http://dx.doi.org/10.5012/bkcs.2013.34.11.3345

Butein Disrupts Hsp90's Molecular Chaperoning Function and Exhibits Anti-proliferative Effects Against Drug-resistant Cancer Cells

Young Ho Seo

College of Pharmacy, Keimyung University, Daegu 704-701, Korea. E-mail: seoyho@kmu.ac.kr
Received August 5, 2013, Accepted August 26, 2013

Hsp90 shows great promise as a therapeutic target due to its potential to disable multiple signaling pathways simultaneously. In this study, we discovered that a natural product, butein moderately inhibited the growth of drug-resistant cancer cells (A2780cis and H1975), and brought about the degradation of oncogenic Hsp90 client proteins. The study demonstrated that butein would be a therapeutic lead to circumvent drug-resistance in cancer chemotherapy. The structure-based screening, synthesis, and biological evaluation of butein are described herein.

Key Words : Butein, Hsp90, Drug-resistance, Cancer, Natural product

Introduction

Cancer is the primary cause of death in many first world countries,[1] and this demands for more efficient strategies in cancer therapy. Although chemotherapy remains the most commonly used treatments for cancers, the resistance to chemotherapy is an increasing problem and represents a major obstacle to the successful treatment of cancer patients. Numerous culprits of drug resistance have been identified, including aberrant regulation of drug transporters that detect and eject anticancer drugs from cells,[2,3] signaling pathway crosstalk that bypasses blocked pathway,[4] and induction of drug-detoxifying mechanisms.[5] Therefore, the majority of patients eventually develop progressive disease after initially responding to treatment of chemotherapy. To circumvent the drug resistance to improve cancer chemotherapy, the design of cancer chemotherapy has become increasingly sophisticated, yet there is no cancer treatment that is 100% effective against disseminated cancer.

Heat shock protein 90 (Hsp90) is an ATP dependent molecular chaperone and has become an attractive cancer target, especially for overcoming the resistance of chemotherapy. Hsp90 is involved in the folding, stabilization, activation, and assembly of their substrate proteins, referred to as "client" proteins. Its client proteins include transmembrane tyrosine kinases (EGFR, Her2), metastable signaling proteins (Akt, Raf-1, IKK), and mutated signaling proteins (p53, v-Src).[6,7] Considering that numerous oncogenic proteins are substrates for Hsp90 mediated protein folding process, inhibition of Hsp90 function simultaneously incapacitates multiple signaling pathways, providing a combinatorial attack on cellular oncogenic processes. More interestingly, Hsp90 accounts for 4-6% of total proteins in tumor cells in contrast with the 1-2% in their normal counterparts. The harsh environmental conditions found in tumors, such as hypoxia, low pH and poor nutrition tend to destabilize proteins, and make tumor cells more dependent on Hsp90's chaperoning function.

Therefore, Hsp90 inhibitors demonstrate selective anti-cancer effects toward cancer cells as compared to normal cells.[8,9] With the notion that natural products are compounds pre-optimized by evolution to act up specific biological targets, natural products provided the promising sources for the majority of the drugs in use today. Several natural products, including geldanamycin,[10] celastrol,[11] novobiocin,[12] and licochalcone A[13] have been discovered to target Hsp90 (Figure 1). We performed a screening aimed to identify a novel class of Hsp90 inhibitors from natural sources and we focused on 3,4,2',4'-tetrahydroxychalcone (butein, **1**) for its structural characteristics and biological profile. Butein is a polyphenolic compound extracted from numerous plants including the stem bark of cashews (*Semecarpus anacardium*), the heartwood of *Dalbergia odorifera*, and the

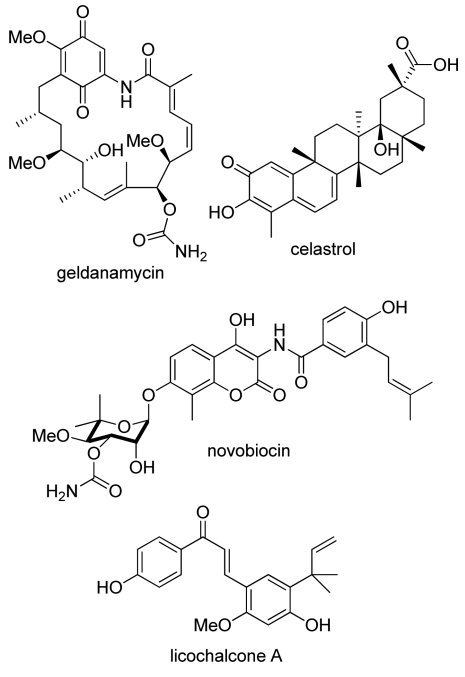


Figure 1. Structure of natural product Hsp90 inhibitors.

