Published in final edited form as:

J Pharm Sci. 2008 December ; 97(12): 5147–5157. doi:10.1002/jps.21394.

Stability of the Hsp90 inhibitor 17AAG hydroquinone and prevention of metal-catalyzed oxidation

Wenchang Guo, David Siegel, and David Ross

Department of Pharmaceutical Sciences, School of Pharmacy and Cancer Center, University of Colorado Denver, Denver, CO 80262 USA

Abstract

17-(allylamino)-17-demethoxygeldanamycin (17AAG) is a benzoquinone ansamycin Hsp90 inhibitor which has promising anticancer activity *in vitro*, in animal models and in clinical trials. 17AAG has poor water-solubility which is a potential problem for clinical formulation. The hydroquinone derivative of 17AAG, 17AAGH₂, is considerably more water soluble and since we previously demonstrated that 17AAGH₂ was a more potent Hsp90 inhibitor than its parent quinone, it is a good candidate for clinical use and is currently in clinical trials. However, 17AAGH₂ can be oxidized back to 17AAG under aerobic conditions so we tested the relative stability of 17AAGH₂ and the effect of different metal ions and metal chelators on the oxidation of 17AAGH₂. We found that copper could accelerate 17AAGH₂ oxidation while copper chelators such as D-penicillamine could inhibit oxidation. Human serum albumin (HA) has copper-binding ability and we found that HA diminished the rate of 17AAGH₂ oxidation. Although we found that 17AAG could associate with HA, no association was observed between 17AAGH₂ and HA. In summary, our data demonstrates that copper chelators can prevent 17AAGH₂ oxidation and suggests that HA prevents 17AAGH₂ oxidation via a copper chelation mechanism. Agents that prevent oxidation may be useful in clinical formulations of 17AAGH₂.

Keywords

17AAG; hydroquinone; Hsp90; copper; oxidation; human serum albumin; NQO1; reduction; stability; solubility

Introduction

Hsp90 is emerging as an interesting anticancer drug target.¹ Hsp90 is a chaperone protein that has ATPase activity which is essential for its chaperone function.²⁻⁴ Hsp90 is expressed at high levels in many tumors including melanoma, lung cancer, breast cancer, colon cancer, prostate cancer and leukemia⁵⁻⁶ and is critical for the folding and stability of a number of oncogenic proteins including Raf-1, mutant p53, ErBb2, Hif-1 α , topoisomerase II and androgen/estrogen receptors.⁷⁻¹¹ Inhibition of Hsp90 leads to proteasome-mediated depletion of these “client” proteins, and therefore many oncogenic signals can be blocked simultaneously by inhibition of Hsp90.¹¹⁻¹³

Benzoquinone ansamycins are a class of Hsp90 inhibitors that inhibit Hsp90 by binding to the N-terminal ATP binding pocket of Hsp90 to block ATPase activity.¹⁴ Geldanamycin (GM) was the first drug in this class and had promising anticancer activity *in vitro* and in animal

models.¹⁵ However, GM was withdrawn from clinical trials due to liver toxicity.¹⁶ 17AAG is an analogue of GM, which maintains Hsp90 inhibitory ability but has greatly decreased liver toxicity.¹⁷⁻¹⁸ Kamal *et al*¹⁹ reported that 17AAG binds more tightly to Hsp90 in cancer cells, which is primarily in the form of a heteroprotein complex, than to homodimeric Hsp90 in normal cells. This could result in the selective accumulation of 17AAG in cancer cells but not in normal cells.¹⁹ 17AAG has promising anticancer effects *in vitro* and *in vivo* and is now in phase II clinical trials.²⁰⁻²² However, 17AAG has very poor water solubility and formulation has been a barrier for its clinical application.²³⁻²⁵

We demonstrated that 17AAG could be reduced by NQO1 to its corresponding hydroquinone 17AAGH₂.²⁴ 17AAGH₂ exhibited superior Hsp90 inhibition compared to its parent quinone, 17AAG.²⁴ Since 17AAGH₂ has increased water solubility, using the hydroquinone directly may be an ideal method to overcome the formulation problems of poor water-solubility for the clinical application of 17AAG. This approach has been employed in the development of a 17AAGH₂ salt as a more soluble and active form of 17AAG.²⁵ This preparation greatly increased water-solubility compared to 17AAG.²⁵ However, 17AAGH₂ is susceptible to auto-oxidation and additional stabilization methods are required in clinical formulations.²⁴⁻²⁵

Metal ions such as copper have been considered as a catalyst in many oxidation processes.²⁶⁻²⁷ and chelators can prevent metal catalyzed oxidation.²⁸ D-penicillamine, a copper chelator, has been used clinically to treat diseases associated with elevated copper such as Wilson's disease.²⁹ In addition, studies have shown that human serum albumin (HA) has antioxidant activity as a result of chelating metal ions and copper binding sites in HA have been characterized.³⁰⁻³²

In this study, the effect of different metal ions and metal chelators on 17AAGH₂ oxidation was characterized. To study the role of HA in 17AAGH₂ oxidation, the ability of HA to prevent 17AAGH₂ oxidation as well as its ability to chelate copper was investigated.

