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Enzymatic reduction and glutathione conjugation of benzoquinone ansamycin Hsp90 inhibitors: Relevance for toxicity and mechanism of action

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Abstract

Two electron reduction of benzoquinone ansamycin (BA) Hsp90 inhibitors by NAD(P)H:Quinone Oxidoreductase 1 (NQO1) to hydroquinone ansamycins (BAH₂) leads to greater Hsp90 inhibitory activity. BA can also be metabolized by one-electron reductases and can interact with glutathione, reactions which have been associated with toxicity. Using a series of BA we investigated the stability of the BAH₂ generated by NQO1, the ability of BA to be metabolized by one electron reductases and their conjugation with glutathione. The BA used were GM (geldanamycin), 17AAG (17-(allylamino)-17-demethoxygeldanamycin), 17DMAG (17-demethoxy-17-[[2-(dimethylamino)ethyl]amino]-geldanamycin), 17AG (17-(amino)-17-demethoxygeldanamycin) and 17AEP-GA (17-demethoxy-17-[[2-(pyrrolidin-1-yl)ethyl]amino]-geldanamycin). The relative stabilities of BAH₂ at pH 7.4 were GMH₂>17AAGH₂>17DMAGH₂>17AGH₂, 17AEP-GAH₂. Using human and mouse liver microsomes, and either NADPH or NADH as cofactors, 17AAG had the lowest rate of one-electron reduction while GM had the highest rate. 17DMAG demonstrated the greatest rate of redox cycling catalyzed by purified human cytochrome P450 reductase while 17AAG again had the slowest rate. GM formed a glutathione adduct most readily followed by 17DMAG. The formation of glutathione adducts of 17AAG and 17AG were relatively slow in comparison. These data demonstrate that GM, the most hepatotoxic BA in the series had a greater propensity to undergo redox cycling reactions catalyzed by hepatic one electron reductases and markedly greater reactivity with thiols when compared to the least hepatotoxic analog 17AAG. Minimizing the propensity of BA derivatives to undergo one-electron reduction and glutathione conjugation while maximizing their two-electron reduction to stable Hsp90 inhibitory hydroquinones may be a useful strategy for optimizing the therapeutic index of BA.

Introduction

Hsp90 is a chaperone protein which 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 (Selkirk et al., 1994; Schulte et al., 1995; Minet et al., 1999; Xu et al., 2002; Xu and Neckers, 2007). Inhibition of Hsp90 leads to depletion of these “client” proteins via the ubiquitin-proteasome pathway (Schulte et al., 1997; Imamura et al., 1998) and therefore, many oncogenic signals can be blocked simultaneously by inhibition of Hsp90 (Powers and Workman, 2006).

Benzoquinone ansamycins (BA) (Scheme 1) are a class of Hsp90 inhibitors that bind to the N-terminal ATP binding pocket of Hsp90 to block Hsp90 ATPase activity (Stebbins et al., 1997). Geldanamycin (GM) was the first drug in this class but was withdrawn from clinical trials due to liver toxicity (Supko et al., 1995). 17AAG and 17DMAG are analogs of GM, which maintained Hsp90 inhibition ability but had decreased hepatotoxicity (Behrsing et al., 2005; Glaze et al., 2005; Xiao et al., 2006). 17AAG is in phase II and 17DMAG is in phase I clinical trials (Ronnen et al., 2006; Shadad and Ramanathan, 2006).

We have previously demonstrated that this series of BA can be reduced by NQO1, an obligate two-electron reductase, to their corresponding hydroquinone ansamycins (Guo et al., 2005 and 2006). BAH₂ were more water-soluble, more potent Hsp90 inhibitors and more active at inducing growth inhibition compared to the respective BA (Guo et al., 2005 and 2006). NQO1 is markedly elevated in many human solid tumors (Siegel and Ross, 2000) as well as in some normal tissues and this work demonstrated that two-electron reduction of BA to BAH₂ is an important component of the mechanism of action of these drugs.

Because of the quinone moiety, these compounds may also be metabolized by one-electron reductases such as NADPH-cytochrome P450 reductase and NADH-cytochrome b5 reductase (Egorin et al., 1998; Dikalov et al., 2002). One electron reduction of quinones generates unstable semiquinones and superoxide radicals may be generated during the oxidation of semiquinones by molecular oxygen (Powis, 1989; Ross et al., 1996). Superoxide can then generate reactive oxygen and nitrogen species capable of injuring cells by damaging critical macromolecules (Monks et al., 1992). Since the liver contains high concentrations of one-electron reductases (Murray, 1992), one-electron metabolism of BA resulting in the generation of reactive oxygen species may contribute to the dose-limiting liver toxicity induced by some BA such as GM. To investigate these potentially toxic metabolic routes mediated by one electron reduction, the metabolism of BA by both human and mouse liver microsomes and purified NADPH cytochrome P450 reductase was studied. Human and mouse liver microsomes were used to provide information for both clinical and pre-clinical studies respectively. One-electron mediated redox cycling reactions were quantified by measuring both pyridine nucleotide utilization and oxygen consumption.