traditional Chinese and Tibetan medicinal herbs *Caragana jubata* and *Rhus verniciflua* Stokes.[14] Butein has been reported to exhibit anticancer activity against several human cancers including breast carcinoma,[15] colon carcinoma,[16] osteosarcoma,[17] lymphoma,[18] acute myelogenous leukemia,[19] and melanoma.[20,21] However, the mechanism of action with respect to the anticancer effects of butein is not fully understood. Herein, we report the synthesis, and biological evaluation of butein (**1**) and 3,4,2,4-tetraallylbutein (**2**) against drug-resistant cancer cell lines (A2780cis and H1975).

Results and Discussion

Structural analysis of Hsp90 inhibitors revealed that majority of Hsp90 inhibitors possessed the resorcinol moiety in their structure, and the resorcinol moiety of these inhibitors bound in the same position and orientation, bridging hydrogen bonds to Asp93 through a conserved water molecule (Figure 2).[22,23] Co-crystal structures of NVP-AUY 922 (PDB code: 2VCI) and radicicol (PDB code: 4EGK) in N-terminal ATP binding pocket of Hsp90 demonstrated that the resorcinol motifs of NVP-AUY 922 and radicicol positioned in the region of the pocket consisted of Asp93 and Asn51, and formed hydrogen-bonds with Asp90 and Asn51 through conserved water molecules. The adenine ring of endogenous ligand, ATP also bound to the same region of the pocket consisted of Asp93 and Asn51. Forming a hydrogen bond to Asp93 residue is believed to be the most important interaction of resorcinol-bearing Hsp90 inhibitors for their inhibitory activities against Hsp90. Regarding that butein

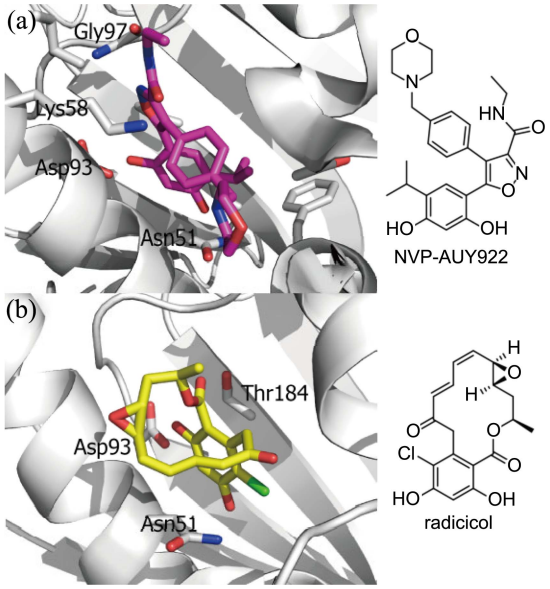
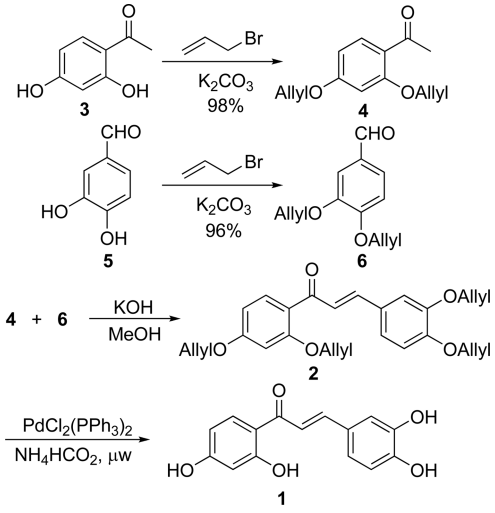


Figure 2. The X-ray crystal structure of (a) NVP-AUY922 (PDB code: 2VCI) and (b) radicicol (PDB code: 4EGK) in ATP-binding pocket of Hsp90. The carbon atoms of NVP-AUY922 and radicicol are illustrated in pink and yellow, respectively. The oxygen, nitrogen and chlorine atoms of NVP-AUY922 and radicicol are shown in red, blue and green, respectively. The side chains of binding site are colored by atom types (carbon, gray; nitrogen, blue; oxygen, red) and labeled with their residue name.