Experiment methods

Materials

17-(Allylamino)-17-demethoxygeldanamycin (17-AAG) was obtained from Invivogen Inc (San Diego, CA). 2,6-Dichlorophenol-indophenol (DCPIP), NADH, NADPH, bovine serum albumin (BSA), human serum albumin (HA), 2, 3-dimethoxy-1, 4-naphthoquinone (DMNQ), D-penicillamine and Aquanal-plus (Cu) detection kit were obtained from the Sigma Chemical Co (St. Louis, MO). Centricon concentrators (10K) were obtained from Millipore Inc. (Billerica, MA). Recombinant human NQO1 (rhNQO1) was purified from *E. coli* as previously described.³³ The activity of rhNQO1 was 4.5 μ moles DCPIP/min/mg protein.

HPLC analysis

17AAG, 17AAGH₂ and NADH oxidation during the metabolism of 17AAG by rhNQO1 were analyzed by high performance liquid chromatography (HPLC). HPLC was performed on a Luna C18 5 μ m, 4.6 $\times$ 250 mm reverse-phase column (Phenomenex, Torrance, CA) at room temperature. For 17AAG and 17AAGH₂ determination, HPLC conditions were as follows: buffer A, 50 mM ammonium acetate (pH 4) containing 10 μ M D-penicillamine; buffer B, methanol (100%). Both buffers were continuously bubbled with argon to prevent 17AAGH₂ oxidation. Gradient, 30% B to 90% B over 10 minutes then 90% B for 5 minutes (flow rate of 1 ml/min). The sample injection volume was 50 μ l and the detection wavelength was 270 nm. For NADH determination, HPLC conditions were as follows: Buffer A, 10 mM potassium phosphate, pH 7.4; buffer B, 50% (v/v) 10 mM potassium phosphate (pH 7.4) and 50% (v/v)

methanol. Gradient, 25% B for 10 minutes (flow rate of 1 ml/min). The sample injection volume was 50 μ l and the detection wavelength was 340 nm.

Prevention of 17AAGH₂ oxidation by metal chelators

The effect of metals and chelators on 17AAGH₂ oxidation was determined by measuring the oxygen consumption rate during the oxidation of 17AAGH₂. Reaction conditions: 17AAG 50 μ M, NADH 500 μ M and rhNQO1 1.5-11.8 μ g were incubated in 50 mM potassium phosphate buffer (pH 7.4, 3 ml) in the presence or absence of BSA 1 mg/ml or D-penicillamine 10 μ M, detapac 10 μ M, desferal 10 μ M, FeSO₄ 10 μ M, FeCl₃ 10 μ M or CuSO₄ 10 μ M at 35°C. Reactions were started with rhNQO1 and oxygen consumption was measured over 10 minutes using a Clark electrode. The reactions were stopped with an equal volume of ice cold methanol and NADH concentrations were analyzed immediately by HPLC at 340 nm. The relative stability of 17AAGH₂ was expressed as nmol oxygen consumption per nmol NADH oxidized.

Stimulation of 17AAGH₂ oxidation by copper

The effect of Cu⁺⁺ on 17AAGH₂ oxidation was determined by measuring the oxygen consumption rate during the oxidation of 17AAGH₂. Reaction conditions: 17AAG 50 μ M, NADH 500 μ M, rhNQO1 1.5-11.8 μ g and CuSO₄ 0-100 μ M were incubated in 50 mM potassium phosphate buffer (pH 7.4, 3 ml) at 35°C. Reactions were started with rhNQO1 and oxygen consumption was measured over 10 minutes using a Clark electrode. The reactions were stopped with an equal volume of ice cold methanol and NADH concentrations were analyzed immediately by HPLC at 340 nm. The relative stability of 17AAGH₂ was expressed as nmol oxygen consumption per nmol NADH oxidized.

Prevention of 17AAGH₂ oxidation by human serum albumin (HA)

The effect of HA on 17AAGH₂ oxidation was determined by measuring the oxygen consumption rate during the oxidation of 17AAGH₂. Reaction conditions: 17AAG 50 μ M, NADH 500 μ M, rhNQO1 1.5-11.8 μ g and HA 0-10% in the presence or absence of CuSO₄ 10 μ M were incubated in 50 mM potassium phosphate buffer (pH 7.4, 3 ml) at 35°C. Reactions were started with rhNQO1 and oxygen consumption was measured over 10 minutes using a Clark electrode. The reactions were stopped with an equal volume of ice cold methanol and NADH concentration was analyzed immediately by HPLC at 340 nm. The relative stability of 17AAGH₂ was expressed as nmol oxygen consumption per nmol NADH oxidation.

Chelation of copper by HA

The ability of HA to chelate copper was measured using an Aquanal-plus (Cu) detection kit. Briefly, CuSO₄ 10 μ M was mixed with HA 0-5% in 50 mM potassium phosphate buffer (pH, 7.4, 1 ml) at room temperature and transferred to a Centricon concentrator (10K) and centrifuged at 10,000 rpm for 20-60 min until all the solution was in the bottom container. Copper concentrations in the protein-free membrane filtrates were determined using the Aquanal-plus (Cu) detection kit according to manufacturer's directions.

