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Synthesis of Butein Analogues and their Anti-proliferative Activity Against Gefitinib-resistant Non-small Cell Lung Cancer (NSCLC) through Hsp90 Inhibition

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Non-small cell lung cancer (NSCLC) is the most common type of lung cancer representing 85% of lung cancer patients. Despite several EGFR-targeted drugs have been developed in the treatment of NSCLC, the clinical efficacy of these EGFR-targeted therapies is being challenged by the occurrence of drug resistance. In this regard, Hsp90 represents great promise as a therapeutic target of cancerous diseases due to its role in modulating and stabilizing numerous oncogenic proteins. Accordingly, inhibition of single Hsp90 protein simultaneously disables multiple signaling networks so as to overcome drug resistance in cancer. In this study, we synthesized a series of 11 butein analogues and evaluated their biological activities against gefitinib-resistant NSCLC cells (H1975). Our study indicated that analogue **1h** inhibited the proliferation of H1975 cells, down-regulated the expression of Hsp90 client proteins, including EGFR, Met, Her2, Akt and Cdk4, and up-regulated the expression of Hsp70. The result suggested that compound **1h** disrupted Hsp90 chaperoning function and could serve a potential lead compound to overcome the drug resistance in cancer chemotherapy.

Key Words : Hsp90, Butein analogues, Gefitinib resistance, Lung cancer

Introduction

Lung cancer is the leading cause of cancer death in the world and there are two major forms of lung cancer, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). The most common type of lung cancer is NSCLC and that accounts for 85% of lung cancer patients. Despite platinum-based standard chemotherapy improves survival, disease progression is inevitable.[1]

For this reason, EGFR-targeted drugs were introduced for the treatment of NSCLC. These include monoclonal antibody, cetuximab (trade name; Erbitux) and small molecule inhibitors, gefitinib (trade name; Iressa) and erlotinib (trade name; Tarceva), and EGFR-targeted drugs have made progress in the treatment of NSCLC. However, after a variable period of time, almost all tumors acquire resistance to EGFR-targeted drugs and tumor progression recurs. Several culprits of this drug resistance have been identified, including the mutation of EGFR T790M, and amplification and activation of Met tyrosine kinase receptor.[2,3]

To overcome drug resistance, the design of cancer chemotherapy has been increasingly sophisticated, yet there is no anti-cancer drug that is 100% effective against disseminated cancer. Therefore, there is a pressing need to discover a new therapeutic agent to defeat drug resistance in the war on cancer.

Heat shock protein 90 (Hsp90) is an ATP dependent molecular chaperone that regulates the maturation, activation, and stabilization of their substrate proteins, referred to as "client" proteins. Many of Hsp90 clients are oncogenic proteins and include transmembrane tyrosine kinases (EGFR, Her2, Met), metastable signaling proteins (Akt, Raf-1, IKK), and mutated signaling proteins (p53, v-Src).[4,5]

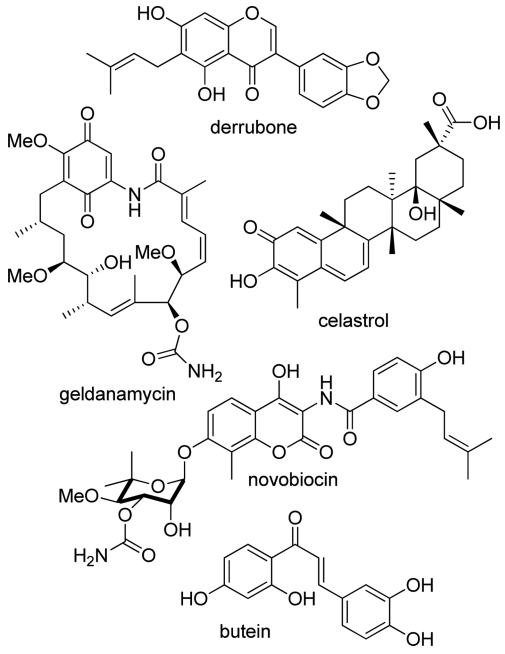


Figure 1. Structure of known natural products targeting Hsp90 and butein.

