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Synthesis and biological evaluation of 2,4-diaminoquinazoline derivatives as novel heat shock protein 90 inhibitors

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ABSTRACT

Novel 2,4-diaminoquinazoline derivatives originating from a virtual screening approach were designed, synthesized and their biological activities as heat shock protein 90 (Hsp90) inhibitors were evaluated. The prepared compounds exhibited significant anti-proliferative activities against DU-145, HT-29, HCT-116, A375P and MCF-7 cancer cell lines. The selected compounds were tested against Her2, a client protein of Hsp90, and showed significant reduction in Her2 protein expression. Compound **6b** was found the most potent, reduced Her2 protein expression levels and induced Hsp70 protein expression levels significantly.

Hsp90 proteins are important molecular chaperones that are responsible for the conformational maturation of numerous oncogenic proteins.[1] The Hsp90 family generally represent 1–2% of total cellular proteins in unstressed cells and up to 4–6% of total cellular proteins in cells under stress.[2] Currently, four Hsp90 chaperones are known, including Hsp90α and Hsp90β, which are found in cytoplasm, Grp94, which is found in the endoplasmic reticulum, and Trap-1, which is found in the mitochondria.[3] The cytoplasmic forms of these chaperones exist predominantly as dimers within the cell, and each subunit is composed of three domains: a 24–28-kDa N-terminal ATP-binding domain, a 38–44 kDa middle domain and a 11–15 kDa C-terminal domain. Conformational changes that occur upon binding and hydrolysis of ATP regulate the ability of the chaperone to bind to its client proteins.[4] Its client proteins include kinases, steroid hormone receptors, transcription factors that are directly involved in malignancy and mutated oncogenic proteins that are required for the transformed phenotype (i.e., Her2, raf-1, Akt, Cdk4, Polo-1-kinase, cMet, mutant B-raf, mutant P53, Ar, ER, Bct-Abl, Hif-1, alpha, and hTERT.[5])

Hsp90 has attracted growing interest over the past few years because of its role in the evolution, development and disease pathology of cancer.[6] Two natural products, radicicol (RDC) (**1** Fig. 1) and geldanamycin (GDC) (**2** Fig. 1), are known to be potent inhibitors of Hsp90 chaperone activity. Inhibition of Hsp90 leads to the degradation of multiple key proteins that depend on the interaction with Hsp90 for maintaining their bioactive conformation. Targeting multiple oncogenic proteins provides an advantage for cancer therapy due to the potential to increase the efficacy of the therapeutic and to overcome the drug resistance that occurs in many cancers including prostate[7] and breast cancer.[8] Most published work show Hsp90 expression in breast cancer cell lines.[9]

In 1994, geldanamycin (GDC) was identified as the first natural product that inhibited of Hsp90.[10] It was shown to bind to Hsp90 and interfere with the Hsp90-v-src heterocomplex formation.[11] GDC alters chaperone function and drives the degradation of many Hsp90 client proteins by stimulating Hsp90-mediated presentation to the ubiquitin-proteosome machinery.[12] Radicicol (RDC), the most potent natural product inhibitor in vitro, is shown to be inactive in vivo.[13] A 17-carbon position derivative, 17-(allylamino)-17-(demethoxygeldanamycin (17-AAG) is currently being tested in ongoing phase 1 and phase 2 clinical trials. Although antitumor activity has been observed in early clinical trials of 17-AAG, this agent is poorly soluble and has limited oral bioavailability. In vivo, RDC is rapidly converted into inactive metabolites that have little or no affinity for Hsp90.

