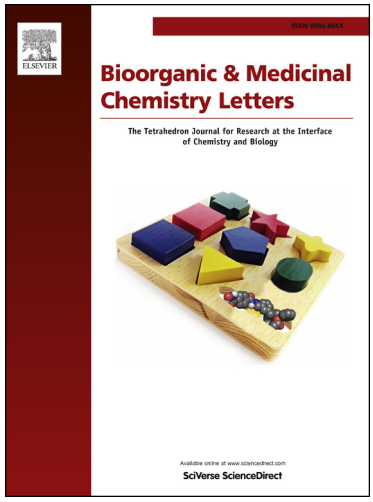

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Taeho Lee[b] and Young Ho Seo[a]*

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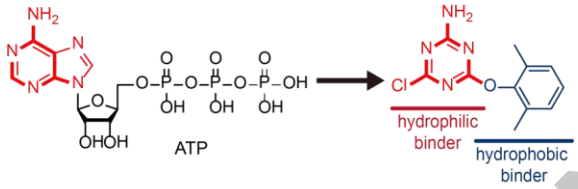

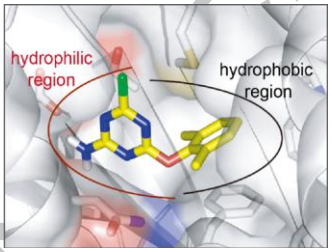

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Targeting the hydrophobic region of Hsp90's ATP binding pocket with novel 1,3,5-triazines

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ABSTRACT

Heat shock protein 90 (Hsp90) is a molecular chaperone that plays an important role in regulating the maturation and stabilization of many oncogenic proteins. In an attempt to discover a new class of Hsp90 inhibitors, a series of 1,3,5-triazine compounds were rationally designed, synthesized, and their biological activities were evaluated. Compound **3b** was found to degrade Hsp90's client proteins of Her2, Met and Akt and to induce the expression level of Hsp70. The binding mode of **3b** in the ATP-binding site of Hsp90 was predicted by the molecular docking.

Heat shock protein 90 (Hsp90) is an ATP-dependent molecular chaperone that plays an important role in regulating many proteins involved in signaling pathway and cell proliferation.[1,2] Once identified in 1987 as one of the most abundant intracellular proteins,[3] Hsp90 has received considerable attention and emerged as an attractive cancer therapeutic target due to its chaperoning function of the substrate proteins. The substrate proteins, referred to as client proteins include many oncology targets such as mutant p53, Raf1, Bcl-Abl, EGFR, Her2, Met, Cdk4, Akt, HIF and MMP2.[4,5] In this regard, the inhibition of Hsp90's chaperoning function can induce simultaneous blockage of several signaling pathways in tumor cells so as to overcome the inevitable drug resistance of conventional cancer therapeutic agents. More interestingly, Hsp90 accounts for 4-6% of total proteins in tumor cells as compared to 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.[5,6] Besides, accumulating evidence in recent studies suggests that Hsp90 is a potential therapeutic target for neurodegenerative diseases, including Alzheimer's, Parkinson's, Prion and Hodgkin's diseases.[7-9] Therefore, the potential therapeutic benefits associated with Hsp90 modulation emphasize the importance of identifying novel Hsp90 inhibitors.

The natural product geldanamycin was first identified as an Hsp90 inhibitor in 1994.[10] Since then, several geldanamycin-based inhibitors, including 17-AAG and 17-DMAG have been developed and represented a major advance in Hsp90-targeted therapy. However, geldanamycin-based inhibitors engendered a number of drawbacks in the clinical applications, such as poor solubility and toxic side effects.

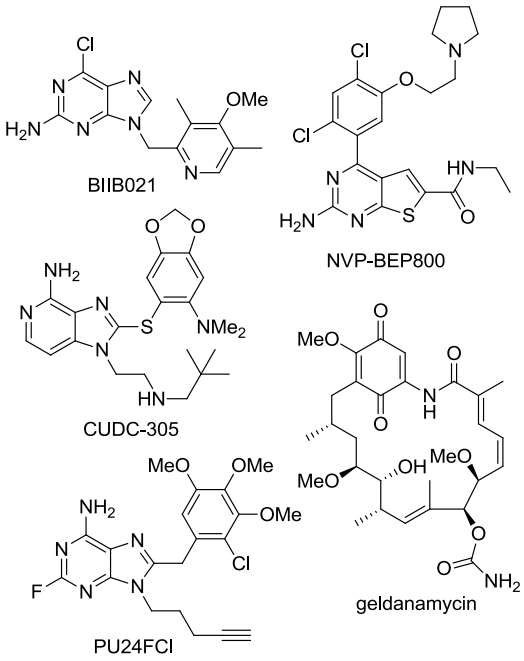


Figure 1. Structures of known Hsp90 inhibitors.