In this regard, Hsp90 has received significant attention and become an attractive cancer target. Inhibition of single Hsp90 protein leads to simultaneously blockage of multiple signaling pathways, providing a combinatorial attack on cellular oncogenic processes, thus reducing the possibility of molecular feedback loops and mutations leading to cancer resistance.[6]

Due to their genetic instability and stressful environment, cancer cells tend to more depend on Hsp90 chaperoning

function than normal cells, that represents 2-10 fold higher expression level of Hsp90 in cancer cells than normal cells. Accordingly, inhibitors targeting Hsp90 demonstrate selective anti-cancer effects toward cancer cells as compared to normal cells.[7,8]

Besides, to target Hsp90 could be best suited to battle against drug resistance from EGFR mutation and Met amplification in the treatment of NSCLC since both EGFR and Met are client proteins of Hsp90.

The discovery of new drugs from natural products has proved to be the single most successful strategy and over 47% of approved anti-cancer drugs are developed from natural origin.[9] Several natural products, including geldanamycin,[10] celastrol,[11] derrubone,[12] and novobiocin[13] have been reported to target Hsp90 folding machinery (Figure 1).

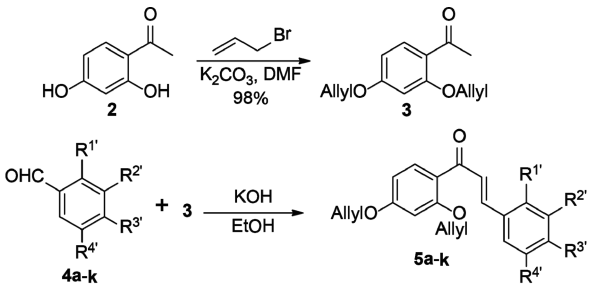
Butein (3,4,2',4'-tetrahydroxychalcone) is a polyphenolic compound extracted from numerous plants including the stem bark of cashews (*Semecarpus anacardium*), the heartwood of *Dalbergia odorifera*, and the traditional Chinese and Tibetan medicinal herbs *Caragana jubata* and *Rhus verniciflua* Stokes. Butein has been reported to exhibit anticancer activity against several human cancers including breast carcinoma,[14] colon carcinoma,[15] osteosarcoma,[16] lymphoma,[17] acute myelogenous leukemia,[18] and melanoma.[19,20]

Recently, we have found that butein disrupts Hsp90 chaperoning function and impaired the growth of cancer cells. The observation prompted us to direct our efforts toward synthesizing butein analogues and investigating their structure-activity relationships. Herein, we describe the synthesis of 11 butein analogues and their biological activities against gefitinib-resistant NSCLC cells (H1975).

Results and Discussion

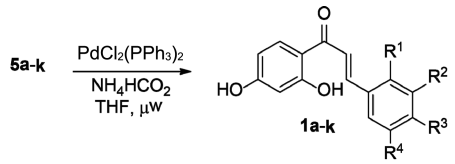
The synthesis of butein analogues (**1a-k**) began with the preparation of allyl-protected ketone **3** (Scheme 1). 2,4-Dihydroxyacetophenone (**2**) were protected with allyl bromide in the presence of potassium carbonate in DMF to furnish compound **3** in 98% yield. With compound **3** in hand, Claisen-Schmidt aldol condensation of compound **3** with aldehydes (**4a-k**) was carried out in the presence of potassium hydroxide in methanol. The condensation reaction successfully provided compound **5a-k** in 20-73% yield. Finally, allyl-protecting groups of compound **5a-k** were cleaved by microwave irradiation in the presence of $PdCl_2(PPh_3)_2$ and ammonium formate to provide butein analogues (**1a-k**) (Scheme 2).[21,22]