To overcome the limitations of natural product inhibitors, synthetic Hsp90 inhibitors have also been reported (**3**–**5**, Fig. 1). Gopalsamy et al. reported the benzisoxazole-based compound **3** with IC_{50} = 30 nM, in comparison to 20 nM for GDC.[14] Hahn and coworkers disclosed the quinazoline-4-one based compound **4** with IC_{50} = 20 μM in an ATPase assay.[15] Chiosis and coworkers

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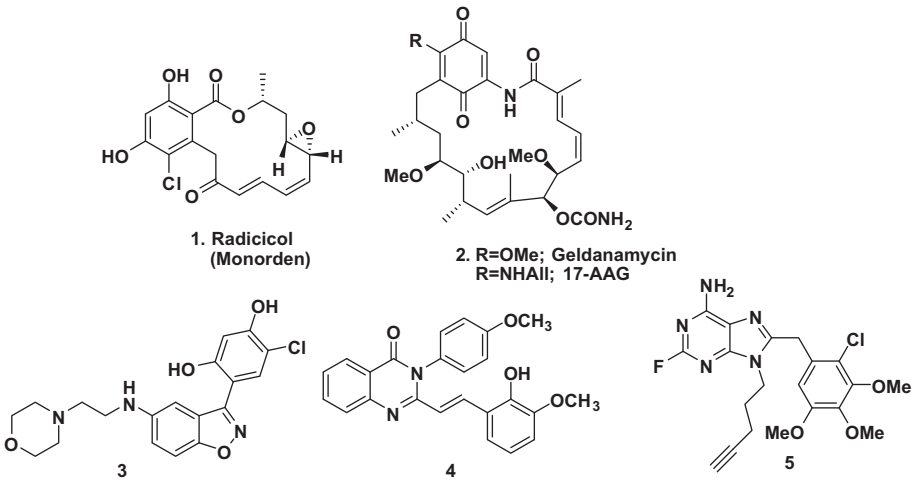


Figure 1. Structures of natural and synthetic inhibitors of Hsp90.

reported a purine-based inhibitor, compound **5** (PU24FCl), with low micromolar in vitro potency.[16] In the present study, we have identified the 2,4-diaminoquinazolines, a new class of Hsp90 inhibitors based on the structure of the hit compound **6a** (Fig. 2) obtained by a virtual screening approach.[17] The compound **6a** showed growth inhibition of human cancer cells with IC_{50} values of 35.01 μM for DU-145, 12.19 μM for HT-29, and 7.25 μM for MCF-7. Synthesis and SAR studies lead to several 2,4-diaminoquinazoline analogs with low micromolar inhibition against various cancer cell lines. Based on the structure of hit compound **6a**, the synthesis and SAR of the 2,4-diaminoquinazoline derivatives are described in detail below.

The 2,4-diaminoquinazoline derivatives (Table 1) were synthesized as shown in Scheme 1. The quinazoline-2,4-(1H,3H)-

