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Conformational significance of EH21A1–A4, phenolic derivatives of geldanamycin, for Hsp90 inhibitory activity

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Abstract—Hsp90 is an attractive chemotherapeutic target because it is essential to maturation of multiple oncogenes. We describe the conformational significance of EH21A1–A4, phenolic derivatives of geldanamycin isolated from *Streptomyces* sp. Their native free structures are similar to the active form of geldanamycin bound to Hsp90 protein. Their conformational character is a probable reason for their high-affinity binding. Lack of toxic benzoquinone in EH21A1–A4 also adds to their potential as lead compounds for anti-tumor drugs.

Heat Shock Protein 90 (Hsp90) is an essential molecular chaperone required for conformational stability and function of multiple growth regulatory signaling proteins. Inhibiting the function of Hsp90 leads to subsequent simultaneous degradation of the client proteins via the ubiquitination-proteosome system.[1] Since the client proteins cover the six hallmarks of cancer,[2] Hsp90 inhibitors are attractive as chemotherapeutic agents.[3] A first-in-class drug, 17-allylamino-17-demethoxygeldanamycin (17-AAG, **2**), a derivative of geldanamycin (**1**), has provided proof of concept for Hsp90 inhibition in clinical trials. However, **2** showed significant hepatotoxicity probably due to the redox-active benzoquinone moiety, and was difficult to administer because of its poor solubility profile.[4] Our aim, therefore, was to find more potent inhibitors with less toxicity and higher solubility.

For screening, we established a dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) using biotinylated radicicol[5] as a probe because radicicol (**8**), another natural Hsp90 inhibitor, shares the common N-terminal ATP binding pocket in Hsp90 with a higher affinity than **1**. In the course of screening, we found that an actinomycete *Streptomyces* sp., isolated from Indonesian soil (see Supplementary data), produced polar inhibitors together with **1**. Jar fermentation culture of the strain (62.7 L), followed by repetitive activity-guided column chromatography, provided four inhibitors; tentatively named EH21A1–A4 (**3**–**6**) with the following yields: 128.9, 34.9, 29.8, and 8.4 mg, respectively (see Supplementary data).

The structures of **3**–**6** were spectroscopically revealed as analogous to **1** with a unique phenol structure rather than the benzoquinone moiety of **1**. The distinctive reduction in C-4/C-5 was also a common feature of **3**–**6**. NMR signals for a methyl group adjacent to C-2 were not observed in **6**. Collectively, **3** was identical to reblastatin,[6] and the structures of **4**–**6** were elucidated as 17-demethoxy, 17-*O*-demethyl,[6] and 17-demethoxy-2-demethyl derivatives of **3**, respectively (Fig. 1). Although production of **4** and **6** has been reported as KOSN1806 and KOSN1559, respectively, in the actinomycetes genetically engineered,[7] the present report would be their first isolation as natural products.

In vitro affinities for a purified Hsp90 protein were compared by compound concentration sufficient to inhibit 50% binding of the probe (EC_{50}) in DELFIA assay

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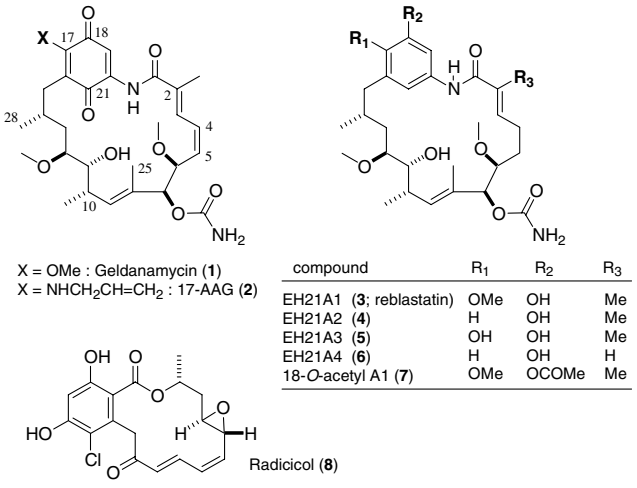

Figure 1. Structures of Hsp90 inhibitors.