Quinones also interact readily with thiols (Ross, 1988; Monks and Lau, 1992) and such reactions can also contribute to toxicity. In 2006, it was reported that GM, 17AAG, 17DMAG and 17AG could form glutathione conjugates at the 19-position on the quinone ring and that interactions with thiols could be important for the mechanism of toxicity of BA (Cysyk et al., 2006). Recently, Lang *et al.* also found that GMH₂ glutathione conjugates were formed during incubation of GM with human liver microsomes and glutathione (Lang et al., 2007). Adduction of glutathione and other thiols in cells may therefore represent another mechanism of BA-induced toxicity. In this study, we have examined the relative rates of both one- and two-electron reduction and rates of glutathione conjugation formation for a series of BA. Our data demonstrate that GM, the most hepatotoxic BA in the series had a greater propensity to undergo redox cycling reactions catalyzed by hepatic one electron reductases and markedly greater reactivity with thiols when compared to the least hepatotoxic analog 17AAG. These data suggest that both one-electron redox reactions and glutathione conjugation may be useful predictors of the potential for hepatotoxicity of this class of drugs.

Experimental methods

Materials

17-(allylamino)-17-demethoxygeldanamycin (17AAG), geldanamycin (GM), 17-demethoxy-17-[[2-(dimethylamino)ethyl]amino]-geldanamycin (17DMAG) and 17-demethoxy-17-[[2-(pyrrolidin-1-yl)ethyl]amino]-geldanamycin (17AEP-GA) were obtained

from Invivogen Inc (San Diego, CA); and 17-(amino)-17-demethoxygeldanamycin (17AG) was provided by the National Cancer Institute and Kosan Biosciences (Hayward, CA). 2, 6-Dichlorophenol-indophenol (DCPIP), NADH, NADPH, glutathione, dicoumarol, potassium phosphate (monobasic and dibasic), β -lapachone, 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) and ammonium acetate were obtained from the Sigma Chemical Co. (St. Louis, MO). [^3H] glutathione was obtained from PerkinElmer Life and Analytical Sciences Inc. (Boston, MA). Methanol and acetonitrile were obtained from Fisher Scientific Inc (Fair Lawn, NJ). 5-methoxy-1,2-dimethyl-3-[(4-nitrophenoxy)methyl]indole-4,7-dione (ES936) was supplied by Professor Christopher J. Moody (School of Chemistry, University of Nottingham, Nottingham, United Kingdom). Recombinant human NQO1 (rhNQO1) was purified after expression in *Escherichia coli* as described previously (Beall et al., 1994). The activity of rhNQO1 was 4.5 $\mu\text{mol DCPIP/min/mg protein}$. NADPH-cytochrome P450 reductase, human and mouse liver microsomes were obtained from BD Biosciences Inc. (Woburn, MA). The activity of NADPH-cytochrome P450 reductase was 39 $\mu\text{mole of cytochrome c reduced per min per mg protein at } 37^\circ\text{C}$.

HPLC analysis

For BA, BAH₂ and BA glutathione conjugates, HPLC conditions were as follows: Buffer A, 50 mM ammonium acetate, pH 4 containing 10 $\mu\text{M D}(-)$ -penicillamine; buffer B, methanol (100%). Both buffers were continuously bubbled with N₂. Gradient, 30% B to 90% B over 10 min, then 90% B for 5 min, flow rate of 1 ml/min. Analysis was performed on a Luna C18 reverse-phase column (5 μm , 4.6 $\times$ 250 mm, Phenomenex, Torrance, CA) with UV detection at 270 nm. The sample injection volume was 50 μl .

For analysis of NADH and NADPH oxidation, 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 min, flow rate of 1 ml/min. Analysis was performed on a Luna C18 reverse-phase column (5 μm , 4.6 $\times$ 250 mm, Phenomenex, Torrance, CA) with UV detection at 340 nm. The sample injection volume was 50 μl .

