Potent Non-Benzoquinone Ansamycin Heat Shock Protein 90 Inhibitors from Genetic Engineering of *Streptomyces hygroscopicus*

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Abstract: Inhibition of the protein chaperone Hsp90 is a promising new approach to cancer therapy. We describe the preparation of potent non-benzoquinone ansamycins. One of these analogues, generated by feeding 3-amino-5-chlorobenzoic acid to a genetically engineered strain of *Streptomyces hygroscopicus*, shows high accumulation and long residence time in tumor tissue, is well-tolerated upon intravenous dosing, and is highly efficacious in the COLO205 mouse tumor xenograft model.

Heat Shock Protein 90 (Hsp90^a) is a protein chaperone that acts to stabilize and/or activate a number of proteins required for cellular signaling pathways, including several known determinants of carcinogenesis.¹⁻³ Hsp90 is effectively inhibited by benzoquinone ansamycins such as geldanamycin, herbimycin, macbecin, and many of their derivatives, which bind to the ATP binding site in the N-terminal domain.^{4,5} Recent results from clinical trials using the geldanamycin derivatives tanespimycin and alvespimycin, also known as 17-AAG (17-allylamino-17demethoxygeldanamycin) and 17-DMAG (17-(2-dimethylamino)ethylamino-17-demethoxygeldanamycin), respectively, suggest that inhibition of Hsp90 could be an important therapeutic mechanism for cancer treatment.⁶ The antiproliferative activity of several benzoquinone Hsp90 inhibitors against cancer cells has been shown in various models to be dependent on reductive activation to the hydroquinone by the enzyme NAD(P)H/ quinone oxidoreductase 1 (NOO1).⁷⁻¹⁰ The activity of this enzyme in cancer patients is variable. The frequency of the most common mutant allele P187S differs in different ethnic groups, but the number of homozygous individuals can be as high as 25% in some populations, resulting in a lack of NQO1 activity in these patients.^{11,12}

Molecules that retain the exquisite specificity of tanespimycin and alvespimycin for the ATP binding pocket of Hsp90 while obviating the need for activation by NQO1 could be very attractive candidates for drug development. Here we report potent, efficacious nonbenzoquinone ansamycin Hsp90 inhibitors that retain robust activity against NQO1 deficient tumor cell lines, produced by genetic engineering of *Streptomyces hygroscopicus* NRRL3602.

The biosynthesis of geldanamycin is initiated by the starter unit 3-amino-5-hydroxybenzoic acid (AHBA). Subsequent extension of this starter unit by the polyketide synthase (PKS) and cyclization produces a macrolactam, which is then subjected to post-PKS carbamoylation, 4,5-dehydrogenation, and several oxidation steps to produce the final product.13-15 The nonbenzoquinone ansamycins reblastatin and autolytimycin have been reported, presumably resulting from natural mutations or deletions in post-PKS modification genes.¹⁶⁻¹⁸ Genetic engineering previously resulted in non-benzoquinone geldanamycin analogues such as KOS-1559 and autolytimycin (KOS-1806) through oxidase knockout or PKS modification.^{13,14} but this approach necessarily produces a very limited number of new compounds, most of which have poor in vitro cytostatic activity. During this previous work, an AHBA-deficient mutant of the geldanamycin producing strain was generated by the Kosan research group to positively identify the geldanamycin biosynthetic gene cluster and the genes involved in its accessory pathways.¹⁴ We surmised that a broader range of diversity could be generated by feeding analogues of the AHBA starter unit to such an AHBA-deficient mutant strain. A panel of ~ 30 substituted 3-aminobenzoic acids and related heterocycles was prepared (Supporting Information Figure 1) and fed to cultures of the AHBA-deficient mutant strain K554-161. Ten of the AHBA analogues were incorporated into novel non-benzoquinone ansamycins as identified by LC/MS/MS analyses of the culture broths (Supporting Information Table 1). Generally, AHBA analogues having small hydrophobic substituents were accepted by the geldanamycin PKS to produce non-benzoquinone ansamycins, while feeding of AHBA analogues with more polar substituents or heterocycles did not lead to detectable ansamycin products. In many cases, more than one product was obtained because of partial post-PKS processing.

Several detected ansamycin products were fermented on a larger scale to provide purified compounds for full structural characterization and further study (Table 1). These compounds were completely characterized by detailed NMR analysis (Supporting Information compound data). Examination of binding to the purified N-terminal domain of human Hsp90 α using isothermal titration calorimetry or a scintillation proximity assay^{19,20} demonstrated that the non-benzoquinone ansamycins are tight binders to the ATP binding site, having affinities between 10 and 10³ times greater than those of the quinone form of tanespinycin (Table 1).

Cell growth inhibition assays demonstrated activities similar to those of tanespimycin and alvespimycin for three of the five isolated molecules (1, 3, and 5) against a panel of cancer cell lines (Table 1). There is no obvious correlation between the binding affinity to the purified N-terminal domain of human Hsp90 α and cytostatic activity, in keeping with earlier observations,^{21–23} suggesting that binding to the purified N-terminal domain is necessary but not sufficient for activity.

As previously reported, cell growth inhibitory activity of the quinone-containing Hsp90 inhibitors tanespimycin and alvespimycin against NQ01-deficient cell lines is relatively poor.⁸ However, the non-benzoquinone analogues described above maintain potency against the NQ01-deficient cell lines MDA-468 and NCI-H596 (Table 1). Further, addition of the NQ01 inhibitor dicumarol to the NQ01 positive cell lines SKBr3 and MCF7 had no effect on the cytostatic potency of **1**, despite significantly reducing the potency of tanespimycin and alvespi-

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^{*a*} Abbreviations: Hsp90, heat shock protein 90; NQO1, NAD(P)H/quinone oxidoreductase 1; AHBA, 3-amino-5-hydroxybenzoic acid; PKS, polyketide synthase; 17-AAG, 17-allylamino-17-demethoxygeldanamycin; 17-DMAG, 17-(2-dimethylamino)ethylamino-17-demethoxygeldanamycin.

