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Discovery of hybrid Hsp90 inhibitors and their anti-neoplastic effects against gefitinib-resistant non-small cell lung cancer (NSCLC)

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ABSTRACT

Heat shock protein 90 (Hsp90) represents an attractive cancer therapeutic target due to its role in the stabilization and maturation of many oncogenic proteins. We have designed a series of hybrid Hsp90 inhibitors by connecting the resorcinol ring of VER-49009 (**2**) and the trimethoxyphenyl ring of PU3 (**3**) using structure-based approach. Subsequent testing established that compound **1f** inhibited gefitinib-resistant H1975 cell proliferation, brought about the degradation of Hsp90 client proteins including EGFR, Met, Her2 and Akt and induced the expression of Hsp70. The design, synthesis, and evaluation of **1f** are described herein.

Conventional drug design embraces the 'one gene, one drug, one disease' philosophy. Over the past two decades, several targeted cancer drugs, including Gleevec, Iressa and Herceptin have been discovered to eradicate tumors in more specific ways and reduce the harmful nonspecific side effects of chemotherapeutics. However, this notion is being challenged by the occurrence of drug resistance. The tumor cells outsmart single-targeted drugs to escape from their destiny by mutating targeted proteins, down-regulating death signals, or up-regulating survival pathways. In this regard, it is being recognized that single-target drugs can be problematic and multi-target drugs have emerged as a new paradigm to overcome the resistance in drug discovery.[1,2] Alternatively, to elucidate a single protein, so called 'nodal' protein that integrates multiple signaling pathways and discover an inhibitor against nodal proteins may be best suited to overcome the genetic and molecular heterogeneity of progressive disease through simultaneously interrupting multiple mechanisms of tumor maintenance.

Heat shock protein 90 (Hsp90) is a cancer nodal protein and has become an attractive therapeutic target in cancer research. Hsp90 is ATP dependent molecular chaperone that is responsible for the stabilization and maturation of their substrate proteins, referred to as 'client' proteins. Disruption of Hsp90 chaperone activity induces client proteins degradation via the ubiquitin–proteasome pathway, which can ultimately lead to cell death. Many Hsp90 client proteins, including Her2, Met, Cdk4, Akt, HIF-1α and MMP2 play significant roles in six essential hallmarks of a cancer cell.[3–5] More interestingly, Hsp90 is constitutively expressed at 2–10 fold higher levels in tumor cells compared to their normal counterparts and Hsp90 inhibitors demonstrate selective anti-proliferative effects toward cancer cells as compared to normal cells, due to the greater dependence of tumor cells on Hsp90's chaperoning function against oncogenic stressors in the hostile hypoxic, acidotic and nutrient-deprived microenvironment.[6,7]

The natural product geldanamycin was first identified as an Hsp90 inhibitor in 1994.[8] Since then, a number of natural products and synthetic small molecules that target Hsp90 have been discovered for the treatment of cancerous diseases, which include radicicol[9], VER-49009,[10] and PU3[11] (Fig. 1). Despite of these advances, none of Hsp90 inhibitors are clinically approved as an anti-cancer chemotherapy until now, and there still remains a need for the discovery of a novel class of small molecule inhibitors against Hsp90. Here, we report the design, synthesis, and anti-cancer effects of a new class of Hsp90 inhibitors.

Structural analysis of Hsp90 revealed that ATP-binding pocket of Hsp90 consisted of a hydrophilic region and a hydrophobic region (Fig. 2). Co-crystal structure of VER-49009[10] (**2**) bound to the N-terminal ATP-binding region of Hsp90 demonstrated that the resorcinol ring of **2** positioned in the hydrophilic region of the pocket. The hydrophilic region of the pocket consisted of Asp93 and Asp54 residues, which typically interact with the adenine ring. The crystal structure of PU3[11] (**3**) indicated that the 3,4,5-trimethoxyphenyl ring of PU3 (**3**) was located in the opposite orientation of the resorcinol ring of VER-49009 (**2**). The π-rich

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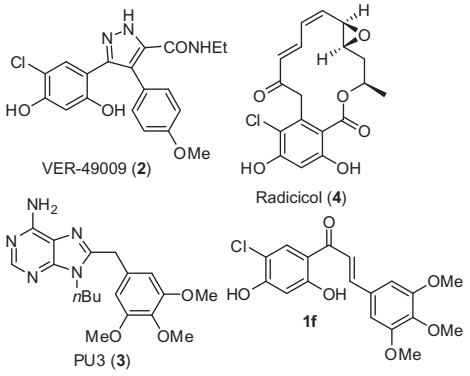


Figure 1. Structures of known Hsp90 inhibitors and **1f**.