In particular, the presence of the reactive benzoquinone moiety in geldanamycin-based molecules brought about hepatotoxicity, which encouraged medicinal chemists to develop non-quinone containing small molecule inhibitors. To this end, the first synthetic purine-based inhibitor, PU24FCl was developed to mimic endogenous ligand ATP binding to Hsp90, by Chiosis et al.[11,12] and demonstrated antitumor efficacy *in vitro* and *in vivo*. Since then, significant progress has been made in identifying purine-based Hsp90 inhibitors and several purine compounds, including BIIB021, CUDC-305, and NVP-BEP800 are currently undergoing either preclinical or clinical trials (Fig. 1).[13] Despite these advances, none of Hsp90 inhibitors are clinically approved as an anticancer drug until now, and there still remains a need for the discovery of novel Hsp90 inhibitors with improved pharmacokinetic properties and safety profiles. Here, we describe the design, synthesis and biological effect of a new class of Hsp90 inhibitors.

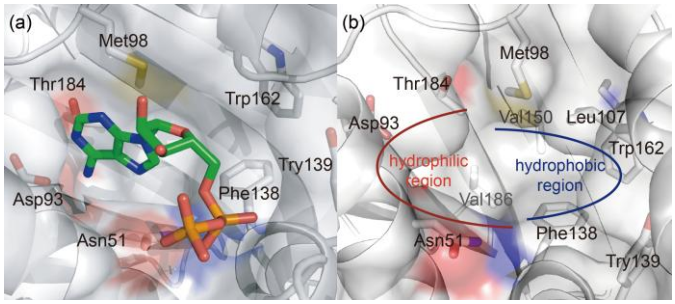
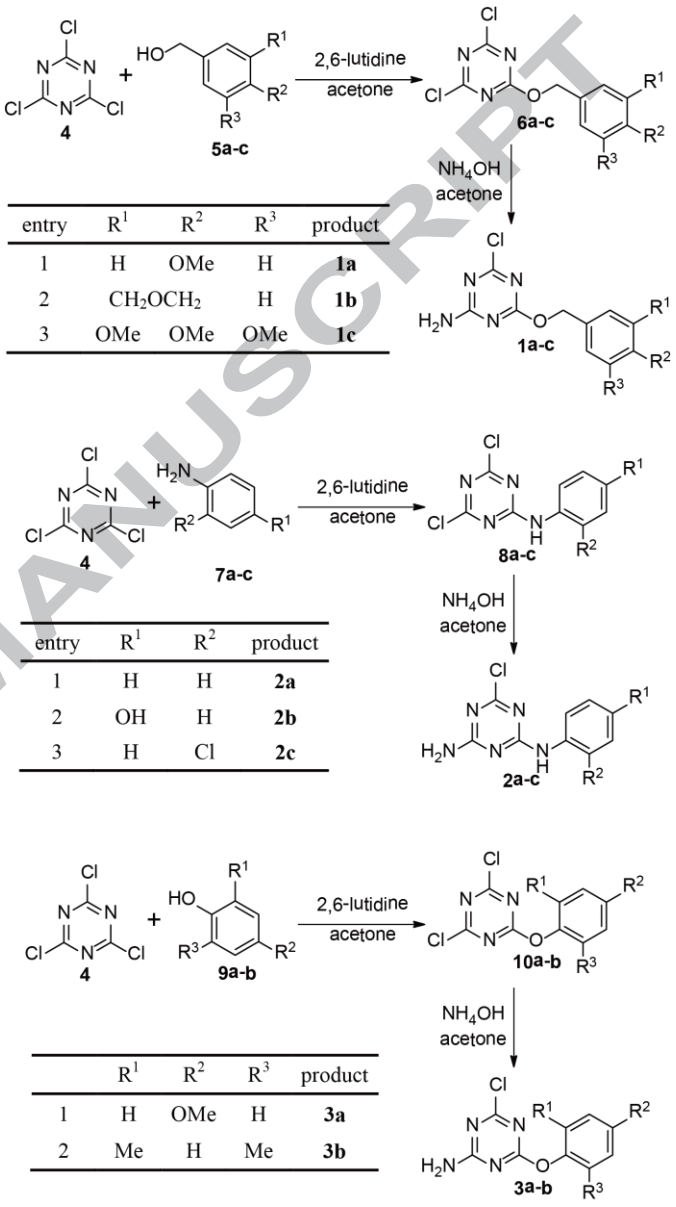


