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Reactive oxygen species mediate hepatotoxicity induced by the Hsp90 inhibiting anti-cancer geldanamycin and its analogs

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Abstract

Geldanamycin (GM), a benzoquinone ansamycin antibiotic, is a natural product inhibitor of Hsp90 with potent and broad anti-cancer properties. Because of its adverse effects on liver, its less toxic derivatives 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) and 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG) are currently being evaluated for the treatment of cancer. Previously, it has been demonstrated that the redox cycling of GM by NADPH-cytochrome P450 reductase leads to the formation of the GM semiquinone and superoxide radicals, the latter being identified using spin-trapping. We hypothesized that the different hepatotoxicity induced by GM, 17-AAG and 17-DMAG reflects the redox active properties of the quinone moiety and possibly the extent of superoxide formation, which may stimulate cellular oxidative injury. Our data demonstrate that superoxide can be efficiently trapped during the reduction of GM, 17-AAG and 17-DMAG by NADPH-cytochrome P450 reductase, and that superoxide formation rate followed the order 17-DMAG > 17-AAG > GM. In the absence of superoxide scavengers, the rate of NADPH oxidation followed the order 17-DMAG > GM > 17-AAG. The half-wave one-electron reduction potentials ($E_{1/2}$) of GM, 17-AAG and 17-DMAG in DMSO have been determined to be -0.37 , -0.13 and -0.015 V (vs. Ag/AgCl), respectively. If the same order of $E_{1/2}$ follows in neutral aqueous media, thermodynamic considerations imply that 17-DMAG is more readily reduced by the P450 reductase as well as by superoxide. The order of the drug cytotoxicity toward rat primary hepatocytes, as determined by their effect on cell viability and on intracellular oxidant level, was opposite to the order of $E_{1/2}$ of the respective quinone/semiquinone couples. These results suggest that hepatotoxicity exhibited by the Hsp90 inhibitors belonging to benzoquinone ansamycins could be attributed to superoxide. The apparent discrepancy between the order of toxicity and the orders of superoxide formation rate, which is correlated with $E_{1/2}$, is discussed.

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Keywords

Quinone; Semiquinone; Reduction potential; 17-AAG; 17-DMAG; hepatocytes; Spin-trapping; Tempol; DMPO; EPR; Kinetics

Introduction

Geldanamycin (GM), a benzoquinone ansamycin antibiotic, interferes with the action of the heat shock protein 90 (Hsp90) leading to its degradation [1]. The latter is a highly abundant protein, essential for cell viability, and plays an important regulatory role by interacting with a range of client proteins [2]. While GM showed promise in preclinical studies, its progression to clinical trials was halted due to unacceptable levels of hepatotoxicity [3]. Consequently, numerous GM analogs, which differ only in their 17-substituent, have been synthesized. These include 17-allylamino-17-demethoxygeldanamycin (17-AAG), which is significantly less hepatotoxic but still maintains its Hsp90-inhibitory qualities [4–6]. While 17-AAG is currently evaluated in clinical trials [7–9], it has several drawbacks including limited aqueous solubility and the potential to form toxic metabolites [10]. To overcome these challenges, a water soluble, stable GM analog, 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG) has recently entered clinical trials [11].

The mechanism underlying the toxicity of GM and its analogs are not fully understood. It is not clear why 17-AAG has a more favorable therapeutic index than that of GM, despite the small difference in chemical structure between the two derivatives and their similar inhibitory effects of the ATPase function of Hsp90. It has been suggested that the chemical reactivity of the quinone moiety could contribute to hepatotoxicity [3] since they are known to be redox-active. In biological systems one-electron reduction of quinone (Q) to semiquinone radical ($Q^{\cdot-}$) and two-electron reduction of quinone to hydroquinone (Q^{2-}) are catalyzed by flavoenzymes using NAD(P)H as electron sources [12]. 17-AAG can undergo two-electron reduction catalyzed by DT-diaphorase to yield toxic metabolites [6]. Interestingly, while DT-diaphorase also metabolizes GM, it has no effect on its anti-tumor activity [13]. Alternatively, GM and its analogs can be metabolized by one-electron reductases such as NADPH-cytochrome P450 reductase ($P_{450}R$) and NADH cytochrome- b_5 reductase (reaction 1) [10, 14].