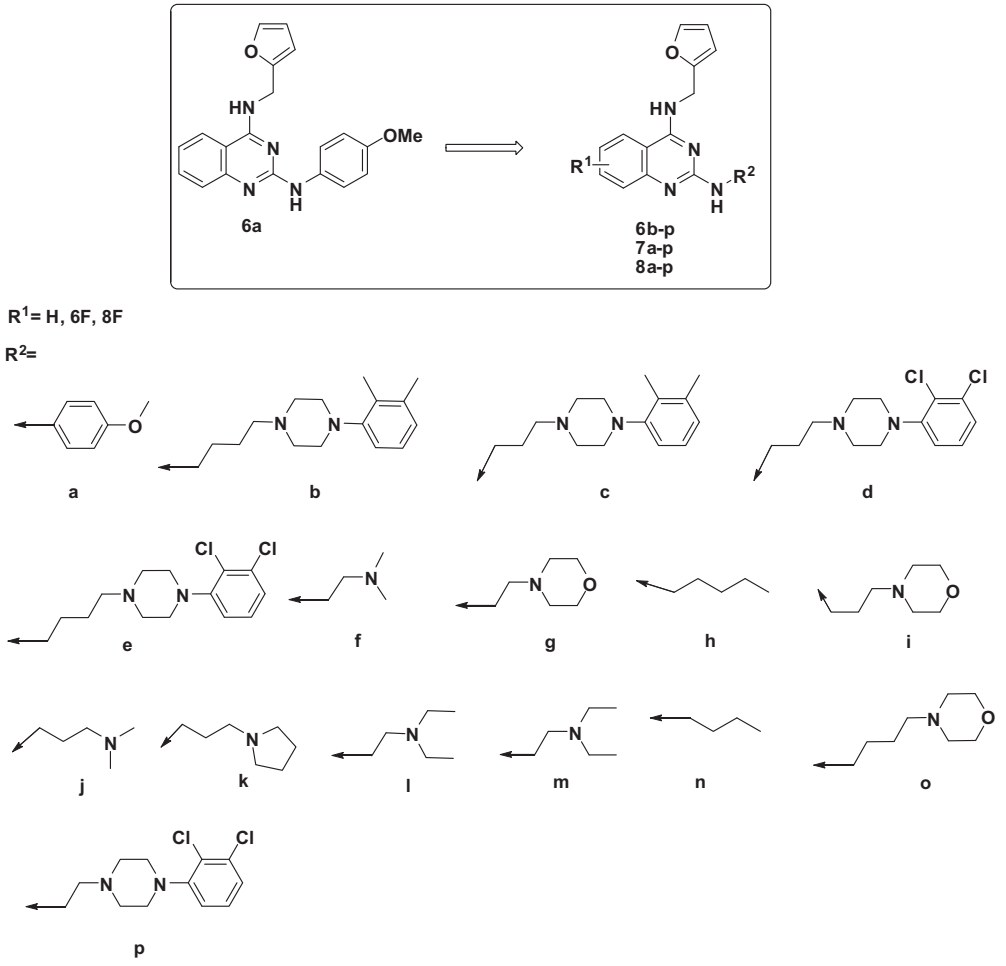
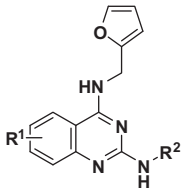


Figure 2. Designed Hsp90 inhibitors.

Table 1
Cytotoxity (% inhibition) and IC_{50} (μM) values of 2,4-diaminoquinazoline derivatives against various cancer cell lines at 10 μM concentration