Figure 2. Analysis of ADP binding to Hsp90. (a) X-ray co-crystal structure of Hsp90 with ADP (PDB code: 1BYQ) and (b) apo-Hsp90. The carbon, oxygen, nitrogen, hydrogen, and phosphorus atoms of ADP are shown in green, red, blue, gray, and orange, respectively. The side chains of Hsp90's binding site are colored by atom types (carbon, gray; nitrogen, red; sulfur, yellow) and labeled with their residue name.

Structural analysis of endogenous ligand ADP binding to Hsp90 has revealed that adenine ring of ADP binds to the hydrophilic region of the pocket in the N-terminal domain of Hsp90, which are composed of Asp93, Asn51, Thr184 (Fig. 2). Considering that 2-amino-1,3,5-triazine moiety is structurally similar to the adenine ring of ADP and likely binds to the hydrophilic region of Asp93, Asn51, and Thr184, we used 2-amino-1,3,5-triazine as a starting fragment structure. Besides, the study done by Miura and co-workers also revealed that 2-amino-1,3,5-triazine played a central role in the affinity of the Hsp90 inhibitor.[14] Guided by the structural information of BIIB021's binding to Hsp90, shown in Figure 5b, we decided to keep chlorine atom at 4-position of 2-amino-1,3,5-triazine.[15] With 2-amino-4-chloro-triazine as a core structure, we attempted to expand the structure by adding hydrophobic arenes through installation of benzyl ethers, aryl ethers, or aryl amines at 6-position of 2-amino-4-chloro-1,3,5-triazine. The structural information of ATP binding pocket in the N-terminal domain of Hsp90 demonstrated that hydrophobic substituents at 6-position could reach to the side chains of Phe138, Try139, and Trp162 residues and formed π–π interactions. Therefore, our medicinal chemistry efforts were focused on synthesizing 6-substituted 2-amino-4-chloro-1,3,5-triazines.

To synthesize compounds with various substituents at 6-position of 2-amino-4-chloro-1,3,5-triazines, we first carried out the reactions of cyanuric chloride (**4**) with benzyl alcohols **5**, anilines **7**, and phenols **9** in the presence of 2,6-lutidine at room temperature for 12 hours, as outlined in Scheme 1. The substitution reactions of cyanuric chloride (**4**) with various nucleophiles (**5a-c**, **7a-c**, and **9a-b**) afforded corresponding 2,4-dichloro-6-substituted-1,3,5-triazines (**6a-c**, **8a-c**, and **10a-b**) in 71-99% yields. These compounds were further subjected to another nucleophilic substitution with ammonium hydroxide in acetone at room temperature for 12 hours to provide 2-amino-4-chloro-6-substituted-1,3,5-triazines (**1a-c**, **2a-c**, and **3a-b**; 61-89% yields from cyanuric chloride).



entry	R^1	R^2	R^3	product
1	H	OMe	H	**1a**
2	CH_2OCH_2		H	**1b**
3	OMe	OMe	OMe	**1c**

entry	R^1	R^2	product
1	H	H	**2a**
2	OH	H	**2b**
3	H	Cl	**2c**

	R^1	R^2	R^3	product
1	H	OMe	H	**3a**
2	Me	H	Me	**3b**

Scheme 1. Synthesis of 1,3,5-triazine-based Hsp90 inhibitors

To investigate whether the synthesized triazines could impair the growth of drug-resistant cancer cells, we selected a gefitinib-resistant non-small cell lung cancer (NSCLC) cell, H1975. NSCLC is the most common type of lung cancer and accounts for 80-85% of lung cancer patients. We first screened the anti-proliferative activities of triazines (**1a-c**, **2a-c**, and **3a-b**) against gefitinib-resistant H1975 cells (Fig. 2a). H1975 cells were incubated with synthesized compounds at various concentrations (0, 1, 10, 30, 50, 100, and, 150 μM) for 72 hours and cell viability was measured using MTS assay. The assay demonstrated that compound **1a** most effectively inhibited cell proliferation among other triazine compounds. Even though most compounds (**1a**, **1c**, **2a**, **2b**, **2c**, **3a**, and **2b**) blocked cell proliferation in a dose-dependent manner, the efficacies of these compounds are modest in cell proliferative assay.

