Enzymatic Reduction and Glutathione Conjugation of Benzoquinone Ansamycin Heat Shock Protein 90 Inhibitors: Relevance for Toxicity and Mechanism of Action

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Received April 23, 2008; accepted July 16, 2008

ABSTRACT:

Two-electron reduction of benzoquinone ansamycin (BA) heat shock protein (Hsp) 90 inhibitors by NAD(P)H:quinone oxidoreductase 1 (NQO1) to hydroquinone ansamycins (BAH₂₃s) leads to greater Hsp90 inhibitory activity. BAs can also be metabolized by one-electron reductases and can interact with glutathione, reactions that have been associated with toxicity. Using a series of BAs, we investigated the stability of the BAH₂₃s generated by NQO1, the ability of BAs to be metabolized by one-electron reductases, and their conjugation with glutathione. The BAs used were geldanamycin (GM), 17-(allylamino)-17-demethoxygeldanamycin (17AAG), 17-demethoxy-17-[(2-[dimethyl amino)ethyl][amino]-geldanamycin (17DMAG), 17-(amino)-17-demethoxygeldanamycin (17AG), and 17-demethoxy-17-[(2-[pyrrolidin-1-yl)ethyl][amino]-geldanamycin (17AEP-GA). The relative stabilities of BAH₂₃s at pH 7.4 were GM hydroquinone > 17AAG hydroquinone > 17DMAG hydroquinone > 17AG hydroquinone and 17AEP-GA hydroquinone. Using human and mouse liver microsomes and either NADPH or NADH as cofactors, 17AAG had the lowest rate of one-electron reduction, whereas GM had the highest rate. 17DMAG demonstrated the greatest rate of redox cycling catalyzed by purified human cytochrome P450 reductase, whereas 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 BAs 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 with 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 BAs.

Hsp90 is a chaperone protein that is critical for the folding and stability of a number of oncogenic proteins, including Raf-1, mutant p53, ErbB2, Hif-1α, 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); therefore, many oncogenic signals can be blocked simultaneously by inhibition of Hsp90 (Powers and Workman, 2006).

This study was supported by National Institutes of Health Grant R01-CA51210 and by a State of Colorado Bioscience, Development, and Evaluation grant.

The authors disclose a patent interest (patent pending) in hydroquinone ansamycins.

Abbreviations: Hsp, heat shock protein; BA, benzoquinone ansamycin; GM, geldanamycin; 17AAG, 17-(allylamino)-17-demethoxygeldanamycin; 17DMAG, 17-demethoxy-17-[(2-[dimethyl amino)ethyl][amino]-geldanamycin; NQO1, NAD(P)H:quinone oxidoreductase 1; BAH₂₃, hydroquinone ansamycin; 17AG, 17-(amino)-17-demethoxygeldanamycin; GMH₂₃, geldanamycin hydroquinone; 17AEP-GA, 17-demethoxy-17-[(2-[pyrrolidin-1-yl)ethyl][amino]-geldanamycin; ES936, 5-methoxy-1,2-dimethyl-3-[4-nitrophenoxy)methyl]indole-4,7-dione; rh, recombinant human; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS, mass spectroscopy; 17AGH₂₃, 17-(allylamino)-17-demethoxygeldanamycin hydroquinone; GSH, reduced glutathione; ANOVA, analysis of variance; 17DMAGH₂₃, 17-demethoxy-17-[(2-[dimethyl amino)ethyl][amino]-geldanamycin hydroquinone; 17AEP-GAH₂₃, 17-demethoxy-17-[(2-[pyrrolidin-1-yl)ethyl][amino]-geldanamycin hydroquinone.

Benzoquinone ansamycins (BAs) (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 because of liver toxicity (Supko et al., 1995). 17-(Allylamino)-17-demethoxygeldanamycin (17AAG) and 17-demethoxy-17-[(2-[dimethyl amino)ethyl][amino]-geldanamycin (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 trial and 17DMAG is in phase I clinical trial (Ronnen et al., 2006; Shadad and Ramathan, 2006).