To investigate the biological effect of butein analogues (**1a-k**), we first measured % inhibitory activity of theses analogues against H1975 cell line, which is *in vitro* model of gefitinib-resistant non-small cell lung cancer (NSCLC). We incubated H1975 cells with 30 μM of butein analogues (**1a-k**) for 72 h, and measured cell viability using MTS colorimetric assay and calculated % inhibition of H1975 cell proliferation by each analogue (**1a-k**), relative to DMSO control (Scheme 2). The assay demonstrated that analogue **1f**, **1h**, and **1j** efficiently inhibited the growth of H1975 cells



entry	$R^{1'}$	$R^{2'}$	$R^{3'}$	$R^{4'}$	yield	product
1	H	OAllyl	OAllyl	H	44	**5a**
2	H	H	H	H	73	**5b**
3	H	H	OMe	H	60	**5c**
4	H	OMe	OMe	H	61	**5d**
5	H	OCH_2O		H	35	**5e**
6	H	OMe	OMe	OMe	64	**5f**
7	Cl	OMe	OMe	OMe	26	**5g**
8	Br	OMe	OMe	OMe	20	**5h**
9	CHCHCHCH		H	H	32	**5i**
10	H	CHCHCHCH		H	52	**5j**
11	CHCHCHCH		OMe	H	48	**5k**

Scheme 1. Claisen-Schmidt aldol condensation of acetophenone **3** with aldehyde **4a-k**.



entry	R^1	R^2	R^3	R^4	product	% inhibition[b]
1	H	OH	OH	H	**1a**[a]	12
2	H	H	H	H	**1b**	35
3	H	H	OMe	H	**1c**	26
4	H	OMe	OMe	H	**1d**	21
5	H	OCH_2O		H	**1e**	22
6	H	OMe	OMe	OMe	**1f**	69
7	Cl	OMe	OMe	OMe	**1g**	39
8	Br	OMe	OMe	OMe	**1h**	61
9	CHCHCHCH		H	H	**1i**	10
10	H	CHCHCHCH		H	**1j**	51
11	CHCHCHCH		OMe	H	**1k**	4

[a]butein. [b]The percent inhibition of H1975 cell proliferation at 30 μM.

Scheme 2. Removal of allyl protecting groups and their inhibition of H1975 cell proliferation.

and displayed 69, 61, and 51% inhibition, respectively.

To precisely determine time and concentration-dependent antiproliferative effect of butein analogues (**1a-k**), we treated H1975 cells with various concentrations (0, 30, 60 and 90 μM) of butein analogues (**1a-k**) for 0, 1, 2 and 3 days, and measured cell viability using MTS assay. In general, all butein analogues (**1b-k**) showed higher antiproliferative activity than butein (**1a**) on H1975 cells (Figure 2). Intriguingly, the