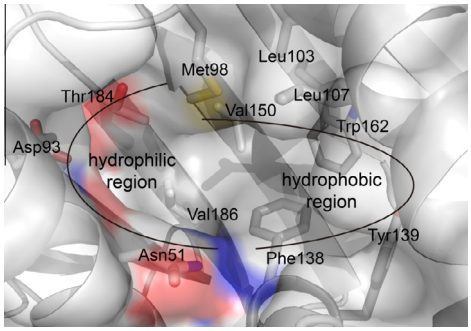
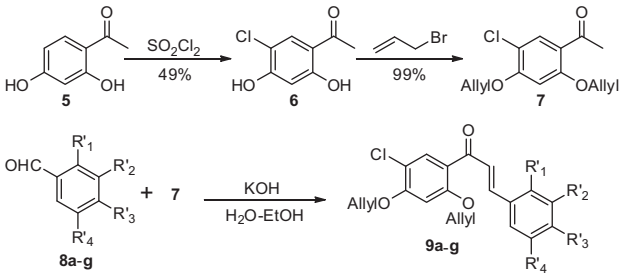


Figure 2. X-ray crystal structure of apo-Hsp90 (PDB code: 1UYM).

amino acids of Phe138, Trp162, and Tyr139 formed the hydrophobic region of the pocket and the trimethoxyphenyl ring of PU3 (**3**) had π–π interaction with those residues. With the aim of maximizing interactions in the ATP binding pocket of Hsp90, we intended to design a molecule by hybridizing the resorcinol ring of VER-49009 (**2**) and the 3,4,5-trimethoxyphenyl ring of PU3 (**3**). To connect the 3,4,5-trimethoxyphenyl ring to the resorcinol ring, we decided to use a chalcone scaffold as a core template. Chalcones are abundant natural products in edible plants such as green tea and exhibit a wide spectrum of biological activities including anti-tumor activities.[12,13] Accordingly, chalcones are an important class of molecules and speculated as promising candidates as anticancer agents. Besides, our recent study has also demonstrated that a natural product, licochalcone A disrupts Hsp90 chaperoning function.[14] With a hybrid inhibitor embedded in the chalcone scaffold, we envision that the resorcinol ring of the inhibitor would make a hydrogen bond with Asp93 involved in ATP binding pocket and the hydrophobic trimethoxyphenyl group of the inhibitor would project into the π-rich lipophilic cavity of the pocket.[15,16]

The synthesis of compounds (**1a**–**g**) began with the preparation of 6-chloro-2,4-dihydroxyacetophenone (**6**) (Scheme 1). Treatment of 2,4-dihydroxyacetophenone (**5**) with sulfuryl chloride provided a chlorinated product **6** as well as its undesired regio-isomer of 3-chloro-2,4-dihydroxyacetophenone in 1:1 molar ratio. After carefully being resolved by silica gel chromatography, the protection reaction of 2,4-dihydroxyl groups of compound (**6**) was carried out. Initial studies to protect 2,4-dihydroxyl groups of **6** with TBSCl or MOMCl using combinations of various bases (TEA, K_2CO_3, and DBU) and solvents (CH_2Cl_2 and DMF) were not fruitful but only to produce a single protected adduct. Consequently, compound **6** was protected with allyl bromide in the presence of K_2CO_3 to furnish the allyl-protected ketone **7** in 99% yield. With ketone **7** in

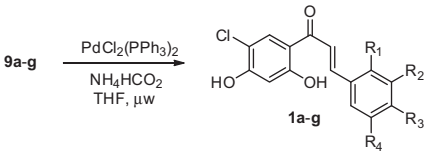


entry	R'_1	R'_2	R'_3	R'_4	aldehyde
1	H	H	OMe	H	**9a**
2	H	H	OAllyl	H	**9b**
3	H	OAllyl	OAllyl	H	**9c**
4	OAllyl	H	OAllyl	H	**9d**
5	OMe	H	OMe	H	**9e**
6	H	OMe	OMe	OMe	**9f**
7	H	OCH_2O		H	**9g**

Scheme 1. Claisen–Schmidt aldol condensation of acetophenone **7** with aldehyde **8a**–**g**.