Table 1. Products isolated from fermentations of K554-161 strain fed with either 3-amino-5-chlorobenzoic acid or 3-aminobenzoic acid^a



tanespimycin	760	160	44	240	202	58	1500	1600
alvespimycin	500	87	58	122	57	71	1600	1100
Compound 1	50	n.d.	55	141	120	57	89	190
Compound 2	n.d	n.d.	394	350	422	330	443	710
Compound 3	n.d.	5.4	96	320	240	139	190	390
Compound 4	n.d.	0.8	835	840	1050	610	420	1200
Compound 5	n.d.	2.5	83	158	179	87	160	325

^{*a*} Hsp90 binding was determined by isothermal titration calorimetry (ITC) or scintillation proximity assay (SPA) using purified recombinant human Hsp90 α N terminal domain, and growth inhibition effects on indicated cancer cell lines were determined after 72 h exposure to the drugs using the CellTiter-Glo cell viability assay from Promega Co. Values shown are means of three independent determinations. The standard deviations were in all the cases less than 10% of the corresponding means. n.d., not determined.



Figure 1. In vivo efficacy of **1**. COLO205 tumor bearing mice were dosed iv with vehicle or **1** or ip, with tanespimycin as indicated. (a) Tumors were collected 4 or 24 h after a single dose of tanespimycin or **1** to determine drug levels in the tumors by LC/MS analysis. Tumor samples were analyzed for levels of Hsp70 (b) or ErbB2 (c) by ELISA assay 4 or 24 h after a single dose of 90 mg/kg tanespimycin or 30 mg/kg **1**. (d) The impact on tumor growth of iv dosing COLO205 tumor-bearing mice with vehicle, 15 mg/kg tanespimycin, or 90 mg/kg tanespimycin, 15 mg/kg of **1**, or 30 mg/kg **1** daily for 5 days with a 2 day holiday for a total of 13 doses (n = 10 mice per group) is shown.

mycin (Supporting Information Table 2). Taken together, these results indicate that the activity of the non-benzoquinone

ansamycins created in this study is independent of reductive activation by NQO1.

Analysis of effects on different Hsp90 client proteins including ErbB2, Raf-1, and pAkt corroborates Hsp90 as the target of the non-benzoquinone ansamycins. Treatment of SKBr3 cells with the potent cytostatic analogues results in the rapid loss of the Hsp90 client ErbB2 and induction of Hsp70, whereas treatment with the less active analogues shows less effect on these markers of Hsp90 inhibition^{24–26} (Supporting Information Figure 2). The response of the client proteins in these cells to treatment by the potent non-benzoquinone ansamycins thus mirrors that observed following treatment with tanespimycin or alvespimycin.

In vivo pharmacokinetic and pharmacodynamic measurements using COLO205 xenograft-bearing mice treated with 1 demonstrated that significant levels of this non-benzoquinone ansamycin are retained in the tumor 24 h after dosing (Figure 1a). Accumulation and retention of 1 in tumor tissue is associated with induction of Hsp70 expression and depletion of the Hsp90 client protein ErbB2, both of which are established pharmacodynamic markers of Hsp90 inhibition (Figure 1b,c).

Consistent with its accumulation in tumor tissue and its ability to induce the hallmark pharmacodynamic signature of Hsp90 inhibition, **1** demonstrated robust and dose-dependent efficacy in the COLO205 mouse xenograft model (Figure 1d). The COLO205 cell line expresses the V600E activating mutant version of BRaf and overexpresses Her2, and treatment with tanespimycin has been shown to induce the degradation of both of these oncogenic proteins.^{27–29} Compound **1** demonstrated significantly greater potency in vivo than tanespimycin as reflected by the observation that 15 mg/kg **1** showed inhibition of the tumor growth greater than that obtained with the same dose of tanespimycin, and 30 mg/kg **1** showed efficacy equivalent to that seen with 90 mg/kg tanespimycin. Compound **1** was well-tolerated upon iv dosing at 15 and 30 mg/kg on a qdx5 schedule in this model (Supporting Information Figure 3).

Feeding AHBA analogues has been successfully used to make pactamycin analogues.¹⁸ Recently the isolation of geldanamycin analogues prepared by mutasynthesis was reported by Kim et al.,³⁰ but no biological characterization of the compounds was described by the authors. Of interest, one of the reported compounds derived from 3-aminobenzoic acid, **3**, was independently produced by us and proved to be potent in the standard 72 h cell growth inhibition assay (Table 1). Compound **3** failed to demonstrate any efficacy in the mouse COLO205 xenograft model, however. Further investigation into this compound is ongoing and will be reported in a subsequent paper.

Precursor-directed biosynthesis using an AHBA-deficient mutant of the geldanamycin producing strain *S. hygroscopicus* NRRL3602 thus provides a unique method of introducing chemical diversity into the ansamycin scaffold. This approach has been used to generate new compounds having in vitro potencies comparable to those of the lead Hsp90 inhibitors under clinical evaluation, higher antiproliferative activity against NQO1 deficient cell lines, potent and long-lasting effects on Hsp90 client proteins in vivo, and robust, superior efficacy in mouse xenograft models.

Supporting Information Available: Experimental details, NMR and LC/MS/MS data for isolated compounds, panel of AHBA analogues used for preparation of compounds, effect of compounds on client proteins, impact of treatment on body weight, effect of dicumarol on cytotocity of isolated compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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