Compds	R^1	R^2	DU145	HT-29	HCT-116	A375P	MCF-7
			%In.(IC_{50})	%In.(IC_{50})	%In.(IC_{50})	%In.(IC_{50})	%In.(IC_{50})
6a	H	a	nd(35.01)	nd(12.19)	nd(nd)	nd(nd)	64.55(7.25)
6b	H	b	93.63(1.07)	94.21(0.86)	94.31(0.84)	nd(3.90)	95.78(3.14)
6c	H	c	91.52(0.93)	93.99(0.84)	93.21(0.77)	nd(3.04)	95.86(3.31)
6d	H	d	93.16(3.71)	94.10(3.37)	94.45(3.36)	Nd(3.36)	95.85(2.98)
6e	H	e	94.32(3.87)	94.75(3.37)	94.73(3.2)	Nd(3.75)	95.82(2.97)
6f	H	f	20.04(nd)	21.69(nd)	nd(nd)	nd(nd)	nd(nd)
6g	H	g	nd(nd)	nd(nd)	nd(nd)	nd(nd)	95.75(2.12)
6h	H	h	nd(11.53)	nd(6.73)	nd(nd)	nd(nd)	nd(nd)
6i	H	i	nd(79.91)	nd(23.93)	nd(nd)	nd(nd)	nd(nd)
6j	H	j	nd(51.03)	nd(4.45)	nd(nd)	nd(nd)	nd(nd)
6k	H	k	40.06(35.27)	87.48(6.01)	67.50(16.12)	nd(19.39)	nd(nd)
6l	H	l	30.25(>100)	75.11(11.26)	62.58(26.14)	nd(>100)	nd(nd)
6m	H	m	28.48(>100)	80.02(10.29)	50.25(25.91)	nd(34.88)	nd(nd)
6n	H	n	89.90(4.99)	92.27(2.94)	94.78(5.47)	nd(9.2)	66.70(nd)
6o	H	o	52.26(6.24)	68.68(1.34)	79.79(2.10)	59.19(5.23)	nd(nd)
7a	6F	a	55.38(11.63)	86.19(4.34)	73.98(6.15)	nd(7.66)	nd(nd)
7b	6F	b	93.40(3.32)	96.12(1.83)	97.39(0.82)	97.55(3.28)	97.65(3.13)
7c	6F	c	95.93(2.66)	96.69(1.05)	97.34(1.14)	97.59(2.83)	nd(nd)
7e	6F	e	95.48(0.67)	96.30(0.39)	97.09(0.52)	97.35(2.65)	nd(nd)
7g	6F	g	27.76(nd)	9.68(nd)	12.76(nd)	28.55(nd)	nd(nd)
7h	6F	h	83.59(7.24)	nd(4.03)	94.17(4.1)	nd(7.34)	94.42(nd)
7i	6F	i	26.72(>100)	83.07(9.32)	52.09(25.28)	nd(22.51)	nd(nd)
7l	6F	l	32.36(nd)	34.83(nd)	27.44(nd)	15.84(nd)	nd(nd)
7m	6F	m	26.59(nd)	27.52(nd)	26.39(nd)	7.67(nd)	nd(nd)
7n	6F	n	75.37(5.91)	88.09(3.04)	87.77(4.31)	nd(8.48)	nd(nd)
7o	6F	o	57.57(6.46)	76.78(1.36)	81.98(2.14)	62.00(4.44)	nd(nd)
7p	6F	p	nd(nd)	nd(nd)	nd(1.01)	nd(2.42)	95.48(nd)
8n	8F	n	nd(nd)	nd(nd)	nd(1.33)	nd(2.63)	86.98(nd)
Doxorubicin			nd(nd)	nd(0.82)	nd(nd)	nd(nd)	nd(nd)

Du-145: Human prostate cancer cell line, HT-29: Human colon cancer, HCT-116: Human colon carcinoma cancer, A375P: Human melanoma cancer, MCF-7: Breast cancer cell line; nd: not determined.

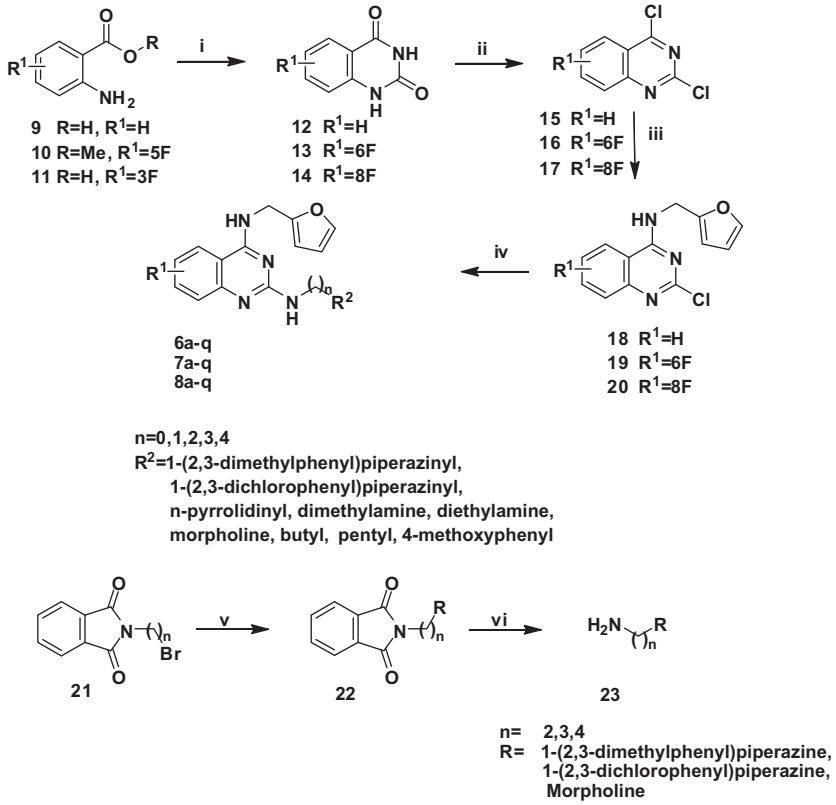
diones[18](**12–14**) were synthesized from 2-amino benzoic acids or methyl-2-amino (**9–11**) benzoates which were treated with phosphoryl chloride in the presence of *N*,*N*-diethylaniline or *N*,*N*-diisopropylethyleneamine to obtain 2,4-dichloroquinazolines[19] (**15**–**17**). Further treatment of the 2,4-dichloroquinazolines with furfurylamine in the presence of ethanol yielded the 2-chloro-*N*-(furan-2-ylmethyl)quinazoline-4-amines[20] (**18–20**).