Association of 17AAG and 17AAGH₂ with HA

The association of 17AAG and 17AAGH₂ with HA was determined using membrane filtration and HPLC. For the association of 17AAG with HA, 17AAG 50 μ M was incubated with HA 0-5% in 50 mM potassium phosphate buffer (pH 7.4, 1 ml) at room temperature for 5 min and transferred to a Centricon concentrator (10K) and centrifuged at 10,000 rpm for 20-60 min until all the solution was in the bottom container. For the association of 17AAGH₂ with HA, 17AAG 50 μ M, NADH 500 μ M and rhNQO1 11.8 μ g were incubated in 50mM potassium phosphate buffer (1 ml), pH 7.4, at room temperature for 10 minutes to generate 17AAGH₂, then incubated with HA 0-5% for 5 min and transferred to a Centricon concentrator (10K) and

centrifuged at 10,000 rpm for 20-60 min until all the solution was in the bottom container. 17AAGH₂ and 17AAG concentrations in the protein-free membrane filtrates were determined by HPLC at 270 nm. The association type of 17AAG (covalent or non-covalent) with HA was also determined by HPLC. Briefly, 17AAG 50 μ M was incubated in 50 mM potassium phosphate buffer (pH 7.4, 1 ml) in the presence or absence of 5% HA at room temperature for 30 min and 17AAG concentrations were analyzed by HPLC at 270 nm.

Stabilization of 17AAGH₂ by HA

The effect of HA on the stability of 17AAGH₂ generated by rhNQO1 was analyzed directly by HPLC. Briefly, 17AAG 50 μ M, NADH 500 μ M and rhNQO1 11.8 μ g in 50 mM potassium phosphate buffer (pH 7.4, 1 ml) were incubated at room temperature for 10 minutes to generate 17AAGH₂ then placed in a Centricon concentrator (10K) with 1 ml of 50 mM potassium phosphate buffer (pH 7.4) in the presence or absence of 5% HA. To analyze the effect of copper concentrations on the stability of 17AAGH₂, experiments were performed in the presence or absence of 10 μ M CuSO₄ in the bottom container of the Centricon concentrator. The samples were centrifuged at 10,000 rpm for 20 minutes. 17AAGH₂ and 17AAG concentrations in the membrane filtrates were determined by HPLC at 270 nm.

Increase in 17AAG solubility by HA

The effect of HA on 17AAG solubility was measured by determining the maximal solubility of 17AAG with 0%-20% HA. Briefly, 17AAG (0.5 mg) was incubated in 50 mM potassium phosphate buffer (pH 7.4, 200 μ l) containing HA (0%-20%) overnight. The saturated solution was centrifuged at 13,000 rpm for 20 min. The supernant was diluted with methanol and 17AAG concentrations were determined by HPLC at 270 nm.

Results

The relative stability of 17AAGH₂ was studied by measuring the ratio of oxygen consumption rate and the NADH oxidation rate during the reduction of 17AAG by rhNQO1 (Scheme 1). In these experiments, 17AAGH₂ was generated by incubation of 17AAG with rhNQO1 and NADH. During the incubation, the oxygen consumption rates were measured using a Clark electrode and the NADH oxidation rates were measured using HPLC. The relative stability of 17AAGH₂ was expressed as nmol oxygen consumed per nmol NADH oxidized. DMNQ generates an unstable hydroquinone after reduction and was used as a positive control in these experiments.³⁴ In 50 mM potassium phosphate buffer, pH 7.4 at 35°C, 17AAGH₂ was relatively stable compared to DMNQ (figure 1). This observation is consistent with our previous report that 17AAGH₂ has appreciable stability with a half-life of 90 min.²⁴ Metal ions Fe²⁺ and Fe³⁺ and metal chelators Detapac and desferal had little or no effect on the oxidation of 17AAGH₂ (figure 1). The rate of 17AAGH₂ oxidation, however, was greatly stimulated by Cu²⁺ and decreased by copper chelators such as D-penicillamine (figure 1). BSA had a similar effect as D-penicillamine and also prevented 17AAGH₂ oxidation (figure 1). The ability of Cu²⁺ to stimulate oxidation has been well-characterized.³⁵ It was reported that Cu²⁺ could stimulate the oxidation of hydroquinones such as the benzene metabolite hydroquinone.³⁶ The ability of Cu²⁺ to stimulate 17AAGH₂ oxidation was further examined using a range of Cu²⁺ concentrations. As expected, the rate of 17AAGH₂ oxidation increased in a dose-dependent manner with increasing Cu²⁺ concentrations (figure 2). The effect was unrelated to the sulfate counter ion since iron sulfate did not stimulate autooxidation.

Based on the documented copper binding ability of HA³⁰⁻³² and the ability of BSA to prevent 17AAGH₂ oxidation (figure 1), the effects of HA on 17AAGH₂ oxidation were examined. The rate of 17AAGH₂ oxidation was prevented by HA in a dose-dependent manner (figure 3). In addition, the stimulation of 17AAGH₂ oxidation by Cu²⁺ was also prevented by HA (figure

3). The ability of HA to prevent 17AAGH₂ oxidation was saturated under these conditions at concentrations >1% HA (figure 3). The ability of HA to chelate Cu²⁺ was examined by incubation of HA with Cu²⁺ and membrane-filtration. Centricon concentrators (10K) were employed to prepare protein free solutions. Prior to these experiments, the efficiency of the Centricon concentrator (10K) to exclude HA was examined. After incubation of HA (0%-20%) in 50 mM potassium phosphate buffer (pH 7.4, 1 ml) and subjecting the solution to membrane-filtration, no protein could be detected in the filtrates using the Lowry protein detection method (data not shown).³⁷ The Cu²⁺ concentrations in the protein-free filtrates were measured using an Aquanal-plus copper detection kit. In this assay, the Cu²⁺ in the protein-free filtrates could complex with cuprizone to form a blue color with an absorbance maximum at 600 nm. In agreement with the previous reports,³⁰⁻³² Cu²⁺ was chelated by HA in a dose-dependent manner (figure 4).