Scheme 1. Synthesis of 3,4,2',4'-tetraallylbutein (**2**) and butein (**1**).

also possessed the resorcinol moiety in its structure, we decided to investigate the inhibitory effect of butein on Hsp90's chaperoning function. Based on the structural feature of butein, we assumed that the resorcinol ring of butein would bind to the region of the pocket having Asp93 and Asn51 residues and form hydrogen-bonds with Asp93 and Asn51, as most resorcinol-bearing Hsp90 inhibitors did.

To assess the hypothesis of butein's binding to ATP-binding pocket of Hsp90, we began to synthesize butein (**1**). Firstly, 2,4-dihydroxyacetophenone (**3**) and 3,4-dihydroxybenzaldehyde (**5**) were protected with allyl bromide in the presence of K_2CO_3 in DMF to provide compound **4** and **6**, respectively (Scheme 1).[24] With ketone **4** and aldehyde **5** in hand, Claisen-Schmidt aldol condensation of ketone **4** with aldehyde **6** was carried out in the presence of KOH in MeOH. The condensation reaction successfully provided 3,4,2',4'-tetraallylbutein (**2**) in 44% yield. Finally, allyl-protecting groups of **2** were removed by microwave irradiation in the presence of $PdCl_2(PPh_3)_2$ and ammonium formate to furnish butein (**1**) in 32% yield.[25]

We first examined the anti-proliferative effects of butein (**1**) and 3,4,2',4'-tetraallylbutein (**2**) on cisplatin-resistant ovarian cancer cells (A2780cis). Cisplatin is the standard chemotherapy for patients with ovarian cancer. However, there is a major problem with this therapy in that ovarian cancer cells are resistant to cisplatin. To evaluate the comparative cytotoxicity of butein (**1**) and 3,4,2',4'-tetraallylbutein (**2**), A2780cis were incubated with **1** and **2** at various concentration (0, 25, 50, 75, and 100 μM) for 3 days and cell viability was measured by MTS assay. The assay revealed that butein moderately inhibited cell proliferation in a concentration-dependent manner, but 3,4,2',4'-tetraallylbutein showed no effect on the cell viability of A2780cis.

Next, we investigated the molecular mechanism of compound **1** and **2**. Regarding that the proteasomal degradation of Hsp90 client proteins and the transcriptional upregulation of Hsp70 are a molecular signature of Hsp90 inhibition, we analyzed the cellular levels of EGFR, Her2, Met, Akt,