To further investigate the molecular mechanism of the observed cytotoxicity, we next assessed the effect of triazine compounds on cellular biomarkers of Hsp90 inhibition (Fig. 3b). Considering the proteosomal degradation of Hsp90 client proteins and the transcriptional upregulation of Hsp70 are molecular hallmarks of Hsp90 inhibition, we measured the expression levels of Her2, Met, Akt, Hsp70, and Hsp90 along with β-actin as an internal standard.

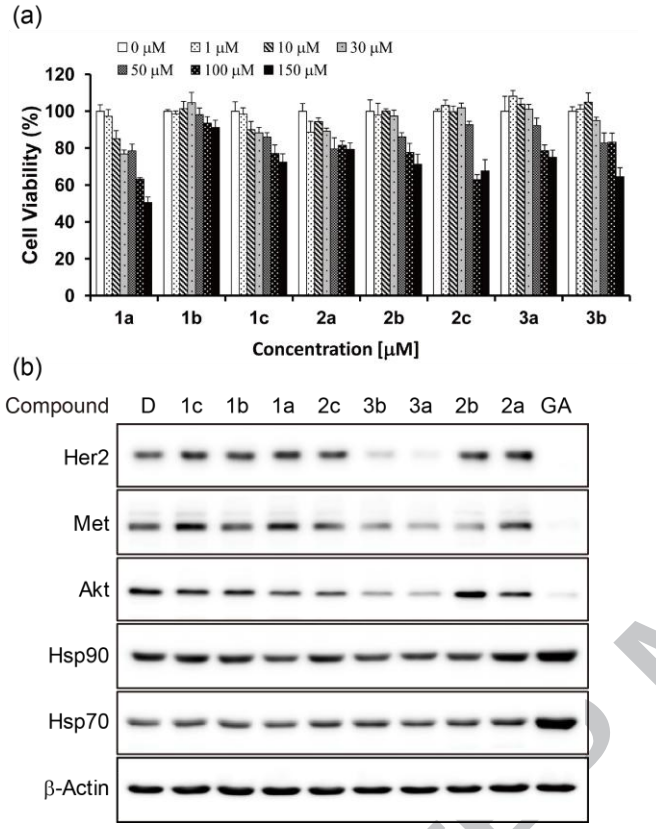


Figure 3. (a) Effects of synthesized compounds (**1a-c**, **2a-c**, and **3a-b**) on cell proliferation of H1975. Cells were treated for 72 hours at 0, 1, 10, 30, 50, 100, and 150 μM of the indicated compounds and the cell viability was measured by MTS assay. Data are presented as mean ± SD (n = 4). (b) Effects of compounds (**1a-c**, **2a-c**, and **3a-b**) on cellular biomarkers of Hsp90 inhibition. H1975 cells were treated for 24 hours with the indicated compounds (100 μM) and the expression of the Hsp90's client proteins was analyzed by western blot. Geldanamycin (GA, 1 μM) and DMSO (D) were employed as positive and negative controls, respectively.

Interestingly, the most toxic triazine **1a** did not induce the degradation of Her2 and Met, or upregulate Hsp70 protein, which suggested that the observed toxicity of **1a** was not associated with Hsp90 inhibition. On the other hand, triazine **3a** and **3b** furnished a robust degradation of Her2, Met and Akt and induction of Hsp70. Both **3a** and **3b** are phenoxy-triazines and structurally different from other synthesized triazines (**1a-c** and **2a-c**), in that triazine **1a-c** and **2a-c** have a benzyloxy-triazine and a phenylamino-triazine scaffold, respectively.

Western blot analysis of **3a** and **3b** demonstrated that **3b** degraded Hsp90's client proteins and induced the expression level of Hsp70 slightly better than **3a** (supplementary data). Therefore, we decided to focus on compound **3b**. To precisely determine the effect of **3b** on the downregulation of Hsp90 client proteins, H1975 cells were incubated with compound **3b** (0, 1, 50, 70, 100, and 150 μM) for 24 hours and the expression of Hsp90 client proteins, such as Her2, Met and Akt, along with Hsp70 and Hsp90 were measured by western blot analysis. As expected, compound **3b** reduced the expression levels of Hsp90 client proteins, including Her2, Met and Akt in a concentration-dependent manner (Fig. 4).

