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Synthesis of Flavokawain Analogues and their Anti-neoplastic Effects on Drug-resistant Cancer Cells Through Hsp90 Inhibition

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Hsp90 is an ubiquitous molecular chaperone protein, which plays an important role in regulating maturation and stabilization of many oncogenic proteins. Due to its potential to simultaneously disable multiple signaling pathways, Hsp90 represents great promise as a therapeutic target of cancer. In this study, we synthesized flavokawain analogues and evaluated their biological activities against drug-resistant cancer cells. The study indicated that compound **1i** impaired the growth of gefitinib-resistant non-small cell lung cancer (H1975), down-regulated the expression of Hsp90 client proteins including EGFR, Her2, Met, Akt and Cdk4, and up-regulated the expression of Hsp70. The result strongly suggested that compound **1i** inhibited the proliferation of cancer cells through Hsp90 inhibition. Overall, compound **1i** could serve as a potential lead compound to overcome the drug resistance in cancer chemotherapy.

Key Words : Flavokawain analogues, Hsp90, Gefitinib resistance, Natural product, Cancer

Introduction

Lung cancer is the leading cause of cancer deaths in both men and women worldwide. Two major forms of lung cancer are small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC is the most common type of lung cancer and that accounts for 85% of lung cancer patients. Long-term exposure to cigarette smoke is a major risk factor for lung cancer and that causes 80-90% of lung cancer cases.[1] Even with surgical resection at early diagnosis, approximately 50% of NSCLC patients face recurring cancers.[2] Moreover, 40-75% of NSCLC patients are unfortunately projected to die within 5 years even with surgery.[3] For this reason, several EGFR-targeted drugs, including small molecule inhibitor, gefitinib (trade name; Iressa) and monoclonal antibody, cetuximab (trade name; Erbitux) have been developed for the treatment of NSCLC and the progress has been made using these EGFR-targeted therapies. However, the clinical efficacies of these EGFR-targeted therapies are limited by the occurrence of drug resistance. Cancer cells circumvent EGFR blockage by mutation of EGFR T790M, and amplification and activation of Met receptor.[4,5]

Hsp90 is a molecular chaperone protein, and many of its clients are oncogenic proteins that play critical roles in cancer progression. These clients include EGFR, Her2, Met, Akt, Raf, HIF-1a and MMP2.[1,6,7] Due to the hostile environment in tumour such as hypoxia, low pH and poor nutrition, cancer cells tend to more depend on Hsp90 chaperoning function than normal cells and that explains 2-10 fold higher expression level of Hsp90 in cancer cells than normal cells.[8-10] Over the past two decades, Hsp90 has received significant attention and has been extensively pursued as a drug target for cancer therapy.

Inhibition of single Hsp90 protein results in simultaneous disruption of multiple signaling pathways in tumor and induces client protein degradation through the ubiquitin-proteasome pathway, which potentially overcomes the inevitable drug resistance of conventional chemotherapies. In particular, inhibition of Hsp90 may be best suited to overcome gefitinib resistance in NSCLC, acquired by EGFR mutation and Met amplification, given that both EGFR and Met are client proteins of Hsp90. Besides, accumulating evidence in recent studies has demonstrated that Hsp90 is a potential therapeutic

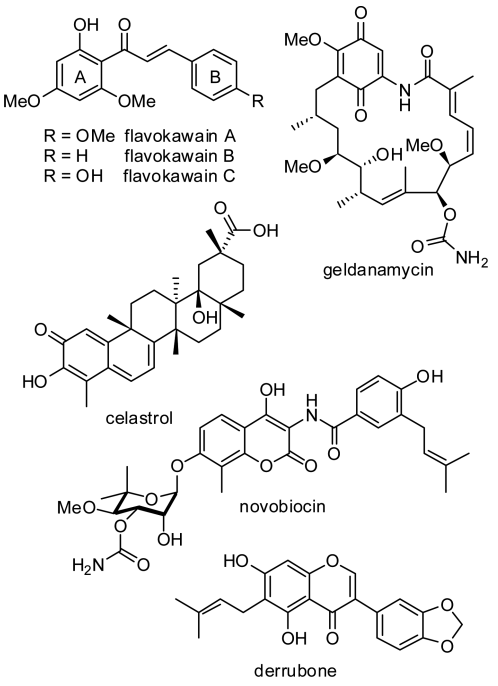


Figure 1. Structures of known natural products targeting Hsp90 and flavokawain A, B and C.

target for neurodegenerative diseases, including Alzheimer's, Parkinson's, Prion and Hodgkin's diseases.[11-13] The rationale has been that inhibition of Hsp90 activates heat shock factor-1 (HSF-1) to induce production of Hsp70 which in turn, promote disaggregation of neuronal toxic proteins, which are implicated in the development of neurodegenerative diseases. Therefore, the potential therapeutic benefits associated with Hsp90 modulation emphasize the importance of identifying novel Hsp90 inhibitors.