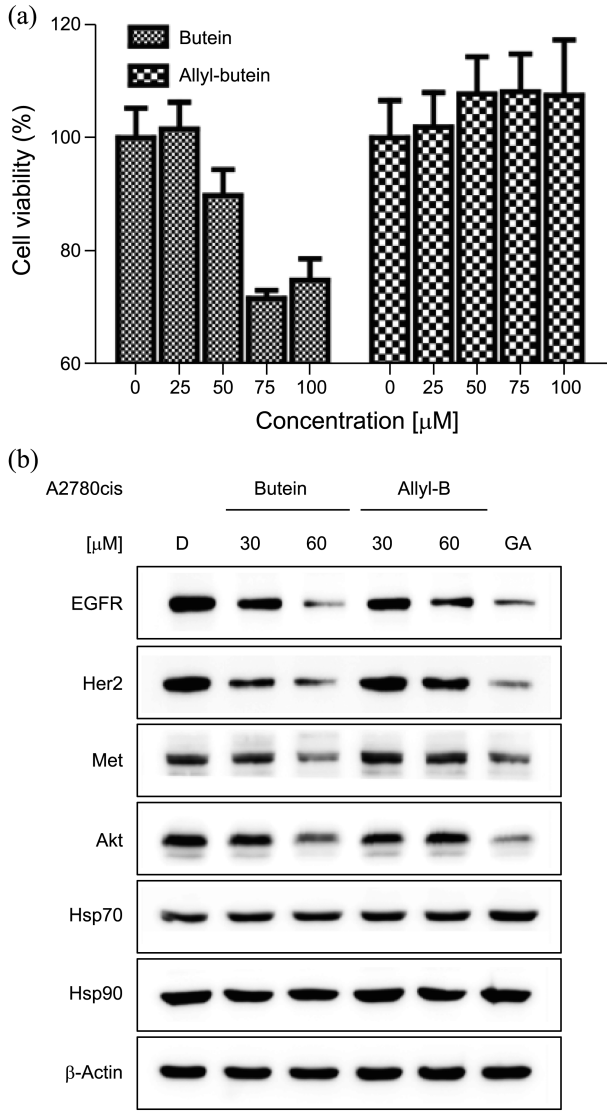


Figure 3. (a) Comparative effect of butein (**1**) and 3,4,2',4'-tetraallylbutein (Allyl-B, **2**) on cell viability of A2780cis cells. Cell viability was determined with compound **1** and **2**. Cells were treated for 3 days at the indicated concentration of compound **1** and **2** and cell viability was measured by MTS assay. Data are presented as mean ± SD (n = 4). (b) Comparative effect of butein (**1**) and 3,4,2',4'-tetraallyl butein (Allyl-B, **2**) on cellular biomarkers of Hsp90 inhibition. A2780cis cells were treated for 24 hours with the indicated concentration of compound **1** and **2** and the expression levels of the Hsp90 client proteins were analyzed by western blot. Geldanamycin (GA, 1 μM) and DMSO (D) were employed, respectively as positive and negative controls.

Hsp70, and Hsp90 along with β-actin as an internal standard. As expected, butein induced the significant degradation of EGFR, Her2, Met, and Akt at 60 μM, compared to DMSO control. In contrast, 3,4,2',4'-tetraallylbutein showed a very weak effect on Hsp90 inhibition. Although the expression levels of EGFR and Her2 at 60 μM of 3,4,2',4'-tetraallylbutein were slightly reduced, Met and Akt remained unchanged even with 60 μM of 3,4,2',4'-tetraallylbutein. Accordingly, MTS assay and western blot demonstrated that butein was a more potent inhibitor against Hsp90 than

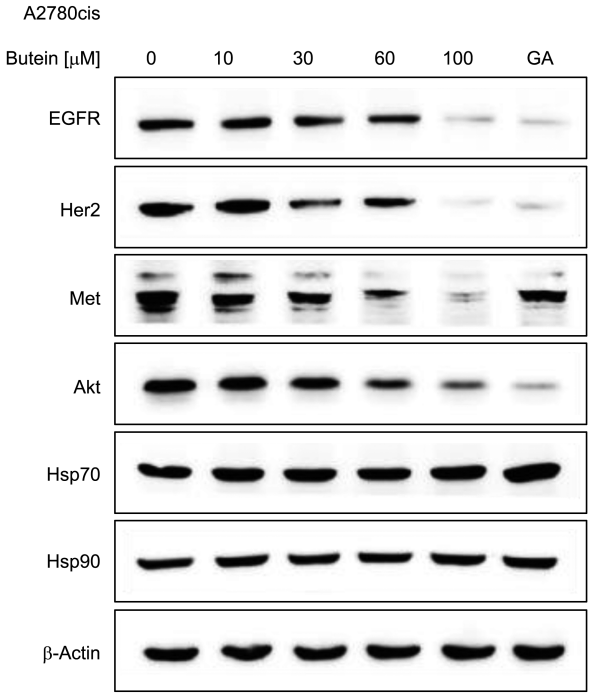


Figure 4. Butein induced the degradation of Hsp90 client proteins of A2780cis cells in a concentration-dependent manner. A2780cis cells were treated for 24 h with the indicated concentration of butein (**1**) and the expression levels of the Hsp90 client proteins were analyzed by western blot. Geldanamycin (GA, 1 μM) and DMSO (D) were employed, respectively as positive and negative controls.

3,4,2,4-tetraallylbutein.