(Table 1). Surprisingly, **3**–**5** exhibited 10- to 25-fold lower EC_{50} values than **1**. This indicates that **3**–**5** are the most potent Hsp90 binders of the ansamycins. Lowering affinity of **6** relative to **4** indicates a partial contribution of 2-methyl moiety for the binding to Hsp90. Then, Hsp90 inhibition of the compounds in cells was assessed by immunoblotting of client proteins to detect depletion. Forty hour treatment of KPL-4, a human breast cancer cell line, with **3**–**6** clearly led to specific depletion of client proteins such as Raf-1 and ErbB2 (see Supplementary data) with minimal effective concentrations (MEC) corresponding to growth inhibitory activities against the cells (Tables 1 and 2). Furthermore, **3**–**6** induced the erythroid differentiation of K562, a human chronic myelogenous leukemia cell line, through probable destabilization of $p210^{Bcr\text{-}Abl}$, also an Hsp90 client protein (Table 1).[8] These functional evaluations demonstrate that a class of ansamycins, with a phenol moiety, is a valid Hsp90 inhibitor.

Table 1. Hsp90 inhibitory activities of **3**–**8** in comparison with the known inhibitors, **1** and **2**[a]

Compound	In vitro affinity EC_{50}[b] (nM)	Client protein depletion MEC (μM)		Induction of K562 differentiation
		ErbB2	Raf-1	EC_{50} (μM)
1	57.0 (19.3)	0.11	0.33	n.d.
2	n.d.[c]	0.03	0.03	n.d.
3	2.3 (0.9)	0.33	0.33	0.76
4	2.2 (0.8)	1.0	1.0	1.9
5	6.0 (0.3)	10	10	21
6	14.7 (2.1)	3.3	3.3	11
7	20.8 (3.0)	0.33	0.11	0.23
8	0.8 (0.5)	n.d.	n.d.	n.d.

[a] See Supplementary data for details.
[b] Values are means of three experiments; standard deviation is given in parentheses.
[c] Not determined.

Subsequently, we carried out crystallization of **3** from DMSO solution to determine stereostructure. The quality of the single crystal obtained was sufficiently high, with a Flack parameter of $x = 0.0(2)$, for determination of the absolute configuration of **3** without introducing a heavy atom. X-ray crystallographic analysis proved that stereochemistry of **3** was identical to reblastatin: enantioselectively synthesized by the Panek group including absolute configuration.[9] Analysis also revealed that the free native state of **3** has a C-clamp conformation in which the benzene ring is almost parallel to lower half of the ansa ring, and the amide bond is in *cis* configuration (Fig. 2). This native state conformation of **3** is significantly different from that of the benzoquinone-type

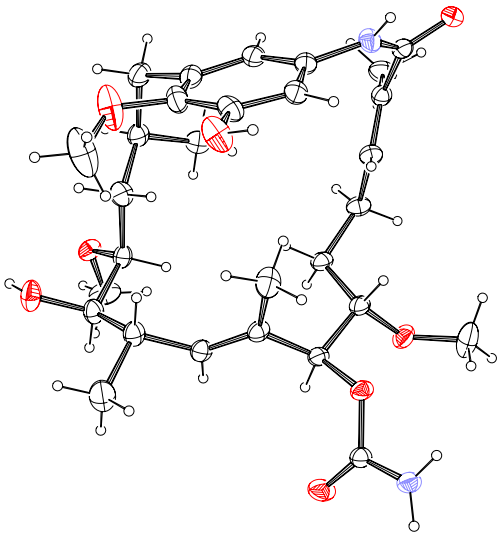

Figure 2. The crystal structure of **3** showing non-hydrogen atoms as 20% probability displacement ellipsoids. H atoms are indicated as circles of arbitrary radii.

Table 2. Growth inhibitory activity of **1**–**8** against various cancer cell lines expressed in GI_{50}[a] (μM)

Compound	HCT116 (colon)	DLD-1 (colon)	A549 (lung)	KPL-4 (breast)
1	0.021 (0.001)	0.037 (0.004)	0.064 (0.012)	0.059 (0.022)
2	n.d.[b]	n.d.	n.d.	0.007 (0.001)
3	0.50 (0.03)	2.08 (0.58)	2.72 (1.53)	0.45 (0.01)
4	0.78 (0.05)	2.35 (0.52)	2.73 (1.34)	0.73 (0.04)
5	8.91 (0.25)	9.40 (0.13)	10.3 (0.45)	4.33 (0.26)
6	>10	>10	>10	3.39 (0.33)
7	0.61 (0.03)	3.52 (0.95)	3.55 (1.97)	0.18 (0.02)
8	0.058 (0.031)	0.076 (0.033)	0.110 (0.016)	n.d.

[a] Values are means of at least three experiments; standard deviation is given in parentheses.
[b] Not determined.