In aerobic systems, the semiquinone radical can reduce O_2 to superoxide (reaction 2), which may stimulate cellular oxidative damage, e.g., *via* the reaction of superoxide with nitric oxide to yield peroxynitrite [15–18].

Equilibrium 2 is established rapidly, and oxidative stress is favored if equilibrium 2 is shifted to the right. The formation of superoxide radicals has been previously demonstrated by EPR during the redox cycling of GM induced by NADPH and $P_{450}R$ using 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide (DEMPO) for trapping superoxide [14].

We hypothesized that the different hepatotoxicity induced by GM, 17-AAG and 17-DMAG reflects the redox active properties of the quinone moiety and possibly the extent of superoxide

formation. However, any reagent that removes efficiently superoxide from the system pulls equilibrium 2 in this direction and perturbs the system. Therefore, different yields of superoxide obtained *via* enzymatic reduction of quinines *in vitro* in the presence of superoxide scavengers cannot be directly correlated with hepatotoxicity. In the present study we investigated the effect of superoxide scavengers on NADPH oxidation rate by GM, 17-AAG and 17-DMAG catalyzed by P₄₅₀R. In addition, the cytotoxicity toward rat primary hepatocytes induced by each drug has been determined and correlated with the respective half-wave one-electron reduction potential and kinetic results.

Materials and Methods

Reagents

Geldanamycin (GM), 17-(allylamino)-17-demethoxygeldanamycin (17-AAG), 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG), 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO), β -Nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Alexis Biochemicals (San Diego, CA, USA). NADPH-cytochrome P450 reductase (P₄₅₀R) and 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (CDCFH₂) were purchased from Invitrogen (Carlsbad, CA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 4-OH-2,2,6,6-tetramethyl piperidine-1-oxyl (Tempol), Cu,Zn-superoxide dismutase (SOD) and tetrabutylammonium perchlorate (TBAP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The drugs were dissolved in DMSO. The concentration of NADPH was spectrophotometrically determined at 340 nm ($\epsilon = 6200 \text{ M}^{-1}\text{cm}^{-1}$).

Tissue culture

Rat primary hepatocytes (Clonetics® rtNHePs) purchased from Cambrex (Walkersville, MD, USA) were grown on collagen-coated 6- or 24-well plates in RPMI media. Cells were incubated for various times with 100 nM, 5 μM or 250 μM drug and then assayed for survival using MTT and for intracellular oxidant level using DCFH₂.

MTT assay

Mitochondrial respiration and cellular activity was measured by incubating the cells with MTT (0.5 mg/mL) for 4 h at 37°C. The water-insoluble formazan product from MTT was dissolved in 0.04 M HCL in isopropanol for 5 min (a test wavelength of 570 nm, a reference wavelength at 630 nm) [19].

Measurement of intracellular oxidant levels

Steady-state oxidant levels were measured using the oxidation-sensitive CDCFH₂ (10 $\mu\text{g/mL}$) fluorescent dye (dissolved in 0.1% DMSO). The cells were washed once with 50 mM PBS (phosphate buffer saline, pH 7.4) and labeled on the culture plates with the fluorescent dye for 30 min at 37°C in PBS. At the end of the incubation time culture plates were placed on ice, trypsinized, re-suspended in ice cold PBS, and analyzed using a FACScan flow cytometer (excitation 488 nm, emission 530 nm band-pass filter). In each replicate experiment the numbers obtained for mean fluorescence intensity (MFI) of 10,000 cells/sample are arbitrary, based on the gain setting of the flow cytometer adjusted to the normal unlabeled cells in that particular experiment. In order to be able to combine the results of replicate experiments that were performed on different days, normalization to the MFI exhibited by the labeled normal cell type in each experiment was done. The MFI from the normal cell type on a given day was used as the denominator and the MFI obtained from each cancer cell type done on that same day was used as the numerator. The data from each experiment were normalized to the corresponding normal cell type and combined for analysis.