We have previously demonstrated that this series of BAs can be reduced by NQO1, an obligate two-electron reductase, to their corresponding hydroquinone ansamycins (Guo et al., 2005, 2006). BAH₂₃s were more water-soluble, more potent Hsp90 inhibitors, and more active at inducing growth inhibition compared with the respective
BAs (Guo et al., 2005, 2006). NQO1 is markedly elevated in many human solid tumors (Siegel and Ross, 2000) and 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). Because 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 BAs such as GM. To investigate these potentially toxic metabolic routes mediated by one-electron reduction, the metabolism of BAs 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 preclinical 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 17-(amino)-17-demethoxygeldanamycin (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 BAs (Cysyk et al., 2006). Recently, Lang et al. (2007) also found that GMH2-glutathione conjugates were formed during incubation of GM with human liver microsomes and glutathione. Therefore, addition of glutathione and other thiols in cells may 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 BAs. 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 with 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.

Materials and Methods

Materials. 17AAG, GM, 17DMAG, and 17-demethoxy-17-[(2-[(pyrrolidin-1-yl)ethyl][amino]-geldanamycin (17AEPA-GA) were obtained from InvivoGen (San Diego, CA), and 17AG was provided by the National Cancer Institute and Kusan Biosciences (Hayward, CA). 2,6-Dichlorophenol-indophenol, NADH, NADPH, glutathione, dicumarol, potassium phosphate (monobasic and dibasic), β-lapachone, 2,3-dimethoxy-1,4-naphthoquinone, and ammonium acetate were obtained from Sigma-Aldrich (St. Louis, MO). [3H] Glutathione was obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). Methanol and acetonitrile were obtained from Thermo Fisher Scientific (Waltham, MA). ES936 was supplied by Professor Christopher J. Moody (School of Chemistry, University of Nottingham, Nottingham, UK). Recombinant human (rh) NQO1 was purified after expression in *Escherichia coli* as described previously (Beall et al., 1994). The activity of rhNQO1 was 4.5 μmol 2,6-dichlorophenol-indophenol/min/mg protein. NADPH-cytochrome P450 reductase and human and mouse liver microsomes were obtained from BD Biosciences (San Jose, CA). The activity of NADPH-cytochrome P450 reductase was 39 μmol cytochrome c reduced/min/mg protein at 37°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 μM D(-)-penicillamine; and buffer B, methanol (100%). Both buffers were continuously bubbled with N2. The gradient was 30% to 90% B over 10 min, then 90% B for 5 min, with a flow rate of 1 ml/min. Analysis was performed on a Luna C18 reverse-phase column (5 μm, 4.6 × 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; and buffer B, 50% (v/v) 10 mM potassium phosphate, pH 7.4, and 50% (v/v) methanol. The gradient was 25% B for 10 min, with a flow rate of 1 ml/min. Analysis was performed on a Luna C18 reverse-phase column (5 μm, 4.6 × 250 mm; Phenomenex), 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 (Agilent Technologies, Santa Clara, CA). Samples were chromatographed on a Luna C18 reverse-phase column (5 μm, 150 × 1 mm; Phenomenex) using a gradient elution consisting of 20 to 80% B over 30 min, then 80% B for 5 min at a flow rate of 50 μl/min and a sample injection volume of 8 μl. HPLC conditions were as follows: buffer A, H2O containing 0.2% (v/v) formic acid; and buffer B, acetonitrile containing 0.2% (v/v) formic acid. The MS settings were as follows: buffer A, 10 mM potassium phosphate, pH 7.4; and buffer B, 50% (v/v) 10 mM potassium phosphate, pH 7.4, and 50% (v/v) methanol. The gradient was 25% B for 10 min, with a flow rate of 1 ml/min. Analysis was performed on a Luna C18 reverse-phase column (5 μm, 4.6 × 250 mm; Phenomenex), with UV detection at 340 nm. The sample injection volume was 50 μl.
The metabolism of BAs by mouse and human liver microsomes was determined using oxygen consumption and NAD(P)H oxidation. Reaction conditions were: 50 μM BA, 500 μM NADPH, and 200 to 600 μg of 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 BAs.** The relative one-electron redox cycling rates of BAs mediated by NADPH-cytochrome P450 reductase were determined by measuring the rates of oxygen consumption. Reaction conditions were: 50 μM BA, 500 μM NADPH, and 3.3 μg of 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 μM GM or 17AAG, 500 μM NADH, and 3.3 μg 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 0.5, 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 were: 50 μM 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 μM of rhNQO1 and 500 μM 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 were: 50 μM GM or 17DMAG and 5 mM GSH (containing 37 kBq [3H] GSH) in the absence and presence of 11.8 μg of rhNQO1 and 500 μM NADH. GM-GSH conjugate formation was analyzed by HPLC at 270 nm. The curves were generated using a Beckman LS 6000IC scintillation counter (Beckman Coulter, Fullerton, CA). 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 analogs 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 determined 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 rhNQO1. Using this measurement, the oxidation rate was 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 (Fig. 1). In 50 mM potassium phosphate buffer, pH 7.4 at 35°C, the relative stability of BAH2 was GMH2 > 17AGH2 > 17DMAGH2 > 17AAGH2 and 17AEP-GAH2 (> p < 0.05). However, all BAH2s 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 h (GMH2).