Historically, natural products have played an essential role in drug discovery, with over 47% of approved anticancer agents being of natural origin, and still provide ample sources of chemical diversity for the discovery of pharmaceutical agents.[14] Several natural products targeting Hsp90 are discovered for the treatment of cancers, which includes geldanamycin,[15] celastrol,[16] derrubone,[17] and novobiocin,[18] illustrated in Figure 1.

Kava (*Piper methylsticum*) is a native plant traditionally used as a medicine and social drink in the South Pacific islands. Intriguingly, epidemiological studies demonstrated that populations in the South Pacific islands that comsume the kava beverage, including smokers, have low incidences of cancer, compared to populations in the non-kava-drinking countries.[19,20] Extracts of kava are classified into two main classes of compounds, kavalactone and chalcone.[21,22] Chalcones from kava have shown anticancer activity against several cancer cell lines.[23-25] The chalcones includes flavokawain A, B, and C.

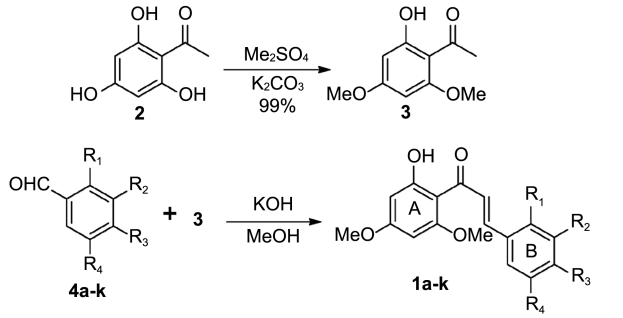
Recently, we have found that flavokawain B disrupts Hsp90's chaperoning function and inhibits the growth of cancer cells. That observation prompted us to direct our efforts toward synthesizing analogues of flavokawain B and evaluating their structure-activity relationships. Herein, we describe the synthesis of flavokawain analogues and their biological activities against gefitinib-resistant non-small cell lung cancer cells (H1975).

Results and Discussion

Flavokawain A, B, and C shared the structural common feature, in that they have 2-hydroxy-4,6-dimethoxybenzene moiety on the A-ring (Figure 1). Therefore, we intended to synthesize 2'-hydroxy-4',6'-dimethoxychalcone analogues with different substituents on the B-ring. To explore the structural modifications of the B-ring geared at improving pharmaceutical potency, methoxy (OMe), hydroxyl (OH), and halide (Cl and Br) groups were chosen as substituents on the B-ring of the chalcone scaffold and 1-naphtalene, 2-naphtalene, and 4-methoxy-1-naphtalene moiety were also selected as a replacement of the B-ring (Scheme 1).

The synthesis of flavokawain analogues (**1a-k**) began with the preparation of 2-hydroxy-4,6-dimethoxyacetophenone (**3**). Compound **3** was quantitatively obtained from the reaction of 2,4,6-trihydroxyacetophenone (**2**) with dimethyl sulfate in the presence of potassium carbonate in acetone.

The corresponding acetophenone **3** was then subjected to the Claisen-Schmidt condensation reaction with aldehydes



Entry	R_1	R_2	R_3	R_4	Yield	Product	% inhibition[d]
1	H	H	H	H	50	**1a**[a]	34
2	H	H	OH	H	35	**1b**[b]	5
3	H	H	OMe	H	58	**1c**[c]	11
4	H	OMe	OMe	H	21	**1d**	5
5	H	OCH_2O		H	69	**1e**	15
6	H	OMe	OMe	OMe	23	**1f**	17
7	Cl	OMe	OMe	OMe	20	**1g**	10
8	Br	OMe	OMe	OMe	15	**1h**	13
9	CHCHCHCH		H	H	32	**1i**	36
10	H	CHCHCHCH		H	70	**1j**	30
11	CHCHCHCH		OMe	H	18	**1k**	22

[a]flavokawain B. [b]flavokawain C. [c]flavokawain A. [d]The percent inhibition of H1975 cell proliferation at 30 μM

Scheme 1. Synthesis of flavokawain A, B, C, and its analogues and their inhibition of H1975 cell proliferation.