LC-MS analysis

For conjugation studies of BA with glutathione, LC-MS was performed using positive ion electrospray ionization and the mass spectra were obtained with an Agilent 1100 series LC/MSD trap MS with a turbo ion spray source interfaced to an Agilent 1100 capillary HPLC system. Samples were chromatographed on a Luna C18 reverse-phase column (5 μm , 150 $\times$ 1 mm, Phenomenex) using a gradient elution consisting of 20% B to 80% B over 30 min then 80% B for 5 min at a flow rate of 50 $\mu\text{l/min}$ and a sample injection volume of 8 μl . HPLC conditions were as follows: buffer A, H₂O containing 0.2% (v/v) formic acid; buffer B, acetonitrile containing 0.2% (v/v) formic acid. The MS settings were turbo ion spray temperature of 350 $^\circ\text{C}$, spray needle voltage at 3,500 V, declustering potential at 35 V, and focus plate at 125 V. Mass spectra were continuously recorded from 150 to 1,000 amu every 3 seconds during the chromatographic analysis.

The relative stability of hydroquinone ansamycin Hsp90 inhibitors

The relative stability of BAH₂ was determined by measuring the oxygen consumption rate of BAH₂ generated by rhNQO1. Reaction conditions: 50 $\mu\text{M BA}$, 500 $\mu\text{M NADH}$ and rhNQO1 (0.059–17.7 μg) were incubated in 50 mM potassium phosphate (pH 7.4, 3 ml) at 35 $^\circ\text{C}$. Reactions were started by adding rhNQO1 and oxygen consumption was measured using a Clark electrode (air tight). After 10 min, the reactions were stopped with an equal volume of ice cold methanol and NADH concentrations were immediately analyzed by HPLC at 340 nm. Dissolved oxygen concentrations were adjusted for altitude (5,280 ft) and temperature (35 $^\circ$

C). The relative stability of BAH₂ was expressed as nmol oxygen consumption per nmol NADH oxidized.

Metabolism of BA by mouse and human liver microsomes

The rate of metabolism of BA Hsp90 inhibitors by mouse liver microsomes was determined using oxygen consumption and NAD(P)H oxidation. Reaction conditions: 50 μ M BA, 500 μ M NAD(P)H and 200–600 μ g microsomes (human or mouse) were incubated in 50 mM potassium phosphate (pH 7.4, 3 ml) at 35 °C and the oxygen consumption was measured using a Clark electrode (air tight). After 10 min, reactions were stopped with an equal volume of ice cold methanol. NAD(P)H concentrations were determined by HPLC at 340 nm.

Analysis of NADPH-cytochrome P450 reductase-mediated one-electron redox cycling of BA

The relative one-electron redox cycling rates of BA mediated by NADPH-cytochrome P450 reductase were determined by measuring the rates of oxygen consumption. Reaction conditions: 50 μ M BA, 500 μ M NADPH and 3.3 μ g NADPH-cytochrome P450 reductase were incubated in 50 mM potassium phosphate buffer (pH 7.4, 3 ml) at 35 °C. The oxygen consumption rate was measured using a Clark electrode (air tight) over 10 min.

Quinone/Hydroquinone cycling in microsomal incubations

The concentration of BA and BAH₂ in microsomal incubations was determined using HPLC. For GM and 17AAG, the concentration of GMH₂ and 17AAGH₂ in microsomal incubations was also determined using standard curves generated by HPLC. Briefly, mixtures containing 50 μ M GM or 17AAG, 500 μ M NADH and 3.3 μ g rhNQO1 were incubated in 50 mM potassium phosphate buffer (pH 7.4, 1 ml). The reactions were stopped with an equal volume of ice cold methanol at 0, 0.5, 1, 2 and 5 min for GM or 0, 1, 5, 10 and 30 min for 17AAG and analyzed using HPLC. The amount of quinone remaining at the various time points was determined using standard curves. The amount of hydroquinone formed at the various time points was obtained by subtraction from the starting concentration of quinone.

Analysis of BA-glutathione conjugate formation

The interaction of BA with glutathione was analyzed by HPLC and LC-MS. Reaction conditions: 50 μ M BA and 5 mM GSH were incubated in 50 mM potassium phosphate buffer (pH 7.4, 1 ml) at room temperature for the indicated times in the absence and presence of 11.8 μ g rhNQO1 and 500 μ M NADH. BA-GSH conjugate formation was analyzed by HPLC at 270 nm and further confirmed by LC-MS.

Prevention of GM and 17DMAG glutathione conjugate formation by rhNQO1

GM and 17DMAG glutathione conjugate formation was quantified using [³H] GSH. Reaction conditions: 50 μ M GM or 17DMAG and 5 mM GSH (containing 37 kBq [³H] GSH) in the absence and presence of 11.8 μ g rhNQO1 and 500 μ M NADH were incubated in 50 mM potassium phosphate buffer (pH 7.4, 1 ml) at room temperature for the indicated times. GM and 17DMAG glutathione conjugate formation was then analyzed by HPLC at 270 nm. HPLC fractions were collected at 1 ml/min/collection using a Gilson 203 fraction collector. ScintiSafe 30% (5 ml) was added to each tube and the radioactivity was counted using a Beckman LS 6000IC scintillation counter. The concentration of GM and 17DMAG glutathione conjugates was determined from a standard curve generated using [³H] GSH.