Lipophilic drugs are known to interact with HA³⁸⁻³⁹ and it is possible that HA could sequester 17AAG or 17AAGH₂ in a hydrophobic interaction. The association of 17AAG and 17AAGH₂ with HA was measured by HPLC after incubation of either 17AAG or 17AAGH₂ (generated by NQO1 and NADH) with HA. In these experiments, 17AAG or 17AAGH₂ were incubated with HA (0-5%) and centrifuged through a Centricon concentrator (10K). 17AAG and 17AAGH₂ concentrations in the protein-free filtrates were measured by HPLC. 17AAG was found to associate with HA in a dose-dependent manner (figure 5). The ability of 17AAGH₂ to associate with HA was greatly decreased compared to 17AAG and only a small amount of association of 17AAGH₂ with HA could be detected at the highest concentration of protein used (figure 5).

In order to determine whether the association of 17AAG with HA was covalent or non-covalent, the recovery of 17AAG on HPLC in the presence and absence of HA was measured. If the association of 17AAG with HA was non-covalent, disassociation would occur on HPLC analysis and free 17AAG would be detected. If the reaction of HA with 17AAG was covalent, 17AAG would be decreased and an adduct would be detected during HPLC analysis. As shown in Figure 6, the recovery of 17AAG after incubation with 5% HA was essentially 100% relative to controls in the absence of HA. These data suggest that the association of 17AAG with HA was non-covalent.

The decreased oxygen consumption detected during the oxidation of 17AAGH₂ in the presence of HA (figure 3) could also be due to sequestration of 17AAG by HA. This would result in less 17AAG being available for NQO1 metabolism and could thus possibly decrease the amount of 17AAGH₂ generation. Therefore, a direct measurement of 17AAGH₂ stability by HPLC was performed to confirm the ability of HA to prevent 17AAGH₂ oxidation. In these experiments, 17AAGH₂ was generated by NQO1 and NADH and then centrifuged through a Centricon concentrator (10K). The 17AAGH₂ containing filtrates were incubated in the presence or absence of 5% HA and in the presence or absence of 10 μM CuSO₄. The oxidation of 17AAGH₂ was measured using HPLC at the indicated time periods. Under aerobic conditions at pH 7.4, 17AAGH₂ was slowly auto-oxidized with a half-life of about 90 min (figure 7A) and this was consistent with our previous report.²⁴ In the presence of 5% HA, the oxidation of 17AAGH₂ was inhibited and its half-life was extended by HA to about 180 min (figure 7B). With the addition of Cu²⁺, 17AAGH₂ was oxidized very rapidly (figure 7C) but the stimulation of 17AAGH₂ oxidation by Cu²⁺ was abrogated in the presence of HA (figure 7D).

Based on the non-covalent association with 17AAG (figure 6), HA may also increase the solubility of 17AAG. The solubility of 17AAG was examined in the presence of varying HA concentrations. As predicted, 17AAG solubility was increased by HA in a concentration-

dependent manner. In phosphate buffer at pH 7.4, HA (20%) could increase 17AAG solubility by about 12 fold (figure 8).

Discussion

We have demonstrated that 17AAGH₂ is a more potent Hsp90 inhibitor than its parent quinone 17AAG.²⁴ 17AAGH₂ has increased water-solubility and this may overcome the solubility problems observed in the clinical use of the parent quinone, 17AAG.²⁴⁻²⁵ The hydroquinone of 17AAG, 17AAGH₂, is currently in clinical trials.⁴⁰ However, 17AAGH₂ is susceptible to auto-oxidation which may cause formulation problems.²⁴⁻²⁵ So the development of stabilization methods for 17AAGH₂ has relevance for clinical applications and the current clinical formulation employs low pH to inhibit 17AAGH₂ oxidation⁴⁰.

Among the factors influencing 17AAGH₂ oxidation, metal ions would be predicted to play an important role.³⁵ Many oxidation processes can be stimulated by metal ions such as Cu²⁺ and Fe³⁺, and metal ions act as catalysts and function as electron transfer mediators in these processes.^{26,36} Metal chelators can chelate metal ions, therefore can inhibit metal-catalyzed oxidation.²⁷⁻²⁸ The copper chelator, D-penicillamine, is used clinically to treat Wilson's disease which is caused by elevated copper levels.²⁹ In this study, the effect of different metal ions and metal chelators on 17AAGH₂ oxidation was studied. As expected, the oxidation of 17AAGH₂ was stimulated by Cu²⁺ and prevented by copper chelating agents such as D-penicillamine. BSA had a similar effect as D-penicillamine on 17AAGH₂ oxidation which may be due to its copper binding ability.⁴¹ Interestingly, Fe²⁺ and Fe³⁺ had little effect on 17AAGH₂ oxidation. As well as BSA, HA has a similar ability to chelate copper and copper binding sites in HA have been characterized.^{30, 32} HA has been found to have nonenzymatic antioxidant properties which are likely related to its ability to chelate metal ions.³¹ We confirmed previous reports³⁰⁻³² that copper was chelated by HA, but in addition, we found that HA prevented both basal and copper-stimulated 17AAGH₂ oxidation.

Many hydrophobic drugs can bind with HA³⁸⁻³⁹ and HA is the primary protein in serum that binds acidic drugs.⁴² Previous studies using mouse models and membrane filtration suggested that >90% of 17AAG in mouse serum was protein bound.²³ In this study, the ability of HA to bind 17AAG and 17AAGH₂ was examined. We found that 17AAG associated with HA but once 17AAG was reduced by NQO1 to 17AAGH₂, association was markedly decreased reflecting the dramatic decrease in hydrophobicity of 17AAGH₂ compared to 17AAG.²⁴⁻²⁵ The association between 17AAG and HA appeared to be non-covalent and this association could increase the solubility of 17AAG.