(**4a-k**) in the presence of potassium hydroxide in methanol, to provide flavokawain analogures (**1a-k**) in 15-70% yield.[26]

To investigate the biological effects of the synthesized compounds (**1a-k**), we first measured anti-proliferative activities of compounds (**1a-k**) against H1975, which is a gefitinib-resistant non-small cell lung cancer cell line. H1975 cells were treated with 30 μM of compound (**1a-k**) for 72 hours, and the inhibitory activities of each compound (**1a-k**) against the growth of H1975 cells were measured using MTS colorimetric assay and expressed as % inhibition, relative to DMSO control (Scheme 1). The assay demonstrated that flavokawain B (**1a**), **1i** and **1j** more efficiently impaired the growth of H1975 cells than other compounds and displayed 34, 36, and 30% inhibition of the H1975 cell growth, respectively. Interestingly, compound **1a**, **1i**, and **1j** had no substituent on the B-ring, but had benzene, 1-naphtalene, and 2-naphtalene moiety as a replacement of the B-ring, respectively.

To determine whether the observed anti-proliferative effects of compounds (**1a-k**) were associated with Hsp90 inhibition, we incubated H1975 cells with compounds (**1a-k**) for 24 hours and analyzed the expression level of Hsp90 client proteins, such as EGFR, Met, Her2, Akt, and Cdk4 along with Hsp70, Hsp90 and β-actin. The molecular biomarker of Hsp90 inhibition includes the proteasomal degradation of Hsp90 client proteins and the transcriptional induction of Hsp70.

As shown in Figure 2, compound **1a** and **1i** demonstrated a robust degradation of EGFR, Met, Her2, Akt and Cdk4

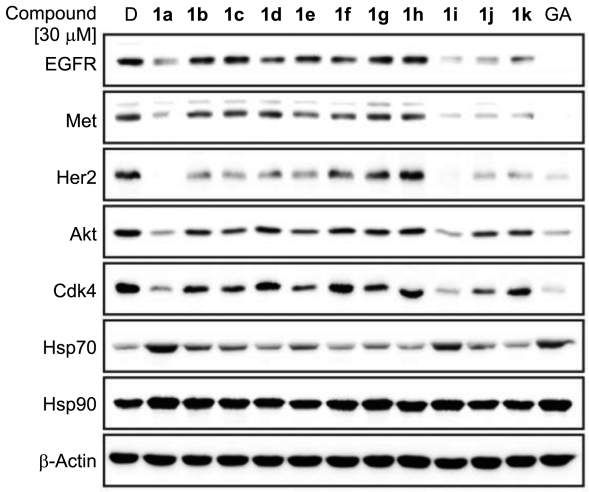


Figure 2. Effects of compounds (**1a-k**) on cellular biomarkers of Hsp90 inhibition. H1975 cells were treated for 24 h with the indicated compound (30 μM) and the expressions of Hsp90 client proteins were analyzed by western blot. Geldanamycin (GA, 1 μM) and DMSO (D) were employed as positive and negative controls, respectively.

and significant induction of Hsp70, suggesting that **1a** and **1i** disrupted Hsp90's chaperoning function. It is noteworthy that compound **1i**, **1j** and **1k** having a naphthalenyl group on the B-ring more efficiently down-regulated the expression levels of EGFR, Met, and Her2, which are members of receptor tyrosine kinase (RTK) proteins. However, **1j** and **1k** had only minor effects on down-regulating Akt and Cdk4, and up-regulating Hsp70.

To precisely determine the concentration and time-dependent efficacy of compound **1a** and **1i**, we treated H1975 cells with compound **1a** and **1i** (0, 10, 20, 30, 50, and 100 μM) for 1, 2, and 3 days and measured the cell proliferation using MTS assay (Figure 3). The assay revealed that compound **1a** and **1i** inhibited the cell proliferation of H1975 in a concentration and time-dependent manner.