EPR measurements

EPR spectra were recorded using a Varian E-9 X-band and JEOL X band JES-RE3X spectrometers. Reaction mixtures were transferred to a gas permeable Teflon capillary (Zeus Industries, Orangeburg, SC, USA) having an inner diameter of 0.81 mm, a wall thickness of 0.38 mm and a length of 15 cm. Each capillary was folded twice, inserted into a narrow quartz tube that was open on both edges (2.5 mm inner diameter) and placed within the EPR cavity.

Cyclic voltammetry

Cyclic voltammetry measurements were performed using a BAS100B Electrochemical Analyzer. A three-electrode system consisting of a platinum working electrode, a platinum wire as the auxiliary electrode and an Ag/AgCl (3.5 M) as a reference electrode. The electrodes were immersed in DMSO containing 0.1 M tetrabutylammonium perchlorate (TBAP) as a supporting electrolyte at 25 °C. Oxygen has been purged from the solutions by bubbling N₂, and an atmosphere of N₂ was maintained over the solution throughout the measurements.

Results

One-electron reduction of GM and its analogs by P₄₅₀R

In the presence of NAD(P)H the SOD-mimic Tempol acts as an efficient superoxide scavenger rapidly reducing HO₂[•] (pK_a = 4.8) to form the respective oxoammonium cation (Tempol⁺), which is reduced by NAD(P)H to the respective EPR-silent hydroxylamine (reactions 3 – 4) [20,21].

The EPR signal of 100 μM Tempol decreased upon the addition of 100 μM GM, 17-AAG or 17-DMAG to aerated solutions containing 1 mM NADPH and 4.5 μg mL⁻¹ P₄₅₀R in 50 mM PBS (pH 7.4). The rate of Tempol consumption followed the order 17-DMAG > 17-AAG > GM (Fig. 1). Addition of SOD completely inhibited the loss of Tempol signal as demonstrated for GM in Fig. 1. Previously, it has been demonstrated that NADPH oxidation by GM catalyzed by P₄₅₀R in the presence of the superoxide spin-trap DEMPO forms the respective GM semiquinone and DEMPO-OOH [14]. In a similar system using DMPO to trap superoxide, the DMPO-OOH signal appeared in the presence of GM, 17-AAG and 17-DMAG as demonstrated for 17-DMAG in Fig. 2b. Omission of the drug from the reaction mixture prevented the appearance of the spin-adduct signal (Fig. 2a) The intensity of the DMPO-OOH signal followed the order 17-DMAG > 17-AAG > GM (Fig. 2c), which is the same order as that obtained for the rates of Tempol loss (Fig. 1).

To obtain the relative rates of the redox cycling of GM and its analogs in the absence of superoxide scavengers, NADPH oxidation rate was measured by monitoring the decay of the absorption at 370 nm upon the addition of P₄₅₀R to aerated solutions containing 200 μM NADPH and 50 μM drug in 36 mM PB (pH 7.4). The choice of 370 nm to monitor NADPH oxidation instead of the widely used wavelength of 340 nm was because of the interfering absorption in this spectral region from GM and its analogs. The concentration of each drug was hardly affected during the consumption of NADPH reflecting their redox cycling. The decay of NADPH absorption obeyed first-order kinetics, and the rate constants followed the

order 17-DMAG > GM > 17-AAG (Fig. 3), which is the same as that previously reported for the rate of O₂ consumption [22].

Cyclic Voltammetry

The cyclic voltammograms of GM, 17-AAG and 17-DMAG in DMSO are shown in Fig. 4. The voltammograms are represented by two irreversible pairs of current peaks defined as I and II. No redox peaks were observed when the potential was cycled between +0.7 and -0.1 V. The first cathodic current peak (labeled Ic) with a related anodic current peak (Ia) represents the reduction of the quinone to the semiquinone radical. The second pair designated IIc (cathodic) and IIa (anodic) reflects the reduction of the semiquinone radical to hydroquinone. Each pair was identified by changing the range of the potential cycle. For example, the peak IIc disappeared when scanning started at -0.8 V in the case of 17-AAG or -0.6 V in the case of GM and 17-DMAG. The measured half-wave potentials ($E_{1/2}$) for the quinone/semiquinone and semiquinone/hydroquinone couples, which have not been previously determined, and the calculated values for the quinone/hydroquinone couples are summarized in Table 1.