The results obtained from this study may be useful for the clinical application of 17AAGH₂. A copper chelator such as HA may be used in formulations of 17AAGH₂ to inhibit auto-oxidation. HA was preferred compared to D-penicillamine because HA not only prevents 17AAGH₂ oxidation but also increases the solubility of any 17AAG generated. The ability of HA to increase the solubility of 17AAG may also be applied in formulations of the quinone, 17AAG. In summary, we found that the oxidation of 17AAGH₂ was stimulated by copper and inhibited by copper chelators. HA has copper binding ability and therefore inhibits the oxidation of 17AAGH₂. In addition, HA associates with 17AAG through a non-covalent interaction and increases 17AAG solubility.

Acknowledgements

This work was supported by National Institute of Health grant R01-CA51210

Abbreviations

Hsp90, heat shock protein 90; GM, geldanamycin; 17AAG, 17-(allylamino)-17-demethoxygeldanamycin; 17AAGH₂, 17-(allylamino)-17-demethoxygeldanamycin hydroquinone; HPLC, high performance liquid chromatography; DMSO, dimethylsulfoxide; BSA, bovine serum albumin; HA, human serum albumin; NQO1, NAD(P)H:Quinone oxidoreductase 1.

References

1. Beliakoff J, Whitesell L. Hsp90: an emerging target for breast cancer therapy. *Anticancer Drugs* 2004;15:651–62. [PubMed: 15269596]
2. Nadeau K, Sullivan MA, Bradley M, Engman DM, Walsh CT. 83-kilodalton heat shock proteins of trypanosomes are potent peptide-stimulated ATPases. *Protein Sci* 1992;1(8):970–9. [PubMed: 1304385]
3. Hutchison KA, Dittmar KD, Pratt WB. All of the factors required for assembly of the glucocorticoid receptor into a functional heterocomplex with heat shock protein 90 are preassociated in a self-sufficient protein folding structure, a “foldosome”. *J Biol Chem* 1994;269(45):27894–9. [PubMed: 7961721]
4. Panaretou B, Prodromou C, Roe SM, O'Brien R, Ladbury JE, Piper PW, Pearl LH. ATP binding and hydrolysis are essential to the function of the Hsp90 molecular chaperone in vivo. *EMBO J* 1998;17(16):4829–36. [PubMed: 9707442]
5. Wataba K, Saito T, Fukunaka K, Ashihara K, Nishimura M, Kudo R. Over-expression of heat shock proteins in carcinogenic endometrium. *Int J Cancer* 2001;91(4):448–56. [PubMed: 11251965]
6. Myung JK, Afjehi-Sadat L, Felizardo-Cabatic M, Slavc I, Lubec G. Expressional patterns of chaperones in ten human tumor cell lines. *Proteome Sci* 2004;2(1):8. [PubMed: 15598346]
7. Schulte TW, Blagosklonny MV, Ingui C, Neckers L. Disruption of the Raf-1-Hsp90 molecular complex results in destabilization of Raf-1 and loss of Raf-1-Ras association. *J Biol Chem* 1995;270(41):24585–8. [PubMed: 7592678]
8. Selkirk JK, Merrick BA, Stackhouse BL, He C. Multiple p53 protein isoforms and formation of oligomeric complexes with heat shock proteins Hsp70 and Hsp90 in the human mammary tumor, T47D, cell line. *Appl Theor Electrophor* 1994;4(1):11–8. [PubMed: 7811761]
9. Xu W, Mimnaugh EG, Kim JS, Trepel JB, Neckers LM. Hsp90, not Grp94, regulates the intracellular trafficking and stability of nascent ErbB2. *Cell Stress Chaperones* 2002;7(1):91–6. [PubMed: 11892991]
10. Minet E, Mottet D, Michel G, Roland I, Raes M, Remacle J, Michiels C. Hypoxia-induced activation of HIF-1: role of HIF-1alpha-Hsp90 interaction. *FEBS Lett* 1999;460(2):251–6. [PubMed: 10544245]
11. Zhang H, Burrows F. Targeting multiple signal transduction pathways through inhibition of Hsp90. *J Mol Med* 2004;82(8):488–99. [PubMed: 15168026]
12. Schulte TW, An WG, Neckers LM. Geldanamycin-induced destabilization of Raf-1 involves the proteasome. *Biochem Biophys Res Commun* 1997;239(3):655–9. [PubMed: 9367823]
13. Imamura T, Haruta T, Takata Y, Usui I, Iwata M, Ishihara H, Ishiki M, Ishibashi O, Ueno E, Sasaoka T, Kobayashi M. Involvement of heat shock protein 90 in the degradation of mutant insulin receptors by the proteasome. *J Biol Chem* 1998;273(18):11183–8. [PubMed: 9556607]
14. Grenert JP, Sullivan WP, Fadden P, Haystead TA, Clark J, Mimnaugh E, Krutzsch H, Ochel HJ, Schulte TW, Sausville E, Neckers LM, Toft DO. The amino-terminal domain of heat shock protein 90 (hsp90) that binds geldanamycin is an ATP/ADP switch domain that regulates hsp90 conformation. *J Biol Chem* 1997;272(38):23843–50. [PubMed: 9295332]
15. Whitesell L, Mimnaugh EG, De Costa B, Myers CE, Neckers LM. Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. *Proc Natl Acad Sci U S A* 1994;91(18):8324–8. [PubMed: 8078881]