Statistical analysis

Statistical analysis was performed using a one way ANOVA followed by a Tukey multiple comparison test. For ordering of stability and rate of reduction within the series of analogues

discussed in the text, > indicates a significant difference at least at $p < 0.05$ (ANOVA, Tukey post-hoc test).

Results

The relative stability of BAH₂

The relative stability of BAH₂ was studied using the ratio between the oxygen consumption rate (reflecting how quickly the BAH₂ is oxidized) and the NADH oxidation rate (reflecting how much BAH₂ is formed) during the reduction of BA by purified recombinant human NQO1 (rhNQO1). Using this measurement, the oxidation rate is corrected for the amount of BAH₂ formed. During the incubation, the oxygen consumption rates were also measured using a Clark electrode and NADH oxidation rates were measured using HPLC (Figure 1). In 50 mM potassium phosphate buffer, pH 7.4 at 35 °C, the relative stability of BAH₂ was GMH₂>17AAGH₂>17DMAGH₂>17AGH₂, 17AEP-GAH₂ (> indicates $p < 0.05$). However, all BAH₂ were susceptible to auto-oxidation and could be oxidized back to the respective quinones over time with half-lives from a few minutes (17AEP-GAH₂) to about 20 hours (GMH₂).

Metabolism of BA by mouse and human liver microsomes

The metabolism of BA Hsp90 inhibitors by human and mouse liver microsomes was examined. In these experiments, either NADH or NADPH were used as cofactors to provide reducing equivalents for one electron reductases present at high concentrations in liver microsomes such as NADPH cytochrome P450 reductase and NADH cytochrome b5 reductase. The rates of oxygen consumption and NAD(P)H oxidation were measured as indicators of the relative redox cycling rates of BA. In both mouse and human liver microsome reactions, all BA in the series except GM were metabolized at a faster rate by NADPH-dependent metabolism compared to NADH-dependent metabolism as indicated by faster NADPH oxidation and oxygen consumption rates (Figure 2 and 3). On the contrary, GM was metabolized more rapidly by NADH-dependent processes in both mouse and human liver microsomes (Figure 2 and 3). In each case, NAD(P)H was oxidized at a faster rate compared to the respective oxygen consumption rate reflecting the fact that the BAH₂ generated have appreciable stability (16, 17). In mouse liver microsomes, the relative oxygen consumption rates during NADH-dependent metabolism of BA, using ANOVA and a Tukey multiple comparison test with a significance level at $p < 0.05$, were GM>17DMAG>17AG, 17AEP-GA, 17AAG (Figure 2A). Oxygen consumption rates during NADPH-dependent metabolism of BA in mouse liver microsomes did not vary significantly between GM, 17DMAG, 17AG and 17AEP-GA but all rates were significantly greater than 17AAG (Figure 2B, $p < 0.05$). In human liver microsomes, the relative oxygen consumption rates during NADH-dependent metabolism of BA, as indicated by ANOVA and a Tukey post-hoc test ($p < 0.05$), were GM>17DMAG, 17AG, 17AEP-GA>17AAG (Figure 3A); the relative oxygen consumption rates (Figure 3B) during NADPH-dependent metabolism of BA were GM, 17DMAG, 17AEP-GA>17AG>17AAG (> indicates $p < 0.05$). In each case, in this series of BA, 17AAG was metabolized at the slowest rate while GM was metabolized at a relatively rapid rate during either NADPH-dependent or NADH-dependent metabolism in human and mouse liver microsomes.

Confirmation of quinone/hydroquinone cycling as the primary mode of metabolism in microsomal preparations

Although NADPH cytochrome P450 reductase and NADH cytochrome b5 reductase are commonly regarded as the major microsomal one-electron reductases, which result in redox cycling, it is possible that other microsomal enzymes may metabolize the BA. Cytochrome P450 itself may oxidize BA (Egorin et al., 1998; Lang et al., 2007); CYP450–3A4 has been reported to metabolize 17AAG and 17DMAG (Egorin et al., 1998 and 2002) and metabolites generated via cytochrome P450 may not be redox active. To confirm quinone/hydroquinone

cycling as the major mechanism of NAD(P)H dependent BA metabolism in microsomes, the metabolites generated were analyzed by HPLC. For 17AAG, 17DMAG and 17AG, although small amounts of hydroquinone and other unidentified metabolites were observed, the vast majority of the compound was in the quinone form on HPLC (quinone >95%, data not shown). For GM, minor unidentified metabolites were observed in incubations either in the presence or absence of NADPH. In addition, more hydroquinone (about 20%) and relatively less quinone (about 70%) was observed on HPLC compared to other BA (data not shown). These results demonstrated that in terms of material balance, the vast majority of compound (>90%) was either in the quinone or hydroquinone form during metabolism of all BA in the series during metabolism in microsomes. These data confirm that the primary mode of metabolism in microsomal systems is quinone/hydroquinone cycling.