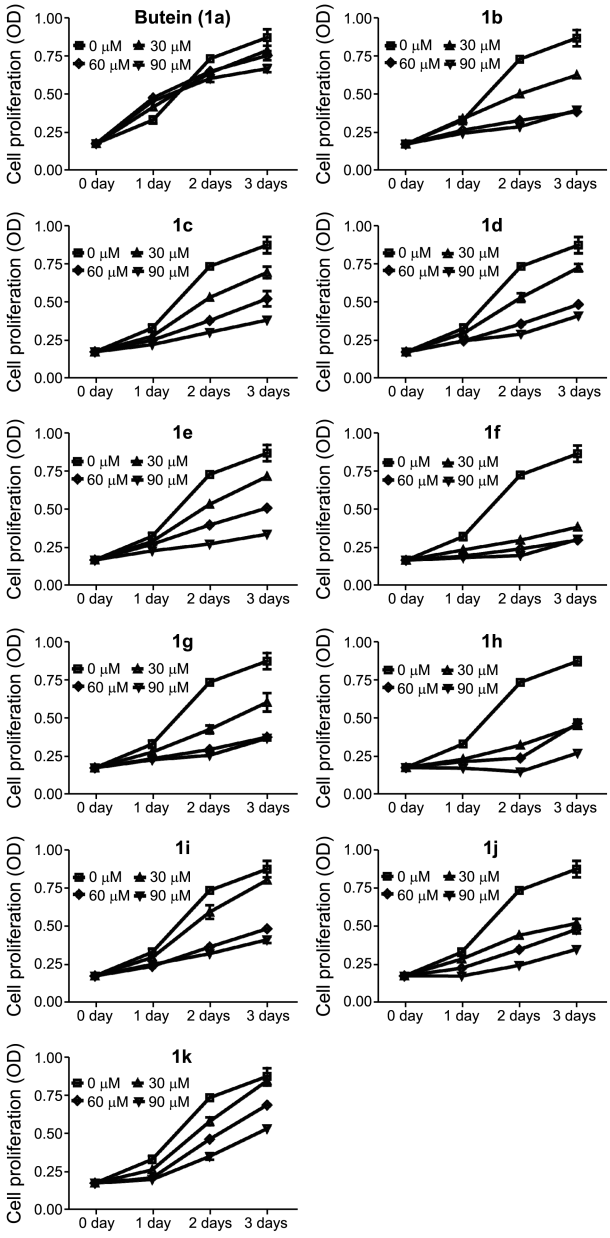


Figure 2. Effect of butein analogues **1a-k** on the cell proliferation of H1975. Cells were incubated with the indicated compound (0, 30, 60 and 90 μM) for 0, 1, 2, and 3 days, and cell viability was measured by MTS assay. Data are presented as mean ± SD (n = 4).

assay revealed that compound **1f** and **1h** more efficiently inhibited cell proliferation than other analogues in a time and concentration-dependent manner. Interestingly, compound **1f**, **1g** and **1h**, possessing 3,4,5-trimethoxy substituents and only differing in substituent at 2-position on the B-ring typically exhibited strong antiproliferative activity on H1975 cells. It is important to mention that compound **1j**, having 2-naphtalene moiety on the B-ring exhibited more potent antiproliferative activity than compound **1i** and **1k**, which contained 1-naphtalene and 4-methoxy-1-naphtalene moiety on the B-ring respectively, in that the structure of compound **1i** and **1k** were similar to compound **1j**.

To further investigate whether the observed cytotoxicity

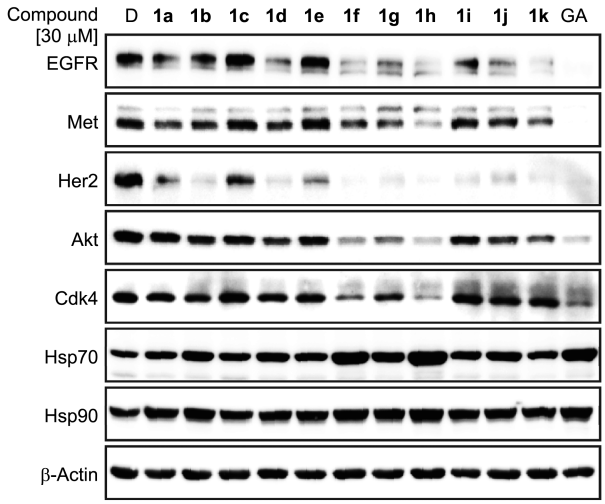


Figure 3. Effect of butein analogue **1a-k** on cellular biomarkers of Hsp90 inhibition. H1975 cells were incubated with the indicated compound (30 μM) for 24 h and the expression of Hsp90 client proteins (EGFR, Met, Her2, Akt, and Cdk4) along with Hsp70, Hsp90, and β-action were analyzed by western blot. Geldanamycin (GA, 1 μM) and DMSO (D) were employed as a positive and a negative control, respectively.