16. Supko JG, Hickman RL, Grever MR, Malspeis L. Preclinical pharmacologic evaluation of geldanamycin as an antitumor agent. *Cancer Chemother Pharmacol* 1995;36(4):305–15. [PubMed: 7628050]
17. Egorin MJ, Rosen DM, Wolff JH, Callery PS, Musser SM, Eiseman JL. Metabolism of 17-(allylamino)-17-demethoxygeldanamycin (NSC 330507) by murine and human hepatic preparations. *Cancer Res* 1998;58(11):2385–96. [PubMed: 9622079]
18. Xu L, Eiseman JL, Egorin MJ, D'Argenio DZ. Physiologically-based pharmacokinetics and molecular pharmacodynamics of 17-(allylamino)-17-demethoxygeldanamycin and its active metabolite in tumor-bearing mice. *J Pharmacokinet Pharmacodyn* 2003;30(3):185–219. [PubMed: 14571691]
19. Kamal A, Thao L, Sensintaffar J, Zhang L, Boehm MF, Fritz LC, Burrows FJ. A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. *Nature* 2003;425(6956):407–10. [PubMed: 14508491]
20. Hostein I, Robertson D, DiStefano F, Workman P, Clarke PA. Inhibition of signal transduction by the Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin results in cytostasis and apoptosis. *Cancer Res* 2001;61(10):4003–9. [PubMed: 11358818]
21. Münster PN, Basso A, Solit D, Norton L, Rosen N. Modulation of Hsp90 function by ansamycins sensitizes breast cancer cells to chemotherapy-induced apoptosis in an RB- and schedule-dependent manner. 2001. See: Sausville EA. Combining cytotoxics and 17-allylamino, 17-demethoxygeldanamycin: sequence and tumor biology matters. *Clin. Cancer Res* 7:2155–2158. [PubMed: 11489788]
22. Ronnen EA, Kondagunta GV, Ishill N, Sweeney SM, Deluca JK, Schwartz L, Bacik J, Motzer RJ. A phase II trial of 17-(Allylamino)-17-demethoxygeldanamycin in patients with papillary and clear cell renal cell carcinoma. *Invest New Drugs* 2006;24(6):543–6. [PubMed: 16832603]
23. Egorin MJ, Zuhowski EG, Rosen DM, Sentz DL, Covey JM, Eiseman JL. Plasma pharmacokinetics and tissue distribution of 17-(allylamino)-17-demethoxygeldanamycin (NSC 330507) in CD2F1 mice. *Cancer Chemother Pharmacol* 2001;47(4):291–302. [PubMed: 11345645]
24. Guo W, Reigan P, Siegel D, Zirrolli J, Gustafson D, Ross D. Formation of 17-allylamino-demethoxygeldanamycin (17-AAG) hydroquinone by NAD(P)H:quinone oxidoreductase 1: role of 17-AAG hydroquinone in heat shock protein 90 inhibition. *Cancer Res* 2005;65(21):10006–15. [PubMed: 16267026]
25. Ge J, Normant E, Porter JR, Ali JA, Dembski MS, Gao Y, Georges AT, Grenier L, Pak RH, Patterson J, Sydor JR, Tibbitts TT, Tong JK, Adams J, Palombella VJ. Design, synthesis, and biological evaluation of hydroquinone derivatives of 17-amino-17-demethoxygeldanamycin as potent, water-soluble inhibitors of Hsp90. *J Med Chem* 2006;49(15):4606–15. [PubMed: 16854066]
26. Houchin OB. A rapid colorimetric method for the quantitative determination of copper oxidase activity (ceruloplasmin). *Clin Chem* 1958;4(6):519–23. [PubMed: 13608914]
27. Chidambaram MV, Barnes G, Frieden E. Inhibition of ceruloplasmin and other copper oxidases by thiomolybdate. *J Inorg Biochem* 1984;22(4):231–40. [PubMed: 6097647]
28. Lin PS, Kwock L, Hefter K, Misslbeck G. Effects of iron, copper, cobalt, and their chelators on the cytotoxicity of bleomycin. *Cancer Res* 1983;43(3):1049–53. [PubMed: 6186368]
29. Osnorn SB, Walshe JM. Effects of D-penicillamine and dimercaprol on turnover of copper in patients with Wilson's disease. *Lancet* 1958;1(7011):70–3. [PubMed: 13503211]
30. Sarkar B, Wigfield Y. Evidence for albumin--cu(II)--amino acid ternary complex. *Can J Biochem* 1968;46(6):601–7. [PubMed: 5660417]
31. Miller NJ, Johnston JD, Collis CS, Rice-Evans C. Serum total antioxidant activity after myocardial infarction. *Ann Clin Biochem* 1997;34(Pt 1):85–90. [PubMed: 9022893]
32. Bal W, Christodoulou J, Sadler PJ, Tucker A. Multi-metal binding site of serum albumin. *J Inorg Biochem* 1998;70(1):33–9. [PubMed: 9661286]
33. Beall HD, Mulcahy RT, Siegel D, Traver RD, Gibson NW, Ross D. Metabolism of bioreductive antitumor compounds by purified rat and human DT-diaphorases. *Cancer Res* 1994;54(12):3196–201. [PubMed: 8205540]
34. Gant TW, Rao DN, Mason RP, Cohen GM. Redox cycling and sulphhydryl arylation; their relative importance in the mechanism of quinone cytotoxicity to isolated hepatocytes. *Chem Biol Interact* 1988;65(2):157–73. [PubMed: 2835188]