Since western blot analysis and anti-proliferative assay demonstrated that compound **1i** exhibited a slightly better potency than **1a** (Figures 2 and 3), we decided to investigate a concentration-dependent effect of compound **1i** on Hsp90 inhibition. H1975 cells were incubated with compound **1i** (0, 5, 10, 20, 30, and 50 μM) for 24 h and the expression level of EGFR, Met, Her2, Akt, Cdk4, Hsp70, Hsp90, and β-actin was measured by western blot analysis (Figure 4). Her2 protein responded more sensitively to the increasing concentration of **1i** than other Hsp90 client proteins. The expression level of Her2 was almost completely depleted even with 20 μM concentration of **1i**. The expression levels of Met, Akt, and Cdk4 appeared to significantly decrease, when exposed to 30 μM of **1i**. In contrast, Hsp70 was dose-dependently induced upon the treatment of compound **1i**. The expression level of non-Hsp90-dependent protein, β-actin remained unchanged as expected. Collectively, the result demonstrated

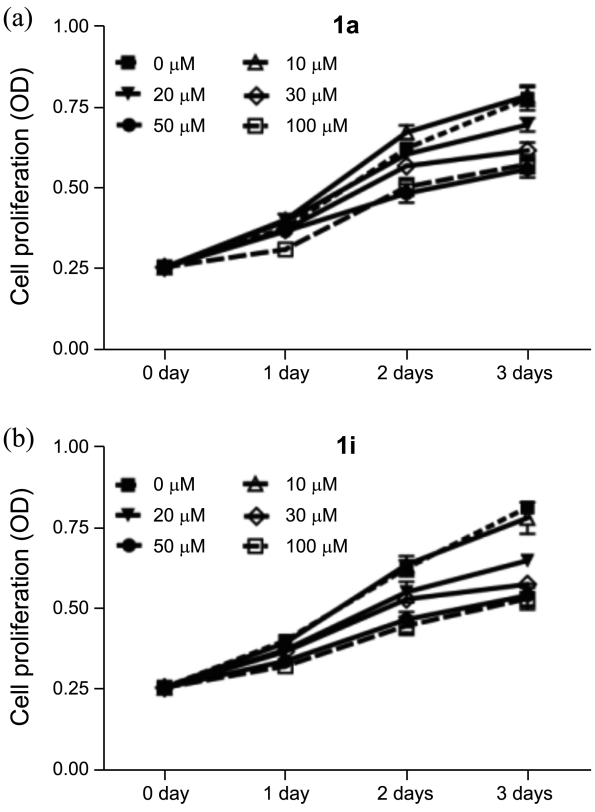


Figure 3. Anti-proliferative effects of (a) compound **1a** and (b) **1i** on H1975 cells. Cell proliferation was measured at 1, 2, and 3 days using MTS assay at the indicated concentrations of each compound. Data are presented as mean ± SD (n = 4).

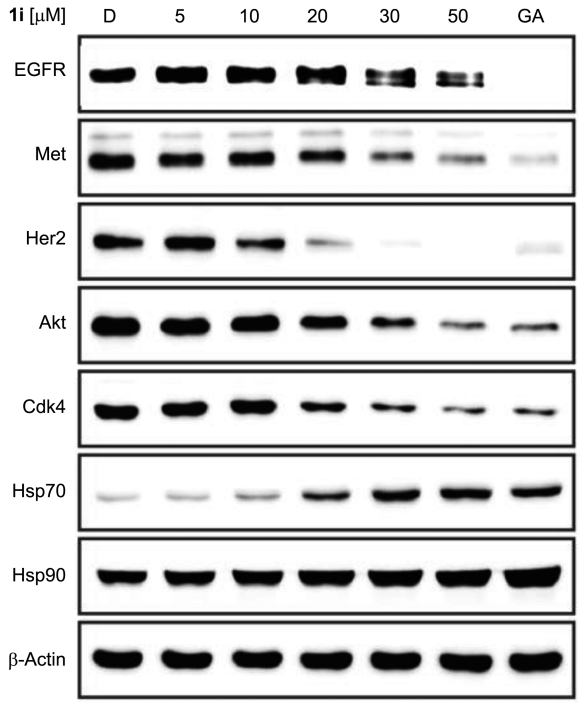


Figure 4. Compound **1i** induced the proteasomal degradation of Hsp90 client proteins (EGFR, Met, Her2, Akt, and Cdk4) and upregulated Hsp70. H1975 cells were treated with compound **1i** (0, 5, 10, 20, 30, and 50 μM) for 24 h and the expressions of Hsp90 client proteins were analyzed by western blot. Geldanamycin (GA, 1 μM) and DMSO (D) were employed as positive and negative controls, respectively.

that compound **1i** down-regulated Hsp90 client proteins, including EGFR, Met, Her2, Akt, and Cdk4 and up-regulated Hsp70 in a concentration-dependent manner, which is consistent with other Hsp90 inhibitors.[27]