The relative one-electron redox cycling rates of BA mediated by human liver NADPH-cytochrome P450 reductase

The one-electron redox cycling properties of BA were further studied using purified NADPH-cytochrome P450 reductase. Oxygen consumption rates were measured using a Clark electrode during the incubation of BA with purified human NADPH-cytochrome P450 reductase as an indication of the relative one-electron redox cycling rates of BA. Prior to these experiments, the cofactor specificity of this enzyme was examined and no oxygen consumption was observed during the incubation of GM, NADH and NADPH-cytochrome P450 reductase, however, oxygen consumption was observed by inclusion of NADPH (Figure 4A). The relative rates of purified NADPH-cytochrome P450 mediated one-electron redox cycling of BA, as indicated by ANOVA and a Tukey post-hoc test ($p < 0.05$), were 17DMAG>GM, 17AEP-GA, 17AG>17AAG (Figure 4B). These data are similar to those obtained in human liver microsomal systems (Figure 3B) indicating that GM and 17DMAG undergo more rapid NADPH dependent redox reactions than 17AAG.

Interaction of BA Hsp90 inhibitors with reduced glutathione

The interaction of BA including GM, 17DMAG, 17AAG, 17AG and 17AEP-GA with glutathione was measured by HPLC and further confirmed by LC-MS (Figure 5, Figure 6). The amount of BA-glutathione conjugate formation was quantified using [^3H] glutathione. In reactions in phosphate buffer at pH 7.4 and room temperature using 5 mM reduced glutathione and 50 μM BA, approximately 45 μM of GMH₂-SG conjugate was formed within 5 min which then slowly oxidized to GM-SG (Figure 5A). This indicates formation via a classic 1,4-reductive Michael addition generating the hydroquinone conjugate intermediates which are then oxidized to quinone conjugates (Monks and Iau, 1992; Eyer, 1994). Under the same conditions, approximately 47 μM of 17DMAG-SG conjugate was formed within 4 hours while 17DMAGH₂-SG was not detected (Figure 6A). This is likely due to the instability of 17DMAGH₂-SG conjugate and its rapid oxidation to 17DMAG-SG during analysis. The identity of glutathione adducts was confirmed by LC-MS analysis (Figure 5D and 6D). Conversely, the formation of 17AAG-SG and 17AG-SG was very slow under these conditions. Even after 24 hours, less than 15% of 17AAG or 17AG was conjugated with glutathione (data not shown). Under the same conditions, about 90% of 17AEP-GA was conjugated with glutathione within 10 hours (data not shown). The relative rate of glutathione conjugate formation in this series of BA was GM>17DMAG>17AEP-GA>17AAG, 17AG. BA glutathione conjugate formation was pH dependent and glutathione conjugates were not formed when the pH was <5.0 (data not shown).

NQO1-mediated protection against glutathione adduction reactions

Since glutathione interacts with BA at the 19-position on the quinone ring (Cysyk et al., 2006), it would be expected that BA reduction to BAH₂ by NQO1 would prevent glutathione

conjugation. In the presence of rhNQO1 and NADH, GM and 17DMAG were reduced to their respective hydroquinones, GMH₂ and 17DMAGH₂, however, these hydroquinones did not react with glutathione (Figure 5B, Figure 6B). In each case, reduction by NQO1 almost totally (>95%) prevented the conjugation of GM and 17DMAG with glutathione (Figure 5B, Figure 6B). The prevention of BA glutathione conjugation by NQO1-mediated reduction can be abrogated by pretreatment with ES936, a mechanism-based inhibitor of NQO1 (Figure 5C, Figure 6C).

Discussion

We have demonstrated that BAH₂ generated by NQO1 were more potent Hsp90 inhibitors than their parent quinones (Guo et al., 2005 and 2006). NQO1 is elevated in many human solid tumors (Siegel and Ross, 2000) which favors improved activity of BAs via efficient reduction to the more active BAH₂ *in-situ* in human tumors. BAH₂ have increased water-solubility and overcome the solubility problems observed in the clinical use of their parent quinones (Guo et al., 2005 and 2006; Ge et al., 2006). In this study, the relative stability of BAH₂ was studied and it was demonstrated that GMH₂ and 17AAGH₂ were relatively resistant to oxidation while 17DMAGH₂, 17 AGH₂ and 17AEP-GAH₂ were more sensitive to oxidation. BAH₂ which are resistant to oxidation are attractive candidates for clinical use to overcome the solubility and formulation difficulties associated with the corresponding parent quinones. A 17AAGH₂ salt has been developed for clinical use and is now in phase I clinical trials (Sydor et al., 2006).