To precisely determine dose-dependent effect of butein on the down-regulation of Hsp90 client proteins, A2780cis cells were incubated with butein (0, 10, 30, 60, and 100 μM) in Figure 4. Western blot analysis indicated that butein dose-dependently induced the degradation of Hsp90 client proteins, EGFR, Her2, Met, and Akt in cisplatin-resistant ovarian cancer cells, A2780cis. However, the expression level of Her2 remained unchanged even with 60 μM of butein. When exposed to 100 μM of butein, a little induction of Hsp70 was observed. The positive control, geldanamycin (GA) also had a very minor effect on the induction of Hsp70. Since β-actin was not dependent on Hsp90 protein folding machinery, β-actin was not affected upon administration of butein. Overall, these results supported that butein disrupted Hsp90 chaperoning function.

To examine the effect of butein on cell viability of H1975, H1975 cells were treated with butein (0, 30, 60, and 90 μM) for 3 days and cell viability was measured using MTS. H1975 is a gefitinib-resistant non-small cell lung cancer cell and its resistance is mediated by the T790M-EGFR mutation and Met amplification.[4,26] The assay indicated that butein also impaired the growth of gefitinib-resistant non-small cell lung cancer cell (H1975) in a concentration dependent manner. To further determine whether the observed anti-proliferative effects was related to Hsp90 inhibition, H1975 cells were incubated with butein (0, 1, 10, 30, 50, 70, and 100 μM) and the expression levels of Hsp90 client protein

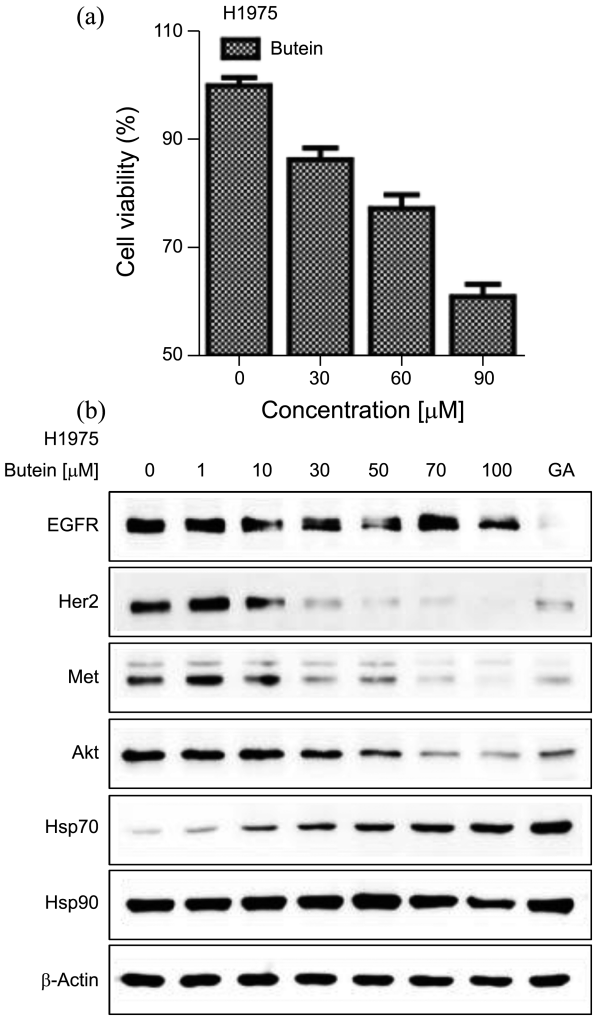


Figure 5. Butein inhibits the growth of gefitinib-resistant non-small cell lung cancer cell (H1975) through Hsp90 inhibition. (a) Effect of butein on cell viability of H1975. Cell viability was determined with butein. H1975 cells were treated for 3 days at the indicated concentration of butein and cell viability was measured by MTS assay. Data are presented as mean ± SD (n = 4). (b) Butein induced degradation of Hsp90 client proteins of H1975 in a concentration-dependent manner. H1975 cells were treated for 24 hours with the indicated concentration of butein and the expression levels of the Hsp90 client proteins were analyzed by western blot. Geldanamycin (GA, 1 μM) and DMSO (D) were employed, respectively as positive and negative controls.

were analyzed. As shown in Figure 5(b), Hsp90 client proteins, Her2, Met, and Akt were significantly depleted in a concentration-dependent manner and the cellular level of Hsp70 was dose-dependently up-regulated when exposed to butein, which that is consistent with other Hsp90 inhibitors.[27]

Conclusion

In conclusion, we have discovered that a natural product, butein inhibits the growth of drug-resistant cancer cells (A2780cis and H1975) with modest potency. Butein induces a significant degradation of Hsp90 client proteins in A2780cis and H1975 cell lines. The biochemical and cellular studies demonstrates that butein inhibits the Hsp90 folding machinery. The result suggests that butein would be a potential therapeutic lead to overcome the drug resistance of cancer. Currently efforts are directed toward synthesizing analogues of butein and SAR exploration to improve the efficacy of the compound. The result will be reported in due course.

