NOTE

New non-quinone geldanamycin analogs from genetically engineered *Streptomyces hygroscopicus*

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The Journal of Antibiotics (2011) **64**, 461–463; doi:10.1038/ja.2011.24; published online 30 March 2011

Keywords: biosynthetic engineering; geldanamycin; Hsp90 inhibitor; non-quinone geldanamycin

Heat shock protein 90 (Hsp90) is emerging as an important target in cancer therapeutics and several other diseases.^{1,2} Hsp90 is an abundant and ubiquitously expressed molecular chaperone that is involved in the maturation of multiple client proteins, many of which are involved in regulating cell signaling, proliferation and survival.^{3,4} Client proteins of Hsp90 include Her-2, Akt, Src, Abl, c-Met and Raf-1, which are currently being targeted for intervention within oncology drug discovery or in clinical development. Although Hsp90 is highly expressed in most cells, Hsp90 inhibitors display remarkable selectivity for cancer cells as compared with normal cells.^{5,6}

Hsp90 is effectively inhibited by benzoquinone ansamycins such as geldanamycin (GM, **1**), herbimycin, macbecin and many of their derivatives, which bind to the ATP-binding site in the N-terminal domain.^{7,8} However, the development of **1** as a clinical agent has so far been limited by its toxicity, especially liver toxicity.⁹ However, the promising antitumor properties of **1** have spurred initiatives to develop biologically active derivatives.^{10,11} 17-Allylamino-17-demethoxyGM (17-AAG) has reduced toxicity compared with **1** and is the most advanced Hsp90 inhibitor in clinical development (Phase II/III).¹² 17-AAG and its benzoquinone analogs conjugate with glutathione, leading to cellular depletion. This conjugation with sulfur-containing nucleophiles may contribute to the dose-limiting hepatotoxicity of these quinone-containing compounds.^{13,14} For these reasons, new non-quinone **1** analogs with improved pharmacological profiles are needed.^{15,16}

Recently, we reported the development of non-quinone **1** analogs by a mutasynthetic approach and directed biosynthetic method.^{16,17} Of these non-quinone **1** analogs, DHQ3 (**3**), a 15-hydroxyl-17-demethoxy non-quinone analog, was found to inhibit Hsp90 ATPase activity more than **1**.¹⁶ Moreover, during these studies, novel tricyclic **1** analogs were prepared from a genetically engineered strain (AC15) of *Streptomyces hygroscopicus*.¹⁸ Presently, we describe the fermenta-

tion of mutant AC15, and the isolation, structural determination and bioactivity of new non-quinone **1** analogs produced by the mutant: DHQ7 (**4**) and DHQ8 (**5**).

AC15 was constructed by a combinational mutation with site-directed mutagenesis of the first dehydratase domain of the geldanamycin polyketide synthase (PKS) gene (*gela*) and a post-PKS modification gene (*gel7*) of *S. hygroscopicus* JCM4427, as previously reported.¹⁶ The seed medium and production medium (YEME) consisted of sucrose 103.0 g, yeast extract 3.0 g, peptone (Difco, Sparks, MD, USA) 5.0 g, malt extract (Difco) 3.0 g, glucose 10.0 g and MgCl₂·6H₂O 1.0 g in 1.0 l of distilled water. Spores that developed during growth on ISP4 medium were harvested and inoculated into 300 ml of YEME in a 1.0 l baffled flask and cultured for 3 days at 28 °C. Equal volumes of the seed culture were inoculated into 25 baffled flasks containing 300 ml of YEME medium. Fermentation was subsequently carried out for 7 days at 28 °C and 160 r.p.m. The resulting culture (~8 l) was extracted twice with an equal volume of ethyl acetate (EtOAc). The extract was filtered through a fritted funnel and the resulting filtrate was evaporated *in vacuo* to yield the EtOAc extract. This extract was partitioned between EtOAc and water. The EtOAc-soluble material (2.7 g) was subjected to silica gel chromatography using a stepwise gradient elution of mixtures of CH₂Cl₂ and MeOH. The fraction eluted with CH₂Cl₂:MeOH of 85:15 was further purified by reverse-phase HPLC using YMC-J'sphere ODS-H80 (10×250 mm, 3 ml min⁻¹; YMC, Kyoto, Japan) with a linear gradient from 30 to 100% acetonitrile (MeCN) containing 0.05% trifluoroacetic acid to yield DHQ7 (**4**; 31.8 mg) and DHQ8 (**5**; 17.6 mg). Compound **3** (317 mg) was purified from the fraction eluted with CH₂Cl₂:MeOH (90:10), subjected to passage over a Sephadex LH-20 (GE Healthcare, Buckinghamshire, UK) column using MeOH as eluant and further purified by HPLC (YMC J'sphere ODS-H80, 10×250 mm, MeOH-H₂O (0.05% trifluoroacetic acid) gradient, 3 ml min⁻¹).