35. Aust SD, Morehouse LA, Thomas CE. Role of metals in oxygen radical reactions. *J Free Radic Biol Med* 1985;1(1):3–25. [PubMed: 3013969]
36. Li Y, Trush MA. Oxidation of hydroquinone by copper: chemical mechanism and biological effects. *Arch Biochem Biophys* 1993;300(1):346–55. [PubMed: 8424668]
37. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193(1):265–75. [PubMed: 14907713]
38. Guentert TW, Oie S, Paalzow L, Frey BM, Brandt R, Aarons LJ, Rowland M. Interaction of mixed micelles formed from glycocholic acid and lecithin with the protein binding of various drugs. *Br J Clin Pharmacol* 1987;23(5):569–77. [PubMed: 3593626]
39. Zini R, Morin D, Jouenne P, Tillement JP. Cicletanine binding to human plasma proteins and erythrocytes, a particular HSA-drug interaction. *Life Sci* 1988;43(25):2103–15. [PubMed: 3210902]
40. Sydor JR, Normant E, Pien CS, Porter JR, Ge J, Grenier L, Pak RH, Ali JA, Dembski MS, Hudak J, Patterson J, Penders C, Pink M, Read MA, Sang J, Woodward C, Zhang Y, Grayzel DS, Wright J, Barrett JA, Palombella VJ, Adams J, Tong JK. Development of 17-allylamino-17-demethoxygeldanamycin hydroquinone hydrochloride (IPI-504), an anti-cancer agent directed against Hsp90. *Proc Natl Acad Sci U S A* 2006;103(46):17408–13. [PubMed: 17090671]
41. Zgierski A, Frieden E. Binding of Cu(II) to non-prosthetic sites in ceruloplasmin and bovine serum albumin. *J Inorg Biochem* 1990;39(2):137–48. [PubMed: 2380706]
42. Du Souich P, Verges J, Erill S. Plasma protein binding and pharmacological response. *Clin Pharmacokinet* 1993;24(6):435–40. [PubMed: 8513647]

Scheme 1.

A proposed model for the reduction of 17AAG by NQO1 and oxidation of 17AAGH₂

Figure 1. Prevention of 17AAGH₂ oxidation by metal chelators

The effect of different metal chelators and metal ions on 17AAGH₂ oxidation was determined by measuring the oxygen consumption rate of 17AAGH₂ generated by rhNQO1 and NADH. Reaction conditions: 17AAG 50 μ M, NADH 500 μ M and rhNQO1 1.5-11.8 μ g were incubated in 50 mM potassium phosphate buffer (pH 7.4, 3 ml) at 35°C in the presence or absence of 1 mg/ml BSA or 10 μ M D-penicillamine, detapac, desferal, FeSO₄, FeCl₃ or CuSO₄. Reactions were started by the addition of rhNQO1 and oxygen consumption was measured using a Clark electrode over 10 minutes. Then the reactions were stopped with an equal volume of ice cold methanol and NADH concentrations were analyzed by HPLC at 340nm. The relative stability of 17AAGH₂ was expressed as nmol oxygen consumption per nmol NADH oxidized. DMNQ is an efficient redox cycling compound and was used as a positive control in these experiments. Mean $\pm$ standard deviation of n = 3. The relative stability of 17AAGH₂ in the presence of CuSO₄, BSA or D-penicillamine was significantly different from 17AAGH₂ only, * p < 0.01. Statistical analysis was performed using a one-way ANOVA with a Tukey multiple comparison test.

Figure 2. Stimulation of 17AAGH₂ oxidation by copper

The effect of copper ions on 17AAGH₂ oxidation was determined by measuring the oxygen consumption rate of 17AAGH₂ generated by rhNQO1 and NADH. Reaction conditions: 17AAG 50 μM, NADH 500 μM, rhNQO1 1.5-11.8 μg and CuSO₄ 0-100 μM were incubated in 50 mM potassium phosphate buffer (pH 7.4, 3 ml) at 35°C. Reactions were started by the addition of rhNQO1 and oxygen consumption was measured using a Clark electrode over 10 minutes. The reactions were stopped with an equal volume of ice cold methanol and NADH concentrations were analyzed by HPLC at 340 nm. The relative stability of 17AAGH₂ was expressed as nmol oxygen consumption per nmol NADH oxidized. Mean ± standard deviation of n = 3. The relative stability of 17AAGH₂ in the presence of 10 μM and 100 μM CuSO₄ was significantly different from no CuSO₄, * p < 0.01. Statistical analysis was performed using a one-way ANOVA with a Tukey multiple comparison test.