Experimental

General Methods. Unless otherwise noted, all reactions were performed under an argon atmosphere in oven-dried glassware. All purchased materials were used without further purification. Thin layer chromatography (TLC) was carried out using Merck silica gel 60 F_{254} plates. TLC plates were visualized using a combination of UV, *p*-anisaldehyde, ceric ammonium molybdate, ninhydrin, and potassium permanganate staining. NMR spectra were obtained on a Bruker 400 (400 MHz for ^{1}H; 100 MHz for ^{13}C) spectrometer. ^{1}H and ^{13}C NMR chemical shifts are reported in parts per million (ppm) relative to TMS, with the residual solvent peak used as an internal reference. Signals are reported as m (multiplet), s (singlet), d (doublet), t (triplet), q (quartet), bs (broad singlet), bd (broad doublet), dd (doublet of doublets), dt (doublet of triplets), or dq (doublet of quartets); the coupling constants are reported in hertz (Hz). Final products were purified by MPLC (Biotage Isolera One instrument) on a silica column (Biotage SNAP HP-Sil). On the basis of NMR and analytical HPLC data (Shimadzu prominence, VP-ODS C18 column), purity for all the tested compounds was found to be > 95%.

Procedure for the Synthesis of Compound 2. A mixture of ketone **4** (0.58 g, 2.5 mmol), aldehyde **6** (0.6 g, 2.75 mmol), KOH (0.31 g, 5.5 mmol) in 10 mL of methanol was stirred at rt for 3 days. The mixture was neutralized with 3 N HCl to pH 6 and then extracted with ethyl acetate. The organic layer was dried over Na_2SO_4, and concentrated under reduced pressure, and purified by column chromatography (20% ethyl acetate in hexane) to afford compound **2** in 44% as yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, J = 8.8 Hz, 1H), 7.62 (d, J = 15.6 Hz, 1H), 7.46 (d, J = 16 Hz, 1H), 7.14-7.12 (m, 2H), 6.87 (d, J = 9.2 Hz, 1H), 6.57 (dd, J = 8.8 Hz, J = 2.4 Hz, 1H), 6.51 (d, J = 2.4 Hz, 1H), 6.12-6.00 (m, 4H), 5.48-5.40 (m, 4H), 5.34-5.24 (m, 4H), 4.65-4.57 (m, 8H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 189.9, 162.6, 158.9, 150.1, 148.1, 141.6, 132.8, 132.6, 132.6, 132.3, 132.2, 128.2, 125.0, 122.8, 122.4, 117.9, 117.6, 117.6, 117.5, 112.9, 112.3, 105.9, 100.0, 69.5, 69.3, 69.0, 68.7. ESI MS (*m/e*) = 433 $[M+1]^+$.

Procedure for the Synthesis of Butein (1). Compound **2** (0.1 g, 0.23 mmol) was stirred under microwave irradiation (Biotage Initiator) for 30 min at 120 °C in the presence of $PdCl_2(PPh_3)_2$ (10 mg) and ammonium formate (150 mg) in 4 mL of THF. The reaction mixture was diluted with ethyl acetate. The organic layer was washed with water, dried over Na_2SO_4,concentrated under reduced pressure, and purified by column chromatography (40% ethyl acetate in hexane) to afford compound **1** in 32% as yellow solid, whose spectral

data were identical to those previously reported.[26]

Materials. 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. A2780cis and 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 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. Butein or 3,4,2',4'-tetraallylbutein was added at the concentrations indicated in Figures 3, 4 and 5, 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 peroxidase, proteins were visualized by ECL chemiluminescence according to the instructions of the manufacturer (Thermo Scientific, USA).