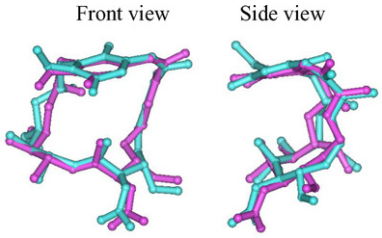

Figure 3. Superimposed structures of **3** in a free native crystalline state (magenta) and **1** in a cocrystal with Hsp90 protein (cyan, PDB ID; 1A4H).

ansamycins such as **1** and **2** whose native conformations are in an extended planar shape with *trans*-amide.[10] NOE correlations observed in NOESY spectra demonstrated that the shape of **3**–**6** were also kinked in solution. For example, correlations among H-3, H-6, H-10, and CH_3-25 in **3** indicated these protons gathered inside the macro ansa ring. In addition, correlations between H-21 and CH_3-28 as well as CH_3-25 and OCH_3-29, bound to C-17, were evident for close proximity of respective protons (see Supplementary data).

The ATP binding motif of Hsp90 in the N-terminal region is characteristic since its natural ligand, ATP, needs to have a bent conformation for binding.[11] As to **1**, a rotation of C–N amide bonds as well as flipping of the ansa ring over the benzoquinone, which results in the compact *cis*-form, occurs during docking to the pocket.[10b,c] Interestingly, the native crystal structure of **3** was in good agreement with 'the active form' of **1** (Fig. 3).[12] Since the overall energy barrier for the *trans*–*cis* isomerization of **1** was estimated as high, a catalytic role for Hsp90 would be required.[10b,c] In contrast, **3**–**6** are able to bind in their native conformation, suggesting that they have an advantage for binding. This simple binding mode of **3**–**6** is similar to that of **8**,[11a] which exhibits 70-fold higher affinity to Hsp90 than **1** (Table 1).

We believe that two chemical features, an elimination of a hydrogen bond between H-amide and ketone oxygen of C-21, and a reduction in C-4/C-5 position, may allow **3**–**6** to increase their conformational flexibility and form the kinked shape in a native state. Ab initio quantum chemical calculation indicates that the C-clamp form with *cis*-amide of **3** is more stable than the planar *trans*-amide form with an enthalpy difference of 3.56 kcal/mol. In addition, with regard to **1**, NMR analysis of molecular flexibility in solution suggests that the most populated conformer of **1** is in a *cis*-amide (22%), while the other conformations are a *trans*-amide (>70%).[13] A hydroxy group at C-18 in **3**–**6** may additionally contribute to their high affinity because a hydrogen bond donor group in the region, originally bound to the triphosphate moiety of ATP, is important for docking.[14] We conclude this is the reason why **3**–**6** exert high affinity for Hsp90 in vitro.

Growth inhibition of **3**–**6** against various cancer cells, however, was no greater than expected from in vitro affinity (Table 2). Although chemical properties of **3**–**6** were thought to be similar to **1** and **2**, their polarities were relatively high judging from chromatographic behaviors. Therefore, we derivatized and evaluated the activity of **7**, an 18-*O*-acetyl derivative of **3**. The client depletion activity of **7** increased slightly in spite of decreased affinity. Membrane permeability of **3**–**6** is a possible reason for modest growth inhibitory activity. Some Hsp90 inhibitors, such as **2**, are reported to accumulate in cancer cells and tissues.[15] Differences in accumulation may, therefore, provide another reason.

Based on molecular dynamics simulations and energetic analysis, Jez et al.[10b] thought that an ansamycin analog with a constrained *cis*-amide bond in the ground state would greatly increase in affinity because the remainder of the molecule would adopt the C-like conformation and bind to Hsp90 without the large energy loss required for conformational change. We believe **3**–**6** are consistent with their findings.

It was recently established that **2** is reduced to its dihydroquinone by cellular enzyme DT-diaphorase,[16] and the resulting dihydroquinone exerts more potent inhibition than the quinone itself.[17] Although a protracted K_{off} rate of the dihydroquinone has been reported,[18] the reason for the potent inhibition is unknown. We believe energetic advantage may explain this since the conformational properties of **3**–**6** presented here may be adapted to the dihydroquinone. Furthermore, unlike the dihydroquinone, a phenol moiety is not easily oxidized to benzoquinone, a possible toxicophore.[19] Therefore, **3**–**6** can be promising lead compounds for anti-tumor drugs targeting Hsp90 inhibition.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.01.072.

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