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Received 25 January 2011; revised 11 February 2011; accepted 4 March 2011; published online 30 March 2011

The structures of known **3** were identified by spectral data interpretation and comparison with literature values.^{16,19} Compound **4** was obtained as a white powder ($[\alpha]_D^{25} +11.1$ (c, 0.10, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 204 (4.55), 260 (3.77), 289 nm (3.69)). The molecular formula was determined as $C_{28}H_{40}N_2O_8$ from HR-ESIMS data (found, m/z 555.2673 $[M+Na]^+$; calculated 555.2677 for $C_{28}H_{40}N_2NaO_8$) in conjunction with NMR data. The ^{13}C NMR and HMQC spectra revealed the presence of 28 carbon signals comprising two carbonyl, six aromatic or olefinic quaternary, six aromatic or olefinic methine, four oxymethine, two aliphatic methine, two aliphatic methylene, two *O*-methyl, and four *C*-methyl carbons. Additionally, four exchangeable proton signals were observed in DMSO- d_6 : two hydroxyl protons (δ 5.10 and 4.76), one phenolic hydroxyl proton (δ 9.13) and one amide proton (δ 9.33). These physicochemical properties and NMR data suggested that **4** was related to **1**. Interpretation of the 2D-NMR data including COSY, HMQC and HMBC spectra enabled the construction of structure of **4**. Compound **4** displayed the characteristic paired signals including those of **3**, suggesting that **4** is a modified **3** and contains a C-15 hydroxylated non-quinone **1** skeleton. Thus, the presence of three olefinic protons ((δ 5.89 (1H, dd, $J = 4.8, 7.2$ Hz, H-3), 5.47 (1H, d, $J = 9.2$ Hz, H-9), and 5.15 (1H, d, $J = 10.4$ Hz, H-12)), and the corresponding carbon signals (δ 136.73 (C-3), 136.10 (C-9) and 111.86 (C-13)), indicated that one more position of **3** is unsaturated. The position of the newly formed double bond was confirmed using the HMBC NMR experiments (H-25/C-13 and C-14, H-14/C-13, H-13/C-12, 12-OCH₃/C-12, H-11/C-12). Therefore, the structure of this new C-15 hydroxylated non-quinone **1** analog **4** was assigned as shown in Figure 1.

DHQ8 (**5**) was also obtained as a white powder ($[\alpha]_D^{25} -24.8$ (c, 0.10, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 203 (4.43), 262 (3.70), 295 nm (3.62)), the molecular formula being established as $C_{29}H_{41}N_3O_9$ on the basis of the HR-ESIMS data (found, m/z 598.2733 $[M+Na]^+$; calculated 598.2735 for $C_{29}H_{41}N_3NaO_9$) and NMR data. The 1H and ^{13}C NMR spectra of **5** were very similar to those of **4** (Table 1), except for the absence of one hydroxyl signal and the additional carbamoyl signal at δ_C 156.95 in the later compound, and were consistent with the molecular formula $C_{29}H_{41}N_3O_9$ obtained by positive HR-ESIMS. The position of these carbamoyl groups was determined by HMBC correlations (H-7/7-OCONH₂ and H-11/11-OCONH₂). Further interpretation of the 2D NMR data led to the structure of **5**.

All isolated compounds (**3**, **4** and **5**) were tested for the ability to inhibit yeast Hsp90 activity using a malachite green ATPase assay. Compounds **4** and **5** showed potent activity of ATPase inhibition with IC₅₀ values of 1.75 and 5.87 μM , respectively. But, the **3** showed stronger ATPase inhibition activity (0.68 μM) compared with the original Hsp90 inhibitor **1** (3.19 μM), as previously demonstrated.¹⁶

To further confirm that **4** and **5** bind and interact with the *N*-terminal nucleotide binding site of Hsp90 as an ATP mimetic, we examined whether these compounds could compete with a bead-immobilized derivative of ATP for Hsp90 binding. It is noteworthy that the compounds demonstrated significant activity compared with the DMSO control. However, the concentration of competitive binding did not exactly match the values of the previous ATPase assay. But, this