was related to Hsp90 inhibition, we next assessed the effect of butein analogues (**1a-k**) on cellular biomarkers of Hsp90 inhibition (Figure 3). H1975 cells were treated with 30 μM of each compound (**1a-k**) for 24 h and the expression level of EGFR, Met, Her2, Akt, Cdk4, Hsp70, Hsp90, and β-actin were analyzed by western blot, in that the proteasomal degradation of Hsp90 client proteins and the transcriptional induction of Hsp70 are considered as cellular biomarkers of Hsp90 inhibition. Consistent with the cell proliferation assay, western blot experiment revealed that compound **1f**, **1g**, and **1h** most efficiently degraded Hsp90 client proteins such as EGFR, Met, Her2, Akt, and Cdk4, and caused a significant induction of Hsp70 cochaperone, suggesting that compound **1f**, **1g**, and **1h** disrupted the Hsp90 protein folding machinery. It is noteworthy that most potent compound **1f**, **1g**, and **1h** structurally contained 3,4,5-trimethoxybenzene moiety on B-ring. Among these three compounds, **1h** showed the most dramatic effect on the downregulation of Hsp90 client proteins and induction of Hsp70, which has a correlation with its high antiproliferative activity.

The most potent compound **1h** was tested for its ability to donwregulate Hsp90 client proteins. We treated H1975 cells for 24 h with compound **1h** (0, 1, 5, 10, 20, and 30 μM) and geldanamycin (GA, 1 μM) as a positive control and measured the expression level of EGFR, Met, Her2, Akt, Cdk4, Hsp70, Hsp90 and β-actin. Western blot analysis demonstrated that compound **1h** induced a significant degradation of Hsp90 client proteins, such as EGFR, Met, Her2, Akt, and Cdk4 in a concentration-dependent manner. The expression level of Her2 most dramatically responded to the exposure of compound **1h** among Hsp90 client proteins. 10 μM concentration of **1h** efficiently downregulated the cellular level of Her2. In contrast, Hsp70 chaperone protein was up-

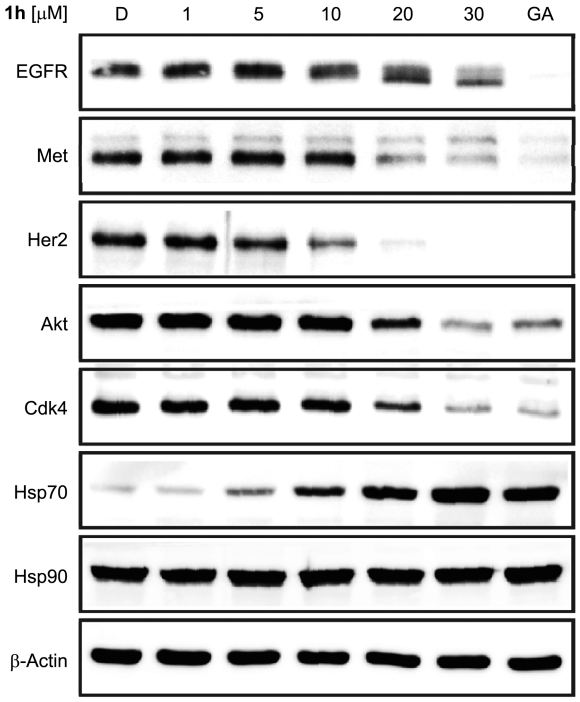


Figure 4. Concentration-dependent effect of compound **1h** on cellular biomarkers of Hsp90 inhibition. H1975 cells were treated for 24 h with the indicated concentration of compound **1h** and the expression of EGFR, Met, Her2, Akt, Cdk4, Hsp70, Hsp90 and β-actin were analyzed by western blot. Geldanamycin (GA, 1 μM) and DMSO (D) were employed as a positive and a negative control, respectively.

regulated when exposed to compound **1h**. As expected, β-actin remained unchanged since the internal standard, β-actin was not a Hsp90-dependent protein.