A broad range of quinone-based compounds can be metabolized by one-electron reductases in liver microsomes leading to the one-electron redox cycling of quinones, the generation of ROS and induction of toxicity (Ross, 1988; Powis 1989; Ross et al., 1996). In this study, the metabolism of BA by both mouse and human liver microsomes was examined using either NADPH or NADH to provide the appropriate co-factors for one electron reductases such as NADPH-cytochrome P450 reductase (NADPH) or NADH-cytochrome b5 reductase (NADH) (Blanck and Smettan, 1978). Analysis of metabolites formed during NADPH-dependent metabolism of BA in human liver microsomes indicated that the vast majority of BA (>90%) was in the form of either quinone or hydroquinone and therefore capable of redox cycling. This data also suggests little biotransformation by cytochrome P450 to non redox-active products under these conditions. Oxygen consumption measurements demonstrated that BA underwent redox cycling during incubation with either mouse or human liver microsomes in the presence of NAD(P)H and results were qualitatively similar using both mouse and human liver microsomes. A proposed model of microsomal redox cycling of BA is shown in Scheme 2. Importantly, 17AAG was relatively resistant to redox cycling as indicated by a relatively slow oxygen uptake rate when compared to GM.

The relative one-electron redox cycling rates of BA in this series were further studied using purified NADPH-cytochrome P450 reductase. Oxygen uptake rates were rapid with GM and 17DMAG but relatively slow with 17AAG. It has been reported that the one-electron redox potential of geldanamycin in water was -0.243 V compared to a lower redox potential of -0.390 V for 17AAG (Lang et al., 2007). These redox potentials for geldanamycin and 17AAG support the observations that geldanamycin would be more easily reduced by NADPH-cytochrome P450 reductase compared to 17AAG. The order of these rates was relatively similar compared to the relative oxygen consumption rates of BA during NADPH-dependent metabolism in mouse and human liver microsomes. In summary, using both mouse and human liver microsomal preparations and purified human liver cytochrome P450 reductase, 17-AAG had the least and geldanamycin the greatest propensity to undergo redox cycling reactions. These observations suggest that the relative abilities of geldanamycin and 17AAG to induce hepatotoxicity (Behrsing et al., 2005) can be explained, at least in part, by their relative abilities to undergo potentially toxic redox cycling reactions catalyzed by hepatic one-electron

reductases. Significant one electron redox cycling rates were also observed using 17DMAG particularly in NADPH catalyzed reactions in either microsomal systems or with purified human cytochrome P450 reductase. 17DMAG has been reported to induce hepatotoxicity in preclinical studies in rats and dogs (Glaze et al., 2005). From a drug development perspective, one strategy to avoid potentially toxic redox cycling reactions would be to design prodrugs of hydroquinone ansamycins which generate the active Hsp90-inhibitory BAH₂ directly in target tumor tissue via prodrug cleavage.

Depletion of glutathione in cells can result in drug-induced arylation of cellular nucleophiles and induction of cellular toxicity (Ross, 1988; Monks and Lau, 1992). Cysyk *et al.* first reported that GM, 17DMAG, 17AAG and 17AG could form glutathione conjugates at the 19-position on the quinone ring (Cysyk et al., 2006). Lang *et al.* obtained GMH₂-SG by incubation of GM, human liver microsomes with glutathione (Lang et al., 2007). We examined the rates of glutathione conjugate formation of GM, 17DMAG, 17AAG, 17AG and 17AEP-GA and from these experiments, the relative rate of glutathione conjugation with BA was found to be GM>17DMAG>17AEP-GA>17AAG, 17AG. Interestingly, the order of this rate of reaction with glutathione also reflects the more pronounced hepatotoxicity of GM relative to 17-AAG. These results suggest that depletion of glutathione by BA may also play a role in BA induced liver toxicity and needs to be considered, in addition to one electron mediated hepatic redox cycling, as an indicator of BA induced liver toxicity.

In addition to hepatotoxicity, the role of thiols in modulation of the therapeutic activity of BA in tumor cells should also be considered since thiol levels in cancer cells have been reported to be inversely related to sensitivity to GM. Cancer cells with high glutathione levels were resistant to GM and their sensitivity to GM was increased by decreasing cellular glutathione levels. Under the same conditions, no relationship was observed for 17AAG between sensitivity and cellular glutathione levels (Huang et al., 2005; Liu et al., 2007). These observations may be explained by the relatively fast conjugation rate of GM with glutathione while 17AAG does not readily conjugate with glutathione. We have performed molecular docking studies (data not shown) using the open and GM-bound human Hsp90 crystal structures (Stebbins et al., 1997) which have indicated that 19-glutathionyl substituted analogs of this series of BAs, in both their *trans*- and *cis*- isomeric forms, are not accommodated in the ATPase active site of Hsp90. This provides a potential explanation for the resistance of cells containing elevated glutathione to BA such as GM. Thus, in addition to potentially predisposing to toxicity, extensive and rapid conjugation of BA with glutathione in tumor cells may result in lack of therapeutic effect and drug resistance.