hand, Claisen–Schmidt aldol condensation of ketone **7** with the corresponding aromatic aldehydes **8a**–**g** was carried out in the presence of KOH in MeOH–H_2O. The condensation reaction successfully provided enones **9a**–**g** in 42–80% yield. Finally, removal of allyl-protecting groups, using $PdCl_2(PPh_3)_2$ and ammonium formate under microwave irradiation produced the resulting 3-chloro-2,4-dihydroxychalcones (**1a**–**g**) (Scheme 2).[20]

To investigate the effects of newly synthesized compounds (**1a**–**g**) for Hsp90 inhibition, we first screened the efficacy of these compounds by measuring anti-proliferative effects against H1975 cell line. H1975 is a gefitinib-resistant non-small cell lung cancer cell line and its resistance is mediated by 'gatekeeper' the mutation T790M-EGFR in combination with L858R,[15–17] The resistance is also related to Met amplification, compensating for the loss of EGFR signals.[18] To test anti-proliferative activities of compounds (**1a**–**g**) against gefitinib-resistant H1975, we treated H1975 cells with various concentrations of compounds (**1a**–**g**) and measured



entry	R_1	R_2	R_3	R_4	product
1	H	H	OMe	H	**1a**
2	H	H	OH	H	**1b**
3	H	OH	OH	H	**1c**
4	OH	H	OH	H	**1d**
5	OMe	H	OMe	H	**1e**
6	H	OMe	OMe	OMe	**1f**
7	H	OCH_2O		H	**1g**

Scheme 2. Removal of allyl protecting groups.

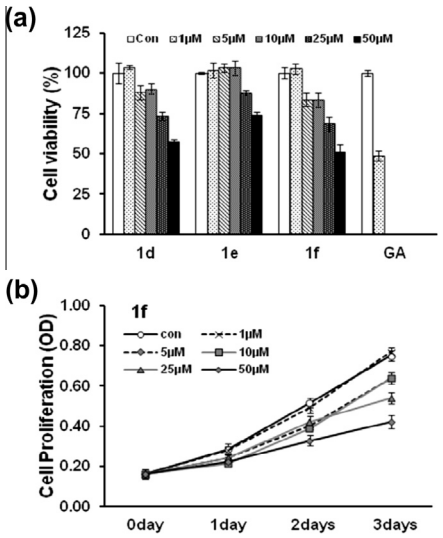


Figure 3. (a) Comparative effects of **1d**, **1e**, and **1f** on cell viability of H1975. Cells were treated for 3 days at the indicated concentrations of each compound. Cell viability was measured by MTS assay. (b) Anti-proliferative effect of **1f** on H1975 cells. Cell proliferation was determined at 1, 2, and 3 days using MTS assay at the indicated concentrations of each compound. Data are presented as mean ± SD ($n = 4$).

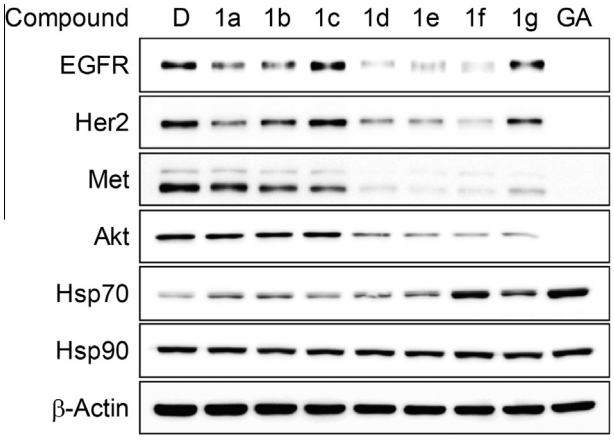


Figure 4. Effects of compounds **1a**–**g** on cellular biomarkers of Hsp90 inhibition. H1975 cells were treated for 24 h with the indicated compound (40 μM) and the expression of the Hsp90's client proteins was analyzed by Western blot. Geldanamycin (GA, 1 μM) and DMSO (D) were employed, respectively, as positive and negative controls.