Conclusion

In summary, a series of 11 butein analogues were synthesized and their biological activities were evaluated against gefitinib-resistant non-small cell lung cancer cells (H1975). It was found that compound **1h** exhibited the most potent antiproliferative effect on H1975 cells and western blot analysis revealed that compound **1h** manifested significant degradation of Hsp90 client proteins such as EGFR, Met, Her2, Akt and Cdk4, and induction of Hsp70 cochaperone in a dose-dependent manner, indicating that compound **1h** disrupts Hsp90 chaperoning function. Currently our efforts are directed toward evaluating its complete mechanism of action, biological profile and safety. 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), q (quartet); 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%.

General Procedure for Preparing Compounds (5a-k and 1a-k), as Exemplified for Compound 5h and 1h. A mixture of compound **3** (0.30 g, 1.29 mmol), 2-bromo-3,4,5-trimethoxybenzaldehyde (0.37 g, 1.42 mmol), KOH (0.5 g) in 10 mL of methanol was stirred at 50 °C for 24 h. The mixture was neutralized with 6 N HCl to pH 6 and then extracted with ethyl aceatate. The organic layer was washed with saturated $NaHCO_3$ solution three times, dried over Na_2SO_4, and concentrated under reduced pressure, and purified by MPLC (Biotage SNAP HP-Sil column) to afford compound **5h** in 20% yield. R_f = 0.11 (1:9 ethyl acetate: hexane). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01 (d, J = 15.6 Hz, 1H), 7.74 (d, J = 8.8 Hz, 1H), 7.43 (d, J = 15.6 Hz, 1H), 7.01 (s, 1H), 6.55 (dd, J = 8.6 Hz, 2.0 Hz, 1H), 6.49 (d, J = 2.0 Hz, 1H), 6.07-5.99 (m, 2H), 5.45-5.38 (m, 2H), 5.31-5.21 (m, 2H), 4.60-4.54 (m, 4H), 3.91 (s, 3H), 3.87 (s, 3H), 3.86 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 190.28, 163.60, 159.60, 153.02, 151.45, 144.98, 140.68, 133.36, 132.88, 132.86, 132.77, 131.08, 129.32, 122.62, 118.54, 118.17, 113.83, 106.77, 106.34, 100.61, 69.68, 69.34, 61.54, 61.28, 56.45. ESI MS (*m/e*) 489 $[M+1]^+$. The resulting compound **5h** was stirred under microwave irradiation (Biotage Initiator) for 30 min at 120 °C in the presence of $PdCl_2(PPh_3)_2$ (13 mg) and ammonium formate (80 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 MPLC to afford compound **1h** in 48% yield. R_f = 0.10 (1:4 ethyl acetate: hexane). ^{1}H NMR (400 MHz, CD_3OD) δ 8.13 (d, J = 15.6 Hz, 1H), 7.97 (d, J = 9.2 Hz, 1H), 7.63 (d, J = 15.6 Hz, 1H), 7.29 (s, 1H), 6.38 (dd, J = 8.8 Hz, 2.0 Hz, 1H), 6.27 (d, J = 2.4 Hz, 1H), 3.94 (s, 3H), 3.88 (s, 3H), 3.84 (s, 3H). ^{13}C NMR (100 MHz, CD_3OD) δ 193.04, 167.77, 166.86, 154.59, 152.43, 146.55, 143.28, 133.93, 131.58, 123.92, 114.77, 114.52, 109.48, 108.16, 103.96, 61.77, 61.62, 57.05. ESI MS (*m/e*) 409 $[M+1]^+$.

Materials. Antibodies specific for EGFR, Her2, Met, Akt, Cdk4, 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).

MTS Assay. 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, the indicated concentration of compound or 1 % DMSO vehicle control was added to the wells. Cells were then incubated at 37 °C for 1, 2, and 3 days. 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. Compound (**1a-k**) was added at the concentrations indicated in Figure 2 or 4, 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, Cdk4, 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).

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Supporting Information. General and analytical data of compounds (**5b-k** and **1b-k**).

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