Since glutathione reacts with BA at the 19-position on the quinone ring (Cysyk et al., 2006), reduction of BA by NQO1 to BAH₂ would be expected to prevent this conjugation. NQO1 and NADH reduced GM and 17DMAG to their corresponding hydroquinones and essentially blocked conjugation with glutathione. Since glutathione conjugation of GM reduces the efficiency of Hsp90 inhibition (Huang et al., 2005; Liu et al., 2007), NQO1 metabolism of GM may not only generate a more active Hsp90 inhibitor, GMH₂, (Guo et al., 2005 and 2006) but may also prevent abrogation of Hsp90 inhibitory activity due to the formation of a glutathione adduct of GM.

In summary, these data indicate that in a series of BA, GM conjugated with glutathione most readily and had the greatest propensity to undergo redox cycling reactions using either mouse or human liver microsomes or purified human liver cytochrome P450 reductase. 17AAG exhibited the lowest potential to interact with glutathione and undergo one electron redox cycling reactions. Since both one electron redox cycling and glutathione adduction are associated with toxicity, these data suggest an explanation for the greater liver toxicity of geldanamycin relative to 17-AAG. Development of new BA analogs that do not conjugate

readily with thiols and demonstrate minimal one electron redox cycling in hepatic systems while maintaining efficient NQO1 mediated two electron reduction may be a useful approach to optimizing the therapeutic index of BA.

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Abbreviations

Hsp90, heat shock protein 90
 BA, benzoquinone ansamycins
 BAH₂, hydroquinone ansamycins
 GM, geldanamycin
 GMH₂, geldanamycin hydroquinone
 17AG, 17-(amino)-17-demethoxygeldanamycin
 17AGH₂, 17-(amino)-17-demethoxygeldanamycin hydroquinone
 17AAG, 17-(allylamino)-17-demethoxygeldanamycin
 17AAGH₂, 17-(allylamino)-17-demethoxygeldanamycin hydroquinone
 17DMAG, 17-demethoxy-17-[[2-(dimethylamino)ethyl]amino]-geldanamycin
 17DMAGH₂, 17-demethoxy-17-[[2-(dimethylamino)ethyl]amino]-geldanamycin hydroquinone
 17AEP-GA, 17-demethoxy-17-[[2-(pyrrolidin-1-yl)ethyl]amino]-geldanamycin
 17AEP-GAH₂, 17-demethoxy-17-[[2-(pyrrolidin-1-yl)ethyl]amino]-geldanamycin hydroquinone
 HPLC, high performance liquid chromatography
 LC-MS, liquid chromatography-mass spectroscopy
 NQO1, NAD(P)H:Quinone oxidoreductase 1
 NADH, nicotinamide adenine dinucleotide, reduced form
 NADPH, nicotinamide adenine dinucleotide phosphate, reduced form
 DCPIP, 2, 6-Dichlorophenol-indophenol
 GSH, reduced glutathione
 DMNQ, 2,3-dimethoxy-1,4-naphthoquinone
 ROS, reactive oxygen species

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Figure 1. The relative stability of hydroquinone ansamycins

The relative stability of BAH₂ was determined by the oxygen consumption rate of BAH₂ generated by rhNQO1 and NADH. After 10 min, reactions were stopped and NADH concentrations were analyzed by HPLC at 340 nm. The relative stability of BAH₂ was expressed as nmol oxygen consumption per nmol NADH oxidized. Results represent mean $\pm$ standard deviation of 3 separate determinations. 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) forms an unstable hydroquinone derivative after two electron reduction (Gant et al., 1988) and was included as a positive control. The relative stability of 17AAGH₂, 17DMAGH₂, 17AGH₂ and 17AEP-GAH₂ was significantly different from GMH₂, * $p < 0.05$. Statistical analysis was performed using a one-way ANOVA with a Tukey multiple comparison test.