Figure 3. Prevention of 17AAGH₂ oxidation by HA

The effect of HA on 17AAGH₂ oxidation was determined by measuring the oxygen consumption rate of 17AAGH₂ generated by rhNQO1 and NADH. Reaction conditions: 17AAG 50 μ M, NADH 500 μ M, rhNQO1 1.5-11.8 μ g and HA 0-10% in the absence (closed bars) or presence of CuSO₄ 10 μ M (hatched bars) were incubated in 50 mM potassium phosphate buffer (pH 7.4, 3 ml) at 35°C. Reactions were started with rhNQO1 and oxygen consumption was measured using a Clark electrode over 10 minutes. The reactions were stopped with an equal volume of ice cold methanol and NADH concentrations were analyzed by HPLC at 340nm. The relative stability of 17AAGH₂ was expressed as nmol oxygen consumption per nmol NADH oxidized. Closed bars, without CuSO₄; open bars, with CuSO₄. Mean $\pm$ standard deviation of n = 3. 0.1%, 1%, 5% and 10% HA were significantly different from no HA, * p<0.01; 0.1 HA+CuSO₄, 1% HA+CuSO₄, 5% HA+CuSO₄ and 10% HA+CuSO₄ were significantly different from CuSO₄, # p<0.001. Statistical analysis was performed using a one-way ANOVA with a Tukey multiple comparison test.

Figure 4. Chelation of copper by HA

The ability of HA to chelate copper was measured using an Aquanal-plus (Cu) detection kit. Briefly, CuSO₄ 10 μM was mixed with HA (0-5%) in 50 mM potassium phosphate buffer (pH 7.4, 1 ml) at room temperature and then transferred to a Centricon concentrator (10K) and centrifuged at 10,000 rpm for 20-60 min until all the solution was in the bottom container. CuSO₄ 10 μM in 50 mM potassium phosphate buffer (pH 7.4, 1 ml) without membrane filtration was used as a control. The copper concentration in the protein-free filtrates was determined using an Aquanal-plus (Cu) detection kit according to manufacturer's instructions. Mean ± standard deviation of n = 3. 0.1%HA, 1%HA and 5%HA was significantly different from control, * p<0.001. Statistical analysis was performed using a one-way ANOVA with a Tukey post-hoc multiple comparison.

Figure 5. Association of 17AAG and 17AAGH₂ with HA

The association of 17AAG and 17AAGH₂ with HA was determined using membrane-filtration and HPLC. For the association of 17AAG with HA, 17AAG 50 μ M was mixed with HA (0-5%) in 50 mM potassium phosphate (pH 7.4, 1 ml) at room temperature and transferred to a Centricon concentrator (10K) and centrifuged at 10,000rpm for 20-60 min until all the solution was in the bottom container. For the association of 17AAGH₂ with human albumin, 17AAG 50 μ M, NADH 500 μ M and rhNQO1 11.8 μ g were incubated in 50 mM potassium phosphate buffer (pH 7.4, 1 ml) at room temperature for 10 minutes to generate 17AAGH₂, then mixed with HA (0-5%) and transferred to the same filter device and centrifuged at 10,000rpm for 20-60 min until all the solution was in the bottom container. 17AAG 50 μ M in 50 mM potassium

phosphate (pH 7.4, 1 ml) without membrane filtration was used as control. 17AAGH₂ and 17AAG concentrations in the protein-free filtrates were determined by HPLC at 270nm. Mean $\pm$ standard deviation of n = 3. For 17AAG, 1% HA and 5% HA were significantly different from control, ** p<0.001. For 17AAGH₂, 5% HA was significantly different from control, * p<0.05. Statistical analysis was performed using a one-way ANOVA with a Tukey post-hoc multiple comparison. Panel A, 17AAG; panel B, 17AAGH₂.

Figure 6. The association of 17AAG with HA was non-covalent

The association of 17AAG with HA was determined by HPLC. Briefly, 17AAG (50 μ M) was incubated in 50 mM potassium phosphate buffer (pH7.4, 1 ml) in the absence (control) or presence of 5% HA at room temperature for 30 min and 17AAG concentrations were determined by HPLC at 270nm. Mean $\pm$ standard deviation of $n = 3$.

Figure 7. Stabilization of 17AAGH₂ by HA

The effect of HA on the stability of 17AAGH₂ generated by rhNQO1 and NADH was analyzed directly by HPLC. Briefly, 17AAG 50 μ M, NADH 500 μ M and rhNQO1 11.8 μ g in 50 mM potassium phosphate buffer (pH 7.4, 1 ml) were incubated at room temperature for 10 minutes to generate 17AAGH₂ then placed in a Centricon concentrator (10K) with 1 ml of 50 mM potassium phosphate buffer, pH 7.4 in the presence or absence of 5% HA or in the presence or absence of 10 μ M CuSO₄ in the bottom container. The samples were centrifuged at 10,000 rpm for 20 minutes. 17AAGH₂ and 17AAG concentrations in the membrane filtrates were determined by HPLC at 270nm. Results are mean $\pm$ standard deviation of n=3. Solid line, 17AAGH₂; dashed line 17AAG. Panel A, no HA; panel B, 5% HA; panel C, 10 μ M CuSO₄; panel D, 10 μ M CuSO₄ + 5% HA.

Figure 8. Increase of 17AAG solubility by HA

The effect of HA on 17AAG solubility was measured using saturated solutions of 17AAG incubated with 0%-20% HA. Briefly, 17AAG (0.5 mg) was incubated in 50 mM potassium phosphate buffer (pH 7.4, 200 μ l) containing HA (0%-20%) overnight. Saturated solutions were centrifuged at 13,000 rpm for 20 min. The supernatant was diluted with methanol and the 17AAG concentration was determined by HPLC at 270 nm. Mean $\pm$ standard deviation of $n = 3$. The solubility of 17AAG in 1%, 5%, 10% and 20% HA was significantly different from no HA, * $p < 0.05$, ** $p < 0.001$. Statistical analysis was performed using a one-way ANOVA with a Tukey post-hoc test for multiple comparisons.