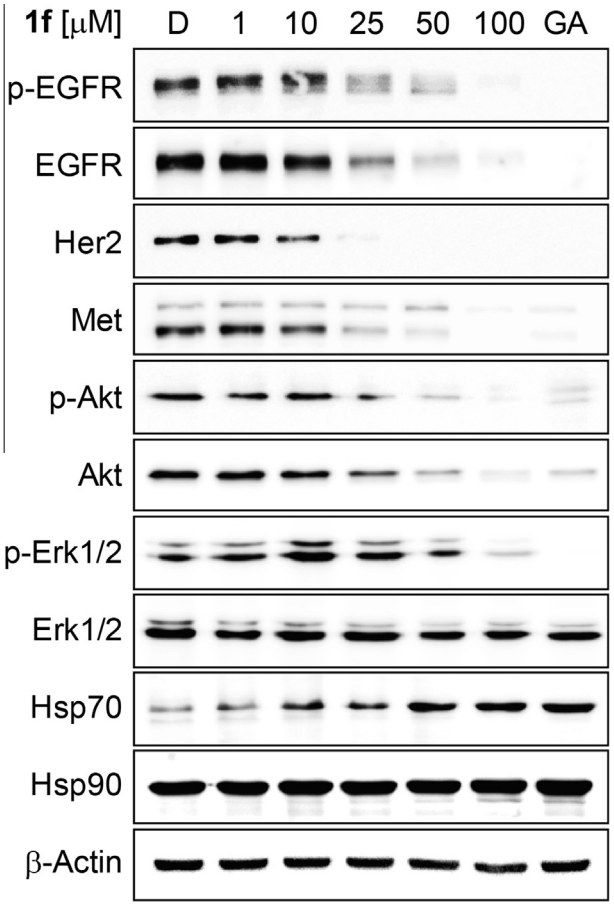


Figure 5. Dose-dependent effect of compound **1f** on cellular biomarkers of Hsp90 inhibition and their downstream signaling. H1975 Cells were treated for 24 h with the indicated concentration of **1f** and the expressions of cellular biomarker proteins of Hsp90 inhibition and their downstream signaling were analyzed by Western blot. Geldanamycin (GA, 1 μM) and DMSO (D) were employed, respectively, as positive and negative controls.

the viability using MTS assay. The result revealed that compounds **1d**, **1e** and **1f** effectively inhibited cell proliferation of H1975 by dose-dependent manners (Fig. 3), whereas others did not (data not shown).

To further determine whether the observed viability was related to Hsp90 inhibition, compounds (**1a**–**g**) were incubated with H1975 cells and screened by analyzing the expression level of Hsp90's clients, EGFR, Her2, Met and Akt along with Hsp70 (Fig. 4). The molecular hallmark of Hsp90 inhibition includes the proteosomal degradation of Hsp90 client proteins and the transcriptional upregulation of Hsp70.[11] As expected, compound **1f** among others revealed a robust degradation of EGFR, Her2, Met and Akt and up-regulated cochaperone Hsp70, suggesting that compound **1f** targets the Hsp90 protein folding machinery.

To precisely determine the degradation of Hsp90's client proteins and their downstream signaling by **1f**, H1975 cells were treated with increasing concentrations of compound **1f** for 24 h, and then the expression levels of Hsp90's client proteins and the downstream signaling proteins were assessed. As shown in Figure 5, compound **1f** resulted in dose-dependent downregulation of Hsp90's client proteins including EGFR, Her2, Met and Akt and up-regulation of Hsp70. In contract, the expression level of Erk1/2 was not depleted, in that Erk1/2 is not a client protein of Hsp90. Moreover, the phosphorylation status of EGFR was reduced according to the increasing concentrations of compound **1f**. Consistently, downstream signaling pathway including the phosphorylation of Akt and Erk1/2 was effectively abolished. Collectively, these results indicate that compound **1f** circumvents gefitinib-resistance by the blockade of EGFR and Met signaling through Hsp90 inhibition, in that gefitinib-resistance in NSCLC is associated with the activation of signaling pathways by the mutation of EGFR T790M and the amplification and activation of Met tyrosine kinase receptor.[15–18]

To investigate the binding pose of **1f** in the Hsp90 active site, molecular docking studies were performed using the human-Hsp90 crystal structure (PDB code: 1UYM). Hybrid inhibitor **1f** was docked with the 3D coordinates of the Hsp90's N-terminal domain using Autodock 4.2 (Molecular Graphics Laboratory). In silico modeling demonstrated that that hybrid inhibitor **1f** bound N-terminal ATP binding pocket of Hsp90 as expected (Fig. 6). The resorcinol ring of **1f** binds to the hydrophilic region of the pocket