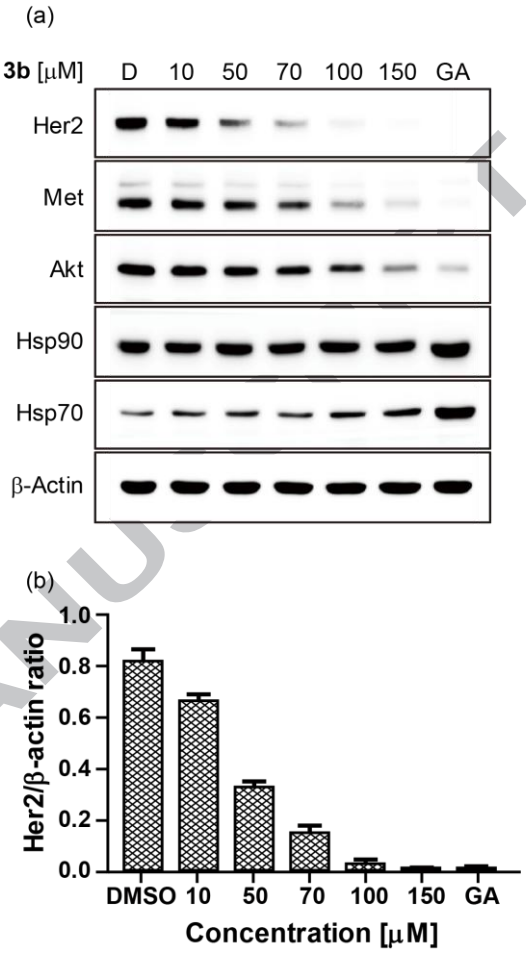


Figure 4. (a) Effects of compound **3b** on expression of Her2, Met, Akt, Hsp70, and Hsp90. β-Actin was used in a loading control. Compound **3b** induced proteasomal degradation of Hsp90 client proteins (Her2, Met, and Akt) and upregulated Hsp70 at various concentrations (μM). Geldanamycin (GA, 1 μM) and DMSO (D) were employed as positive and negative controls, respectively. (b) Densitometry analysis of Her2. The expression of Her2 was normalized to β-actin ratio

The expression level of Her2 was substantially decreased upon the treatment of cells with **3b** at a concentration of 50 μM, while the incubation with 100 μM of compound **3b** almost completely depleted Her2 protein and significantly degraded Met and Akt. In addition, the treatment with **3b** increased the protein level of Hsp70 in a dose-dependent manner. Collectively, the results strongly suggested that compound **3b** disrupted Hsp90 chaperoning machinery in H1975 cell line.

To test whether compound **3b** directly bound to ATP-binding pocket of N-terminal domain of Hsp90, we performed a fluorescence polarization assay with recombinant human Hsp90α (supplementary data, Fig 1S).[16] Human Hsp90α protein (30 nM) was incubated with compound **3b** (0.001, 0.01, 0.1, 1, 10, 50, 100, and 200 μM) in the presence of geldanamycin-FITC (5 nM) for 4 hours and the fluorescence polarization values were measured with an excitation wavelength at 495 nm and an emission wavelength at 530 nm. The assay demonstrated that compound **3b** inhibited Hsp90 with IC_{50} (FP) value of 5 μM and K_i value of 2.8 μM. The hydrophobic moiety of 2,6-dimethylbenzyloxy group at the 6 position of 2-amino-4-chloro-1,3,5-triazine led to a

significant improvement in the potency, and the result indicated the importance of this interaction in the hydrophobic region of ATP binding pocket, since 2-amino-1,3,5-triazine was reported to have 10 mM of K_d values using surface Plasmon resonance (SPR)-based binding assay.[14]

To predict the binding pose of **3b** in the N-terminal domain of Hsp90, the inhibitor **3b** was docked to Hsp90 using Autodock 4.2. The crystal structure of Hsp90 (PDB code: 3QDD) in complex with BIIB021 was selected as the receptor model for the docking of **3b**. After removing the ligand of BIIB021 and solvent molecules, molecular docking of **3b** was carried out in the ATP-binding site of N-terminal Hsp90. The docking study revealed that compound **3b** fits the ATP binding pocket in a similar fashion as BIIB021. 2-Amino-4-chloro-triazine ring and 2,6-dimethylphenyl ring of **3b** were positioned in the hydrophilic and the hydrophobic pocket of Hsp90, respectively (Fig. 5a).