Conclusion

In conclusion, a series of flavokawain analogues were synthesized and evaluated against gefitinib-resistant non-small cell lung cancer cells (H1975). Compound **1i** appears to be most potent to inhibit H1975 cell proliferation. Consequently, compound **1i** manifests significant degradation of Hsp90 client proteins, including Met, Her2, Akt, and Cdk4, and up-regulation of Hsp70 in a concentration-dependent manner. The combining evidence of biochemical and cellular studies indicate that compound **1i** disrupts the Hsp90 chaperoning function. Our finding provides a new molecular scaffold and strategy to circumvent the drug resistance in cancer chemotherapy. Currently 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 (1a-k), as Exemplified for Compound 1k. A mixture of compound **3** (0.2 g, 1.02 mmol), 4-methoxy-1-naphthaldehyde (0.19 g, 1.02 mmol), and potassium hydroxide (1.0 g) in 10 mL of methanol was stirred at 50 °C for 12 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 to afford compound **1k** in 18% yield as yellow solid. R_f = 0.23 (2:8 ethyl acetate: hexane). ^{1}H NMR (400 MHz, $CDCl_3$) δ 14.48 (s, 1H), 8.59 (d, *J* = 15.6 Hz, 1H), 8.33-8.28 (m, 2H), 7.92 (d, *J* = 15.2 Hz, 1H), 7.83 (d, *J* = 8.0 Hz, 1H), 7.63–7.58 (m, 1H), 7.55–7.51 (m, 1H), 6.86 (d, *J* = 8.0 Hz, 1H), 6.13 (d, *J* = 2.4 Hz, 1H), 5.96 (d, *J* = 2.4 Hz, 1H), 4.04 (s, 3H), 3.90 (s, 3H), 3.84 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.9, 168.7, 166.4, 162.8, 157.7, 139.9, 133.1, 127.9, 127.7, 126.6, 125.9, 125.9, 125.6, 123.7, 122.9, 106.7, 104.2, 94.1, 91.5, 56.2, 56.0, 55.9. ESI MS (m/e) = 365 $[M+1]^+$.

(*E*)-3-(2-Chloro-3,4,5-trimethoxyphenyl)-1-(2-hydroxy-4,6-dimethoxyphenyl)prop-2-en-1-one (1g): 20% yield. R_f = 0.24 (2:8 ethyl acetate: hexane). ^{1}H NMR (400 MHz, $CDCl_3$) δ 14.25 (d, *J* = 0.4 Hz, 1H), 8.08 (d, *J* = 15.6 Hz, 1H), 7.77 (d, *J* = 15.6 Hz, 1H), 6.98 (s, 1H), 6.08 (d, *J* = 2.4 Hz, 1H), 5.92 (d, *J* = 2.0 Hz, 1H), 3.93 (s, 3H), 3.91 (s, 3H), 3.90 (s, 3H), 3.87 (s, 3H), 3.81 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.4, 168.8, 166.4, 162.7, 152.3, 150.5, 145.2, 138.4, 129.6, 129.5, 122.7, 106.5, 106.2, 94.1, 91.6, 61.6, 61.4, 56.4, 56.1, 55.9. ESI MS (m/e) = 409 $[M+1]^+$.

(*E*)-3-(2-Bromo-3,4,5-trimethoxyphenyl)-1-(2-hydroxy-4,6-dimethoxyphenyl)prop-2-en-1-one (1h): 15% yield. R_f = 0.16 (2:8 ethyl acetate: hexane). ^{1}H NMR (400 MHz, $CDCl_3$) δ 14.24 (s, 1H), 8.08 (d, *J* = 15.2 Hz, 1H), 7.72 (d, *J* = 15.6 Hz, 1H), 7.01 (s, 1H), 6.09 (d, *J* = 2.4 Hz, 1H), 5.94 (d, *J* = 2.4 Hz, 1H), 3.93 (s, 3H), 3.92 (s, 3H), 3.90 (s, 3H), 3.88 (s, 3H), 3.82 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.4, 168.8, 166.7, 162.7, 153.0, 151.5, 145.0, 141.0, 131.4, 129.8, 113.7, 106.7, 106.6, 94.2, 91.6, 61.7, 61.3, 56.4, 56.1, 55.9. ESI MS (m/e) = 455 $[M+1]^+$.

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 g 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 (**1b-f**, **1i-j**)

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