Acknowledgments. The present research has been conducted by the Settlement Research Grant of Keimyung University in 2011.

References

1. Runowicz, C. D. *Science* **2006**, *313*, 171.
2. Stewart, D. J. *Crit. Rev. Oncol. Hematol.* **2007**, *63*, 12.
3. Gately, D. P.; Howell, S. B. *Br. J. Cancer* **1993**, *67*, 1171.
4. Paez, J. G.; Janne, P. A.; Lee, J. C.; Tracy, S.; Greulich, H.; Gabriel, S.; Herman, P.; Kaye, F. J.; Lindeman, N.; Boggon, T. J.; Naoki, K.; Sasaki, H.; Fujii, Y.; Eck, M. J.; Sellers, W. R.; Johnson, B. E.; Meyerson, M. *Science* **2004**, *304*, 1497.
5. Gottesman, M. M. *Annu. Rev. Med.* **2002**, *53*, 615.
6. Li, Y.; Zhang, T.; Schwartz, S. J.; Sun, D. *Drug. Resist. Updat.* **2009**, *12*, 17.
7. Kamal, A.; Boehm, M. F.; Burrows, F. J. *Trends Mol. Med.* **2004**, *10*, 283.
8. Whitesell, L.; Lindquist, S. L. *Nat. Rev. Cancer* **2005**, *5*, 761.
9. Ferrarini, M.; Heltai, S.; Zocchi, M. R.; Rugarli, C. *Int. J. Cancer* **1992**, *51*, 613.
10. Whitesell, L.; Mimnaugh, E. G.; De Costa, B.; Myers, C. E.; Neckers, L. M. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 8324.
11. Zhang, T.; Hamza, A.; Cao, X.; Wang, B.; Yu, S.; Zhan, C. G.; Sun, D. *Mol. Cancer Ther.* **2008**, *7*, 162.
12. Marcu, M. G.; Chadli, A.; Bouhouche, I.; Catelli, M.; Neckers, L. M. *J. Biol. Chem.* **2000**, *275*, 37181.
13. Seo, Y. H. *Bull. Korean Chem. Soc.* **2013**, *34*, 1917.
14. Roux, D. G.; Paulus, E. *Biochem. J.* **1961**, *80*, 62.
15. Wang, Y.; Chan, F. L.; Chen, S.; Leung, L. K. *Life Sci.* **2005**, *77*, 39.
16. Yit, C. C.; Das, N. P. *Cancer Lett.* **1994**, *82*, 65.
17. Jang, H. S.; Kook, S. H.; Son, Y. O.; Kim, J. G.; Jeon, Y. M.; Jang, Y. S.; Choi, K. C.; Kim, J.; Han, S. K.; Lee, K. Y.; Park, B. K.; Cho, N. P.; Lee, J. C. *Biochim. Biophys. Acta* **2005**, *1726*, 309.
18. Lee, J. C.; Lee, K. Y.; Kim, J.; Na, C. S.; Jung, N. C.; Chung, G. H.; Jang, Y. S. *Food Chem. Toxicol.* **2004**, *42*, 1383.
19. Kim, N. Y.; Pae, H. O.; Oh, G. S.; Kang, T. H.; Kim, Y. C.; Rhew, H. Y.; Chung, H. T. *Pharmacol. Toxicol.* **2001**, *88*, 261.
20. Iwashita, K.; Kobori, M.; Yamaki, K.; Tsushida, T. *Biosci. Biotechnol. Biochem.* **2000**, *64*, 1813.
21. Pandey, M. K.; Sung, B.; Ahn, K. S.; Aggarwal, B. B. *Mol. Pharmacol.* **2009**, *75*, 525.
22. Roughley, S. D.; Hubbard, R. E. *J. Med. Chem.* **2011**, *54*, 3989.
23. Janin, Y. L. *Drug. Discov. Today* **2010**, *15*, 342.
24. Takahashi, T.; Miyazawa, M. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 1983.
25. Yamakawa, J. T. A. T. *Tetrahedron Lett.* **1979**, *20*, 613.
26. Sordella, R.; Bell, D. W.; Haber, D. A.; Settleman, J. *Science* **2004**, *305*, 1163.
27. Chiosis, G.; Vilenchik, M.; Kim, J.; Solit, D. *Drug. Discov. Today* **2004**, *9*, 881.