**Metabolism of BAs 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-c oxidase. The rates of oxygen consumption and NAD(P)H oxidation were measured as indicators of the relative redox cycling rates of BAs. In both mouse and human liver microsome reactions, all BAs in the series except GM were metabolized at a faster rate by NADPH-dependent metabolism compared with NADH-dependent metabolism as indicated by faster NADPH oxidation and oxygen consumption rates (Figs. 2 and 3). On the contrary, GM was metabolized more rapidly by NADH-dependent processes in both mouse and human liver microsomes (Figs. 2 and 3). In each case, NAD(P)H was oxidized at a faster rate compared with the respective oxygen consumption rate reflecting the fact that the BAH2s generated have appreciable stability (Guo et al., 2005, 2006). 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, and 17AAG (Fig. 2A). Oxygen consumption rates during NADPH-dependent metabolism of BAs in mouse liver microsomes did not vary significantly among GM, 17DMAG, 17AG, and 17AEP-GA, but all rates were significantly greater than 17AAG (Fig. 2B, p < 0.05). In human liver microsomes, the relative oxygen consumption rates during NADH-dependent metabolism of BAs, as indicated by ANOVA and a Tukey post hoc test (p < 0.05), were GM > 17DMAG, 17AG, and 17AEP-GA > 17AAG (Fig. 3A); the relative oxygen consumption rates (Fig. 3B) during NADPH-dependent metabolism of BAs were GM, 17DMAG, 17AEP-GA > 17AG > 17AAG (>, p < 0.05). In each case, in this series of BAs, 17AAG was metabolized at the slowest rate, whereas GM was metabolized at a relatively rapid rate during either NADPH-
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 BAs. 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, 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%) were observed on HPLC compared with other BAs (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 BAs 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 BAs 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 BAs with purified human NADPH-cytochrome P450 reductase as an indication of the relative one-electron redox cycling rates of BAs. 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 (Fig. 4A). The relative rates of purified NADPH-cytochrome P450-mediated one-electron redox cycling of BAs, as indicated by ANOVA and a Tukey post hoc test (p < 0.05), were 17DMAG > GM, 17AEP-GA, and 17AG > 17AAG (Fig. 4B). These data are similar to those obtained in human liver microsomal systems (Fig. 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 BAs, including GM, 17DMAG, 17AAG, 17AG, and 17AEP-GA, with glutathione was measured by HPLC and further confirmed by LC-MS (Figs. 5 and 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 GMH2-SG conjugate was formed within 5 min, which then slowly oxidized to GM-SG (Fig. 5A). This indicates formation via a