Finally, the 2-chloro-*N*-(furan-2-ylmethyl)quinazoline-4-amines (**18**–**20**) were treated with the appropriate amines in the presence of ethanol and catalytic amounts of concentrated hydrochloric acid to yield the target 2,4-diaminoquinazoline derivatives **(6b**–**q**, **7a**–**q**, **8a**–**q**).[21] The amines that are not commercially available were synthesized as per Scheme 1.[22]

The inhibitory activities of the 2,4-diaminoquinazoline derivatives were measured against various cancer cell lines including DU-145 (prostate), HT-29 (colon), HCT-116 (colon carcinoma), A375P (melanoma). The IC_{50} values were also measured.[23] As the Human Epidermal growth Factor (Her2) is mainly found in breast cancer cells, which are client proteins of Hsp90, the selected compounds were tested for their anti-cancer activities against the MCF-7 breast cancer cell line.

To improve the activity, a variety of different linkers were introduced between the quinazoline and terminal amine moieties. In general, compounds with phenylpipierazine moiety (**6b–e, 7b, 7c, 7e, 7p**) showed enhanced potency (Table 1) over the compounds without phenylpiperazine moiety (**6h**–**o**, **7h**– **n**). The compounds which shown better potency against DU-145 (prostate), HT-29 (colon), HCT-116 (colon carcinoma), A375P (melanoma) were chosen to tested against MCF-7 breast cancer cell line. As seen in Table 1, all the selected compound (**6b–e** and **7b**) having phenylpiperazine moiety exhibited more potent activity against MCF-7 breast cancer cell line compared to lead compound **6a**.

The compound which shown the better potency against MCF-7 breast cancer cell line were tested for their affects on Her2 degradation in MCF-7 breast cancer cell line,[24] in addition we tested for the induction of Hsp70 to evaluate Hsp90 inhibitors. Since heat shock transcription factor (Hsf1) is inactivated by Hsp90, most of the Hsp90 inhibitors such as geldanamycin activate expression of Hsp1 target genes including Hsp70.[25] MCF-7 cells were treated with selected compounds for 24 h and the expression levels of Her2 and Hsp70 were detected by western blotting analysis. We found compounds **6b**, **6d** and **6e** conferred more than 50% reduction of Her2 protein levels compared to vehicle control where as compounds **6c** and **7b** showed less than 50% reduction of Her2 protein levels. Compounds **6b–e**, **7b** induced Hsp70 expression and compound **6b** was found the most potent one for both Her2 protein degradation and Hsp70 protein induction (Fig. 3), whereas β-actin levels remained unchanged which is not dependent on the Hsp90 protein folding machinery. Both, Her2 client protein degradation and heat shock protein70 induction indicates that 2,4-diaminoquinazoline acts as Hsp90 inhibitors, which is consistent with other Hsp90 inhibitors.