Intracellular oxidant level and cell toxicity

The ability to generate reactive oxygen species and the consequent cytotoxic effects of GM and its analogs were tested using primary rat hepatocyte cultures. Different concentration ranges were used in these experiments to obtain reliable end-points experimentally. The intracellular oxidant levels in primary rat hepatocytes incubated for 30 min with 0.1 or 5 μ M drug were determined using the fluorescent dye CDCFH₂. The results presented in Fig. 5 demonstrate that GM induced an increase in fluorescence when compared to the same concentration of 17-DMAG or 17-AAG treated or control cells.

To determine the consequence of reactive oxygen species generation by redox-cycling of the drug, survival of primary rat hepatocytes was estimated using the MTT assay following incubation with the drug for 4 h. Incubation with 0.1 μ M drug led to a small decrease in viability. Incubation with 250 μ M drug diminished cell survival where GM was more cytotoxic than either 17-AAG or 17-DMAG (Fig. 6).

Discussion

While the mechanism(s) underlying the toxicity of GM and its analogs are not fully understood, it has been suggested that the reactivity of the benzoquinone moiety could contribute to their hepatotoxicity. Since quinones are reduced to their respective semiquinone radicals followed by reduction of O₂ to superoxide, we postulated that hepatotoxicity could be associated with the production of reactive oxygen species.

In agreement with a previous report for GM [14], we found that superoxide can be scavenged during the redox cycling of GM and its analogs exposed to NADPH and P₄₅₀R (Figures 1 – 2). In the case of Tempol, the rates of reactions 3 and 4 exceed by far that of the reduction of the drug by P₄₅₀R, which is the rate-determining step in this system. Therefore, the rate of Tempol loss, which follows the order 17-DMAG > 17-AAG > GM, reflects the rate of NADPH oxidation rather than superoxide formation. In contrast, the rate of NADPH oxidation in the absence of superoxide scavenger was the lowest in the case of 17-AAG.

We determined $E_{1/2}(Q/Q^{\cdot-})$ in DMSO, which follows the order 17-DMAG > 17-AAG > GM. Previously, the one-electron reduction potentials of GM and 17-AAG in water at pH 7 were calculated to be -0.243 and -0.390 V (vs. NHE), respectively [23]. This calculation was based on the Hammett equation where substitution into the ring by electron-donating or -withdrawing groups reduces or increases, respectively, the one-electron reduction potential of the quinone

in a predictable manner [24]. It was assumed that the allylamino group in 17-AAG is in its deprotonated form, i.e. electron-donating substituent [23]. However, the allylamino group is likely to be protonated at pH 7, i.e., electron-withdrawing substituent, and the one-electron reduction potential of 17-AAG could be higher than that of GM. The same considerations apply also for dimethylaminoethylamino group in 17-DMAG. The effect of the terminal dimethylamino function, which is also likely to be protonated at pH 7, could raise the effective Hammett constant in spite of the two-carbon 'insulation' between the protonated terminal amine moiety and the ring amino substituent leading to a higher one-electron reduction potential compared to that of 17-AAG.

If the same order of $E_{1/2}(Q/Q^{\cdot-})$ in DMSO follows in neutral aqueous media, as is the case with other quinones [25,26], thermodynamic considerations imply that 17-DMAG is more readily reduced. Hence, the reduction rate of GM and its analogs by $P_{450}R$ should follow the same order as $E_{1/2}(Q/Q^{\cdot-})$ as is the case in the presence of Tempol. In the absence of superoxide scavengers, a different order of NADPH oxidation rates was obtained suggesting that the rate-determining step is not the reduction of the quinone by $P_{450}R$. The order of $E_{1/2}(Q/Q^{\cdot-})$ also implies that O_2 is more readily reduced to superoxide by the semiquinone radical of GM than by the other analogs. The apparent contradiction between the order of hepatotoxic effect following $GM > 17-AAG > 17-DMAG$ (Figures 5, 6), and that of $E_{1/2}(Q/Q^{\cdot-})$ is reconciled if hepatotoxicity is determined by the extent of superoxide formation rather than by the in vitro enzymatic reduction rate of the drug. Our results show that all three quinones are capable of participating in futile redox cycling by redox activation through the semiquinone intermediate to generate reactive oxygen species which can account for the oxidative stress when using these drugs.