Results represent mean ± S.D. of three separate determinations. The oxygen consumption rate was measured using a Clark electrode for 5 min, then 500 μM NADPH was added, and the oxygen consumption rate was measured for another 5 min. B. 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. A. oxygraph of NADPH-dependent redox cycling of GM mediated by NADPH-cytochrome P450 reductase as described under Materials and Methods. The oxygen consumption rate was measured using a Clark electrode for 5 min, then 500 μM NADPH was added, and the oxygen consumption rate was measured for another 5 min. B. one-electron redox cycling of BA mediated by NADPH-cytochrome P450 reductase as indicated by oxygen consumption rates. Results represent mean ± S.D. of three separate determinations. The oxygen consumption rate of 17AAG was significantly slower compared with GM, *p < 0.05. The oxygen consumption rate of 17DMAG was significantly faster compared with GM; #p < 0.05. Statistical analysis was performed using a one-way ANOVA with a Tukey multiple comparison test.

Discussion

We have demonstrated that BAH2s generated by NQO1 were more potent Hsp90 inhibitors than their parent quinones (Guo et al., 2005, 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. BAH2s have increased water solubility and overcome the solubility problems observed in the clinical use of their parent quinones (Guo et al., 2005, 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, whereas 17DMAGH2, 17AGH2, and 17AEP-GAH2 were more sensitive to oxidation. BAH2s, 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 reactive oxygen species, and induction of toxicity (Ross, 1988; Powis, 1989; Ross et al., 1996). In this study, the metabolism of BAs by both mouse and human liver microsomes was examined using either NADPH or NADH to provide the appropriate cofactors for one-electron reductases such as NADPH-cytochrome P450 reductase (NADPH) or NADH-cytochrome-b reductase (NADH) (Blanck and Smetian, 1978). Analysis of metabolites formed during NADPH-dependent metabolism of BAs 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. These data also suggest little biotransformation by cytochrome P450 to non-redox-active products under these conditions. Oxygen consumption measurements demonstrated that BAs 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 with GM.

The relative one-electron redox cycling rates of BAs 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 with 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 with 17AAG. The order of these rates was relatively similar compared with the relative oxygen consumption rates of BAs during NADPH-dependent metabolism in mouse and human liver microsomes. In summary, using both mouse and human liver microsomal preparations and purified human

Fig. 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.
Fig. 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 were: 50 μM 17DMAG, 500 μM NADH, and 5 mM glutathione in the absence and presence of 11.8 μg rhNQO1 and in the absence or presence of 2 μM 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). A, 17DMAG and glutathione; B, 17DMAG, NADH, rhNQO1, and glutathione; C, 17DMAG, NADH, rhNQO1, ES936, and glutathione; D, LC-MS confirmed 17DMAG-SG as the product of the interaction of 17DMAG and glutathione.

Fig. 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 μM GM, 500 μM NADH, and 5 mM glutathione in the absence and presence of 11.8 μg rhNQO1 and in the absence or presence of 2 μM 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). A, GM and glutathione; B, GM, NADH, rhNQO1, and glutathione; C, GM, NADH, rhNQO1, ES936, and glutathione; D, LC-MS confirmed GMH2-SG and GM-SG as the product of the interaction of GM and glutathione.
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. (2006) first reported that GM, 17DMAG, 17AAG, and 17AG could form glutathione conjugates at the 19-position on the quinone ring. Lang et al. (2007) obtained GMH2-SG by incubation of GM, human liver microsomes with glutathione. We examined the rates of glutathione conjugate formation of GM, 17DMAG, 17AAG, and 17AEP-GA, and from these experiments, the relative rate of glutathione conjugation with BA was found to be GM > 17DMAG > 17AEP-GA > 17AAG and 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 because 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, whereas 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 BAs 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 a lack of therapeutic effect and drug resistance.

Because 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. Because 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, 2006) but may also prevent abrogation of Hsp90 inhibitory activity because of the formation of a glutathione adduct of GM.

In summary, these data indicate that in a series of BAs, 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. Because 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 BAs.

References

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