Figure 2. Metabolism of BA by mouse liver microsomes

The metabolism of BA Hsp90 inhibitors by mouse liver microsomes was determined by measuring the rates of oxygen consumption and NAD(P)H oxidation as described in Experimental Methods. Results represent mean $\pm$ standard deviation of 3 separate determinations. NAD(P)H (open column) and oxygen consumption (closed column). Panel A, NADH; panel B, NADPH. For NADH, the NADH and oxygen consumption rates of 17AAG, 17DMAG, 17AG and 17AEP-GA were significantly slower compared to GM (*, $p < 0.05$ for oxygen consumption; #, $p < 0.05$ for NADH oxidation). For NADPH, the NADPH and oxygen consumption rates of 17AAG were significantly slower compared to GM (*, $p < 0.05$ for oxygen

consumption; #, $p < 0.05$ for NADPH oxidation). Statistical analysis was performed using a one-way ANOVA with a Tukey multiple comparison test.

Figure 3. Metabolism of BA by human liver microsomes

The metabolism of BA Hsp90 inhibitors by human liver microsomes was determined by measuring the rates of oxygen consumption and NAD(P)H oxidation, as described in Experimental Methods. Results represent mean $\pm$ standard deviation of 3 separate determinations. NAD(P)H (open column) and oxygen (closed column). Panel A, NADH; panel B, NADPH. For NADH, the NADH and oxygen consumption rates of 17AAG, 17DMAG, 17AG and 17AEP-GA were significantly slower compared to GM (*, $p < 0.05$ for oxygen consumption; #, $p < 0.05$ for NADH oxidation). For NADPH, the NADPH and oxygen consumption rates of 17AAG and 17AG were significantly slower compared to GM (*, $p < 0.05$ for oxygen consumption; #, $p < 0.05$ for NADPH oxidation). Statistical analysis was performed using a one-way ANOVA with a Tukey multiple comparison test.

Figure 4. One-electron redox cycling of BA mediated by NADPH-cytochrome P450 reductase as indicated by oxygen consumption rates

The relative one-electron redox cycling rates of BA mediated by NADPH-cytochrome P450 reductase were determined by measuring the rates of oxygen consumption. Panel A, Oxygraph of NADPH-dependent redox cycling of GM mediated by NADPH-cytochrome P450 reductase as described in Experimental Methods. The oxygen consumption rate was measured using a Clark electrode for 5 min, then 500 μ M NADPH was added and the oxygen consumption rate was measured for another 5 min. Panel B, One-electron redox cycling of BA mediated by NADPH-cytochrome P450 reductase as indicated by oxygen consumption rates. Results represent mean $\pm$ standard deviation of 3 separate determinations. The oxygen consumption

rate of 17AAG was significantly slower compared to GM, *, $p < 0.05$. The oxygen consumption rate of 17DMAG was significantly faster compared to GM, #, $p < 0.05$. Statistical analysis was performed using a one-way ANOVA with a Tukey multiple comparison test.

Figure 5. HPLC and LC/MS analysis of the formation of GM glutathione conjugates

GM glutathione conjugate formation was analyzed by HPLC and LC-MS. Briefly, 50 μ M GM, 500 μ M NADH and 5 mM glutathione in the absence and presence of 11.8 μ g rhNQO1 and in the absence or presence of 2 μ M ES936, were incubated in 50 mM potassium phosphate buffer (pH 7.4, 1 ml) at room temperature for 5 min. GM-glutathione conjugate formation was analyzed by HPLC at 270 nm (5 min). Panel A, GM and glutathione; panel B, GM, NADH, rhNQO1 and glutathione; panel C, GM, NADH, rhNQO1, ES936 and glutathione; Panel D, LC-MS confirmed GMH₂-SG and GM-SG as the product of the interaction of GM and glutathione.

Figure 6. HPLC and LC/MS analysis of the formation of 17DMAG glutathione conjugates
17DMAG glutathione conjugate formation was detected by HPLC and LC-MS. Reaction conditions: 50 μ M 17DMAG, 500 μ M NADH and 5 mM glutathione in the absence and presence of 11.8 μ g rhNQO1 and in the absence or presence of 2 μ M ES936, were incubated in 50 mM potassium phosphate buffer (pH 7.4, 1 ml) at room temperature for 3 h. 17DMAG glutathione conjugate formation was analyzed by HPLC at 270 nm (3 h). Panel A, 17DMAG and glutathione; panel B, 17DMAG, NADH, rhNQO1 and glutathione; panel C, 17DMAG, NADH, rhNQO1, ES936 and glutathione; Panel D, LC-MS confirmed 17DMAG-SG as the product of the interaction of 17DMAG and glutathione.

	R	MW
GM	OCH₃	560
17AAG	NHCH₂CHCH₂	586
17DMAG	NHCH₂CH₂N(CH₃)₂	616
17AG	NH₂	545
17AEP-GA	NHCH₂CH₂NC₄H₈	643

Scheme 1.
Structures of BA Hsp90 inhibitors

Scheme 2.

A proposed model of the metabolism of BA by both one and two electron reductases and conjugation with glutathione