Table 1 NMR data of DHQ7 (**4**) and DHQ8 (**5**) in DMSO- d_6

	δC		δH	
	4	5	4	5
1	168.32 s	168.45 s		
2	132.43 s	132.05 s		
3	136.73 d	136.90 d	5.89 dd (4.8, 7.2)	6.01 t (6.0)
4	23.75 t	23.92 t	2.12 m; 2.23 m	2.15 m
5	30.64 t	30.80 t	1.42 m; 1.62 m	1.33 m; 1.45 m
6	79.89 d	80.60 d	2.96 t (8.4)	3.22 m
7	81.14 d	81.40 d	4.80 d (4.8)	4.79 d (8.8)
8	130.02 s	131.93 s		
9	136.10 d	133.47 d	5.47 d (9.2)	5.31 d (10.4)
10	32.66 d	31.60 d	2.57 m	2.82 m
11	71.83 d	73.70 d	3.88 d (4.8)	4.98 brs
12	154.98 s	150.26 s		
13	111.86 d	112.64 d	5.15 d (10.4)	5.01 d (10.4)
14	37.35 d	37.39 d	2.57 m	2.53 m
15	76.75 d	76.75 d	4.39 brs	4.34 brs
16	146.80 s	146.86 s		
17	108.07 d	108.42 d	6.27 s	6.26 s
18	156.86 s	156.93 s		
19	103.76 d	103.48 d	7.49 s	7.48 s
20	139.42 s	139.50 s		
21	110.25 d	109.35 d	6.73 s	6.48 s
22	12.62 q	12.48 q	1.78 s	1.75 s
23	11.47 q	11.64 q	1.50 s	1.59 s
24	12.17 q	12.80 q	0.77 d (6.4)	0.88 d (7.2)
25	18.71 q	19.07 q	1.10 d (6.8)	1.11 d (6.8)
6-OCH ₃	59.21 q	58.74 q	3.38 s	3.37 s
12-OCH ₃	56.57 q	56.66 q	2.68 s	2.77 s
7-OCONH ₂	156.05 s	155.99 s		
11-OCONH ₂		156.95 s		
11-OH			4.76 d (5.6)	
15-OH			5.10 d (2.8)	5.07 d (3.2)
18-OH			9.13 s	9.19 s
NH			9.33 s	9.22 s

Chemical shifts are shown in the δ scale with J values (Hz) in parentheses.

Figure 1 Chemical structures of geldanamycin (**1**), 4,5-dihydrogeldanamycin (**2**), DHQ3 (**3**), DHQ7 (**4**) and DHQ8 (**5**).

Table 2 Comparison of biological activities of geldanamycin, DHQ3, DHQ7 and DHQ8

Compounds	Yeast Hsp90 ATPase inhibition assay (IC ₅₀)	Cell viability assay (IC ₅₀)		
		A2780	SK-Br3	BT474
Geldanamycin (1)	3.19 μ M	7 nM	9 nM	5 nM
DHQ3 (3)	0.68 μ M	24 μ M	40 μ M	16 μ M
DHQ7 (4)	1.75 μ M	13 μ M	15 μ M	8 μ M
DHQ8 (5)	5.87 μ M	17 μ M	173 μ M	63 μ M

A2780, human ovarian cancer cell; SK-Br3 and BT474, human breast cancer cell.

Figure 2 Non-quinone geldanamycin derivatives (DHQ3 (**3**) and DHQ7 (**4**)) destabilize Hsp90 client proteins (Her2 and c-Raf). SK-Br3 cells were treated with **3** and **4** for 24 h at increasing doses. Her2 and c-Raf levels were analyzed by western blotting.

finding confirmed that non-quinone **1** analogs also interact with the nucleotide-binding site of Hsp90 (Supplementary data).

The non-quinone **1** analogs were evaluated for their anti-proliferation activity using tumor cell growth inhibition assays in human ovarian A2780 and breast SK-Br3 and BT474 cancer cell lines, following the standard procedures. **4** showed anti-proliferative activity against A2780, SK-Br3 and BT474 cells at 13, 15 and 8 μ M, respectively, whereas **5** was less effective in the cancer cell lines. In contrast, **1** showed sub- μ M cytotoxic activity against several human cancer cells (Table 2). The anti-proliferation activity data showed that C-15 hydroxylated non-quinone **1** analogs exhibited a potency that was weak and less than that of **1**. Further carbamoylation at the C11 hydroxy position did not show improvement in the anti-proliferative activities. In addition to anti-proliferation activity studies, we further examined whether Her2 and c-Raf, well-documented clients of Hsp90, were degraded by **3** and **4**. As shown in Figure 2, western blot analysis indicated that Her2 and c-Raf were degraded by the inhibitors in a concentration-dependent manner. Therefore, the non-quinone **1** seemed to have an anti-proliferative effect via Hsp90 inhibition in SK-Br3 cells.

Although these non-quinone **1** derivatives showed favorable potency in the ATPase assay, its lack of potency in the cellular assays

was found. The reason for this discrepancy between ATPase inhibition activity and anti-proliferative activities is not clear. But, we expect that further development of semi-synthetic derivatives with more diverse residues, such as diaminoalkyl functionality introduced at the benzene ring and/or C15-position,²⁰ may exhibit improved cellular potency over **4** or **5**.

ACKNOWLEDGEMENTS

This work was supported in part by the 21C Frontier Microbial Genomics and Application Center and Global R&D Center program, the Ministry of Science and Technology, Republic of Korea and by a grant from KRIBB Research Initiative Program. We thank the Korea Basic Science Institute for the NMR measurements

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (<http://www.nature.com/ja>)