Scheme 1. Reagents and conditions: (i) Urea, phenol, 150 °C; (ii) $POCl_3$, *N*,*N*-diisopropylethylamine or *N*,*N*-diethylaniline; (iii) furfurylamine, ethanol, reflux; (iv) appropriate amine, ethanol, concn HCl, reflux; (v) Appropriate amine, *N*-bromoethylphthalimide or *N*-bromopropylphthalimide or *N*-bromobutylphthalimide, acetomitrile, potassium carbonate, reflux; (vi) hydrazine hydrate, ethanol, reflux.

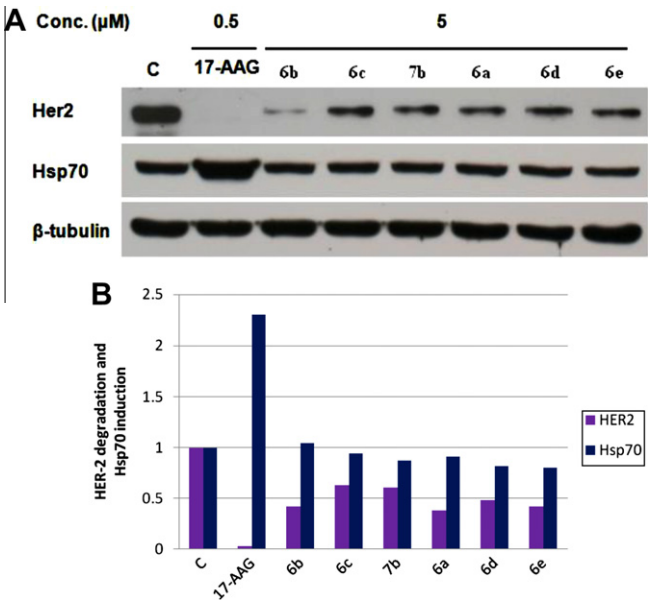


Figure 3. (A) Effects of 2,4-diaminoquinazolines on Her2 degradation and Hsp70 induction. MCF-7 cells were treated with 0.5 μM of 17AAG and 5 μM of all other 2,4-diaminoquinazoline compounds, dissolved in 0.4% DMSO, for 24 h, DMSO (C) for control. The protein level of Her2 and Hsp70 were detected by western blotting. β Tubulin was used as a loading control. (B) Quantification of Her2 degradation and Hsp70 induction.

In conclusion, we have reported a novel series of 2,4-diaminoquinazolines as Hsp90 inhibitors. Several compounds shown excellent growth inhibition activity against cancer cell lines. It was found that the extension of the methylene linker chain enhances the inhibitory activity. Furthermore, the replacement of the terminal dialkylamine moiety with phenyl piperazine led to new derivatives with 10- to 30-fold enhanced anti-proliferative activity than the hit compound **6a**. Western blot analysis supports that 2,4-iaminoquinazoline derivatives acts as Hsp90 inhibitors by degrading client protein, Her2 and increasing heat shock protein70 level. The data resulted from compounds **6b**–**e**, **7b** seems promising and have prompted further evaluation of this 2,4-diaminoquinazoline scaffold and additional work in improving the results is in progress.