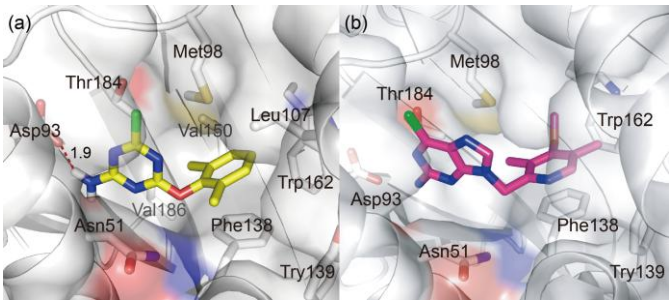


Figure 5. Comparative binding modes of **3b** and BII021. (a) Docking model of **3b** in the N-terminal of human Hsp90. (b) X-ray co-crystal structure of Hsp90 with BIIB021 (PDB code: 3QDD). The carbon atoms of **3b** and BIIB021 are shown in yellow and pink, respectively. The oxygen, nitrogen, hydrogen, and chlorine atoms of **3b** and BIIB021 are shown in red, blue, gray, and green, respectively. The side chains of Hsp90's binding site are colored by atom types (carbon, gray; nitrogen, red; sulfur, yellow) and labeled with their residue name.

As expected, the amino group at 2-position of 1,3,5-triazine interacted with Asp93 by hydrogen bonding and the hydrogen bonding distance between the hydrogen atom at amino group and the oxygen atom of Asp93 was calculated in 1.9Å. Despite 2,6-dimethylphenyl moiety of **3b** was located in the hydrophobic cavity of Hsp90 and formed proximal contacts with Phe138, Leu107 and Val150, the π–π interactions between 2,6-dimethylphenyl group and Phe138 was modest. The angle and the distance between 1,3,5-triazine and 2,6-dimethylphenyl ring didn't seem to be optimally shaped so as to negate the proximal π–π contact between 2,6-dimethylphenyl group and Phe138. That may explain the modest anti-proliferative effect of **3b** against H1975. Collectively, the hydrogen bonding of triazine ring and Van der Waals interactions of phenyl ring contributed to the binding of **3b** to ATP binding pocket of Hsp90 and the estimated binding energy (ΔG_b) and inhibition constants (K_i) of **3b** using the Lamarckian genetic algorithm result in -6.78 kcal/mol and 10.71 μM, respectively.

In summary, we rationally designed and synthesized a series of 1,3,5-triazine compounds. To create additional hydrophobic interactions in ATP binding pocket of Hsp90, we expanded 2-amino-4-chloro-1,3,5-triazine structure by adding substituents of benzyloxy, phenylamino and phenoxy groups at 6-postion of 2-amino-4-chloro-1,3,5-triazine. The biological assays demonstrated that the phenoxy-triazine was the most effective substituent to inhibit Hsp90 chaperoning machinery among others. The cell viability assay indicated that **3b** has an anti-proliferative activity against H1975. Western blot analyses revealed that **3b** dose-dependently downregulated the expression of Hsp90 client proteins of Her2, Met and Akt, and upregulated Hsp70 expression level. Computational modeling indicated that 2-amino-4-chloro-1,3,5-triazine moiety of **3b** bound to the hydrophilic pocket of Hsp90 through hydrogen bonding, and 2,6-dimethylphenyl moiety of **3b** reached the hydrophobic region of Hsp90 for Van der Waals interactions. Further SAR efforts of 6-phenoxy-1,3,5-triazines are currently underwent to improve the efficacy and the pharmacokinetic properties and the result will be reported in due course.

Acknowledgments

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Supplementary data

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17. Procedure for the synthesis of **3b**: A mixture of cyanuric chloride (**4**) (0.5 g, 4.1 mmol), 2,6-xylenol (0.75 g, 4.1 mmol), and 2,6-lutidine (0.53 g, 4.9 mol) in acetone was stirred for 12 h at rt. The solvent was removed under reduced pressure, and the remaining residue was dissolved in ethyl acetate. The organic layer was washed with water, dried over Na_2SO_4, and concentrated under reduced pressure to give compound **10b**, quantitatively. Compound **10b** was used in the following step without further purification. A mixture of **10b** (0.4 g, 1.5 mmol) and ammonium hydroxide (0.68 g, 11.2 mmol) in acetone was stirred for 12 h at rt. The solvent was removed under reduced pressure, and the remaining residue was purified by silica gel column chromatography using hexane-ethyl acetate (4:1) as eluent, to give compound **3b** in 89% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 2.07 (s, 6H), 7.07-7.15 (m, 3H), 8.10 (br s, 1H), 8.13 (br s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm) 16.43, 126.30, 129.17, 130.26, 149.14, 168.74, 170.10, 170.83; ESI-MS (*m/e*) = 251 ([M+1]$^+$)