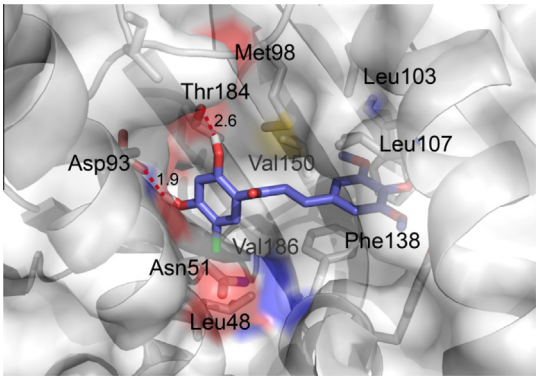


Figure 6. Molecular docking model of **1f** with Hsp90 (PDB code: 1UYM). The carbon, oxygen, hydrogen atoms of **1f** are illustrated in blue, red, and gray, respectively. The side chains of binding site are colored by atom types (carbon, gray; nitrogen, blue; oxygen, red) and labeled with their residue name. Hydrogen bonds are shown in dashed red lines.

and the 3,4,5-trimethoxyphenyl ring of **1f** binds to π-rich hydrophobic region of the pocket. The hydroxyl groups at C2 and C4 of the resorcinol ring formed hydrogen-bonding with Thr184 and Asp93, respectively in the hydrophilic region of the pocket, while the chlorine atom of the resorcinol ring formed Van der Waals contacts with Leu48 and Val186. On the other hand, the phenyl group of the 3,4,5-trimethoxyphenyl ring made π–π interaction with Phe138 and formed Van der Waals contact with Leu107 in the hydrophobic region of the pocket. Collectively, the estimated binding energy (ΔG_b) and inhibition constants (K_i) using the Lamarckian genetic algorithm result in 7.91 kcal/mol and 1.59 μM, respectively.

In conclusion, a series of hybrid Hsp90 inhibitors were rationally designed, synthesized, and evaluated against gefitinib-resistant non-small cell lung cancer cell line (H1975). Compound **1f** appears to overcome gefitinib resistance and inhibit H1975 cell proliferation. The hybrid inhibitor **1f** exerts a modest inhibitory activity (48 μM of GI_{50}) against the growth of gefitinib-resistant cancer cells, considering that known Hsp90 inhibitors, VER-49009 and PU3 are reported to have 1–5 μM and 50 μM of GI_{50} in human cancer cell lines, respectively.[11,19] Consequently, **1f** manifests considerable degradation of Hsp90's client proteins and induction of Hsp70, supporting that **1f** targets the Hps90 chaperone machinery. Currently, our efforts are directed toward exploring further structure–activity relationships and investigating the complete mechanism, biological profile and safety of the hybrid inhibitor. The result will be reported in due course.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.11.034.

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20. General procedure for preparing compounds (**9a–g** and **1a–g**), as exemplified for compound **9f** and **1f**.
*Procedure for the synthesis of compound **9f***: A mixture of compound 7 (0.1 g, 0.37 mmol), 3,4,5-trimethoxybenzaldehyde (0.16 g, 0.93 mmol), KOH (4.0 g) in 2 mL of water and 10 mL of ethanol was stirred at rt for 12 h. The mixture was neutralized with 6 N HCl to pH 6 and then extracted with ethyl acetate. 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 column chromatography (20% ethyl acetate in hexane) to afford compound **9f** (0.21 g, 63.7%) as yellow solid. ^1H NMR (400 MHz, $CDCl_3$) δ 7.77 (s, 1H), 7.55 (d, J = 15.6 Hz, 1H), 7.44 (d, J = 15.6 Hz, 1H), 6.76 (s, 2H), 6.46 (s, 1H), 6.08–5.59 (m, 2H), 5.49–5.38 (m, 2H), 5.32–5.21 (m, 2H), 4.60 (dd, J = 5.2 Hz, J = 18.0 Hz, 4H), 3.83 (s, 9H). ESI MS (m/e) = 445 $[M+1]^+$.
*Procedure for the synthesis of compound **1f***: The resulting compound **9f** 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 (20% ethyl acetate in hexane) to afford compound **1f** (0.12 g, 69.7%) as yellow solid. ^1H NMR (400 MHz, $CDCl_3$) δ 13.20 (s, 1H), 7.09 (s, 1H), 7.83 (d, J = 15.2 Hz, 1H), 7.35 (d, J = 15.6 Hz, 1H), 6.88 (s, 2H), 6.65 (s, 1H), 6.20 (s, 1H), 3.95 (s, 6H), 3.91 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.1, 164.1, 160.0, 153.4, 145.2, 140.5, 130.9, 130.1, 119.1, 113.9, 112.3, 106.0, 104.1, 60.9, 56.2. ESI MS (m/e) = 365 $[M+1]^+$.