Acknowledgement

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21. 2*N*-(3-(4-(2,3-dichlorophenyl) piperazine-1-yl)propyl)-8-fluoro-4*N*-(furan-2-ylmethyl)quinazoline-2,4-diamine (**8d**):
To a solution of 2-chloro-8-fluoro-*N*-(furan-2-ylmethyl)quinazolin-4-amine **20** (0.03 g, 0.1 mmol) and 3-(4-(2,3-dichlorophenyl)piperazin-1-yl)propan-1-amine **23d** (0.057 g, 0.2 mmol) in ethanol (2 mL), a catalytic amount of concn HCl was added and the reaction mixture was refluxed for 22 h. The ethanol was then removed under reduced pressure and the residue was extracted with dichloromethane, dried over magnesium sulfate, concentrated and purified by column chromatography on silica gel to obtain 2*N*-(3-(4-(2,3-dichlorophenyl)piperazine-1-yl)propyl)-8-fluoro-4*N*-(furan-2-yl methyl)quinazoline-2,4-diamine **8d** in 18% (0.01 g) yield.
^{1}H NMR (400 MHz, CD_3OD): δ 8.15 (q, J = 8.5 Hz, 1H), 7.45 (s, 1H), 7.24 (m, 2H), 7.09 (m, 3H), 6.38 (s, 2H), 4.87 (s, 2H), 3.63 (t, J = 7.4 Hz, 2H), 3.14 (s, 4H), 2.93 (s, 4H), 2.82 (s, 2H), 2.01 (quin, J = 7.4 Hz, 2H).
^{13}C NMR (400 MHz, CD_3OD): 150.98, 150.54, 142.16, 133.56, 127.73, 127.03, 124.83, 118.84, 110.17, 107.48, 55.25, 52.65, 49, 92, 39.19, 37.78.
IR (KBr):3244, 2948, 2823, 1650, 1579 cm^{-1}.
HRMS: $C_{26}H_{27}Cl_2FN_6O$, calcd: 528.1607, found: 529.1688$[M+H]^+$.
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23. Cytotoxic activities of the anticancer drugs against human cancer cell lines were investigated using the MTT assay. Human prostate cancer (DU145), Human colon cancer (HT-29), Human colon carcinoma cancer (HCT-116), Human melanoma cancer (375P) and Human breast cancer (MCF-7) were supplied from the Korean Cell Line Bank at Seoul National University. All cell lines were grown in RPMI 1640 medium (Gibco BRL) supplemented with 10% (v/v) heat inactivated Fetal Bovine Serum (FBS) and maintained at 37 °C in a humidified atmosphere with 5% CO_2. The cells (3×10^3 cells/well) were seeded into 96-well plates. Various concentrations of samples were added to each well in duplicates and then incubated at 37 °C with 5% CO_2 for two days such that time cells are in the exponential phase of growth at the time of drug addition. Then 15 μL of the Dye Solution (Promega, CellTiter96) was added to each well. The plate was incubated at 37 C for up to 4 h in a humidified, 5% CO_2 atmosphere. After incubation, 100 μL of the Solubilization Solution/Stop Mix (Promrga, CellTiter96) was added to each well. The plates were allowed to stand overnight in a sealed container with a humidified atmosphere at room temperature to completely solubilize the formazan crystals. The optical density was measured by using a microplate reader (Versamax, Molecular Devices) with a wavelength of 570 nm and the anticancer concentration was expressed as an IC_{50}.
24. *Her2 degradation assay*: MCF-7 cells were seeded into 6-well plates at a density of 10^5 cells/well and grown up to 60% confluency. Grown cells were treated with the compounds, 17-AAG which was dissolved in DMSO, or DMSO alone as a control. Cells were incubated for an additional 24 h and then harvested and lysed in TNES buffer [50 mM Tris–HCl, pH 7.4, 1% NP-40, 2 mM EDTA, 100 mM NaCl, 1 mM PMSF, 0.1% protease inhibitor cocktail solution]. The clarified cell lysates were collected after centrifugation and the protein concentration of each lysates were determined by Protein assay solution (Bio-rad). Each lysates containing 50 μg of total protein was loaded, subjected to SDS–PAGE and transferred to nitrocellulose membrane. After being probed with an antibody against Her2-neu (Santa Cruz Biotechnology), the membrane was stripped and also probed with antibodies against β-tubulin (Santa Cruz Biotechnology) and HSP70 (Stressgen Bioreagents).
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