich hydrophobic region of Phe138, Trp162, Leu107, Val150 and Tyr139 residues. Based on the *In silico* modeling, *para*-hydroxy group at C4' and the carbonyl oxygen atom of licochalcone A formed hydrogen-bonding with Asp93 and Asn51, respectively in the hydrophilic region of the pocket, which typically the adenine ring of ATP and the resorcinol of NVP-AUY922 bind with.[24] Meanwhile, the prenylated phenyl ring of licochalcone A projected into the hydrophobic region, where the hydroxy group at C4 interacted with Tyr139 by hydrogen bonding, the prenyl group of licochalcone A stretched inside of the hydrophobic region and formed Van der Waals interaction with Leu107, and the phenyl group had π-π interaction with Phe138 residue. Collectively, three hydrogen bonds and two hydrophobic interactions contributed to the binding of licochalcone A to Hsp90 and the estimated binding energy (ΔG_b) and inhibition constants (K_i) using the Lamarckian genetic algorithm result in −8.84 kcal/mol and 329 nM, respectively.

In conclusion, we have discovered that a natural product, licochalcone A inhibits the growth of gefitinib-resistant non-small cell lung cancer (H1975) with modest potency. Licochalcone A causes a significant degradation of EGFR, Met, Her2, and Akt and it suggests that licochalcone A would be a potential therapeutic lead to circumvent the cancer drug resistance due to EGFR/Met-Akt signaling axis. The combining evidence of biochemical and cellular studies indicates that licochalcone A disrupts the Hsp90 chaperoning function. Moreover, molecular modeling manifests that licochalcone A would bind to the N-terminal ATP binding site of Hsp90. The significance of our study for pharmacological and clinical medicine lies in finding a novel Hsp90 inhibitor, which also provides a new molecular scaffold and strategy to overcome the drug resistance in cancer therapy. Efforts are currently directed toward synthesizing analogues of licochalcone A and SAR exploration to improve the efficacy and the pharmacokinetic properties of the compound and the result will be reported in due course.

Experimental

Materials. Licochalcone A was purchased from Sigma-Aldrich. Antibodies specific for EGFR, Her2, Met, Akt, Hsp90, Hsp70, and β-actin were purchased from Cell Signaling Technology. Goat *anti*-rabit IgG horseradish peroxidase conjugate was purchased from Santa Cruz Biotechnology. Cell Titer 96 Aqueous One Solution cell proliferation assay kit was purchased from Promega.

Cell Culture. H1975 cells were grown in RPMI 1640 with L-glutamine supplemented with streptomycin (500 mg/mL), penicillin (100 units/mL), and 10% fetal bovine serum (FBS). Cells were grown to confluence in a humidified atmosphere (37 °C, 5% CO_2).

Effect of Licochalcone A on Cell Proliferation. Cells were seeded at 3000 cells per well in a clear 96-well plate, the medium volume was brought to 100 μL, and the cells were allowed to attach overnight. The next day, varying concentrations of compound or 1% DMSO vehicle control was added to the wells. Cells were then incubated at 37 °C for 24, 48, and 72 h. Cell viability was determined using the Promega Cell Titer 96 Aqueous One Solution cell proliferation assay. After incubation with compounds, 20 μL of the assay substrate solution was added to the wells, and the plate was incubated at 37 °C for an additional 1 h. Absorbance at 490 nm was then read on Tecan Infinite F200 Pro plate reader, and values were expressed as percent of absorbance from cells incubated in DMSO alone.

Western Blot. Cells were seeded in 60 mm culture dishes (5×10^5/dish), and allowed to attach overnight. Licochalcone A was added at the concentrations indicated in Figure 3, and the cells were incubated for an additional 24 h. For comparison, cells were also incubated with DMSO (1%) or geldanamycin (1 μM) for 24 h. Cells were harvested in ice-cold lysis buffer (23 mM Tris-HCl pH 7.6, 130 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS), and 20 μg of lysate per lane was separated by SDS-PAGE and followed by transferring to a PVDF membrane (Bio-Rad). The membrane was blocked with 5% skim milk in TBST, and then incubated with the corresponding antibody (EGFR, Her2, Met, Akt, Hsp90, Hsp70, or β-Actin). After binding of an appropriate secondary antibody coupled to horseradish per-

oxidase, proteins were visualized by ECL chemiluminescence according to the instructions of the manufacturer (Thermo Scientific, USA).

Docking Studies. *In silico* docking of licochalcone A with the 3D coordinates of the X-ray crystal structures of the N-terminal domain of human Hsp90 (PDB, 1UYM) was accomplished using the AutoDock program downloaded from the Molecular Graphics Laboratory of the Scripps Research Institute. The AutoDock program was chosen because it uses a genetic algorithm to generate the poses of the ligand inside a known or predicted binding site utilizing the Lamarckian version of the genetic algorithm where the changes in conformations adopted by molecules after in situ optimization are used as subsequent poses for the offspring. In the docking experiments carried out, water was removed from the 3D X-ray coordinates while Gasteiger charges were placed on the X-ray structures of the N-terminal domain of HSP90 along with licochalcone A using tools from the AutoDock suite. A grid box centered on the N-terminal HSP90 domain with definitions of 60_60_60 points and 0.375 Å spacing was chosen for ligand docking experiments. The docking parameters consisted of setting the population size to 150, the number of generations to 27000, and the number of evaluations to 25000000, while the number of docking runs was set to 50 with a cutoff of 1 Å for the root-mean-square tolerance for the grouping of each docking run. The docking model of human Hsp90 with licochalcone A was depicted in Figure 4 and rendering of the picture was generated using PyMol (DeLano Scientific).

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