Abbreviations

17-AAG	17-(allylamino)-17-demethoxygeldanamycin
CDCFH ₂	5-(and-6)-carboxy-2', 7'-dichlorodihydrofluorescein diacetate
DEMPO	5-(diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide
17-DMAG	17-(dimethylaminoethylamino)-17-demethoxygeldanamycin
DMPO	5,5-Dimethyl-1-pyrroline-N-oxide, DMPO
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
DMSO	dimethylsulfoxide
EPR	electron paramagnetic resonance
GM	geldanamycin
NADPH	β-Nicotinamide adenine dinucleotide phosphate
$P_{450}R$	NADPH-cytochrome P450 reductase
PB	phosphate buffer
PBS	phosphate buffer saline
TBAP	tetrabutylammonium perchlorate
Tempol	4-OH-2,2,6,6-tetramethyl piperidine-1-oxyl, Tempol
PB	phosphate buffer
PBS	phosphate buffer saline
SOD	Cu,Zn-superoxide dismutase

TBAP

tetrabutylammonium perchlorate

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Figure 1. Loss of Tempol during the reduction of GM, 17-AAG or 17-DMAG by P₄₅₀R
 The loss of Tempol EPR signal was followed upon the addition of 100 μM drug to solutions containing 100 μM Tempol, 1 mM NADPH, 4.5 μg mL⁻¹ P₄₅₀R and 50 mM PBS (pH 7.4). Addition of 1000 U mL⁻¹ SOD completely inhibited the decrease of the signal.

(a)

(b)

(c)

Figure 2. Spin-trapping by DMPO of superoxide formed during the reduction of GM, 17-AAG or 17-DMAG by P₄₅₀R

Spin trapping of superoxide ion by DMPO (100 mM) in PBS (50 mM, 0.9% NaCl, pH 7.4) containing 1 mM NADPH, 2.27 µg/ml P₄₅₀R and 200 µM of 17-DMAG (b). Omission of the drug from the reaction mixture prevented the appearance of the spin-adduct signal (a). The EPR spectra were recorded using a Varian E-9 X-band spectrometer operating at 0.8 G modulation amplitude, 0.32 s time constant, 3354 G field set, 100 kHz modulation frequency and 10 mW microwave power. (c) The relative intensities of DMPO-OOH obtained in the presence of 0.5 M DMPO, 50 µM drug, 8.5 µg/ml P₄₅₀R and 40 mM PB (pH 7.0). The EPR spectra were recorded using a JEOL X band JES-RE3X spectrometer operating at 2 G modulation amplitude, 0.1 s time constant, 3287 G field set, 100 kHz modulation frequency and 16 mW microwave power. At this high modulation, the 12 lines, usually resolved at lower modulations, merged into 4 lines.

Figure 3. Oxidation of NADPH by GM, 17-AAG or 17-DMAG catalyzed by P₄₅₀R

The absorbance was monitored at 370 nm upon the addition of $5.7 \mu\text{g mL}^{-1}$ P₄₅₀R to aerated solutions containing $200 \mu\text{M}$ NADPH, $50 \mu\text{M}$ drug and 36 mM PB (pH 7.4). The fit of first-order kinetics to the data resulted in $k = 0.143 \pm 0.003$, 0.113 ± 0.004 and $0.055 \pm 0.002 \text{ min}^{-1}$ for 17-DMAG, GM and 17-AAG, respectively.

Figure 4.

Cyclic voltammograms of 3.4 mM GM, 17-AAG or 17-DMAG in deaerated DMSO solutions containing 0.1 M TBAP at positive scan rate of 500 mV s^{-1} .

Figure 5. Intracellular oxidant levels in primary rat hepatocytes incubated with GM, 17-AAG, and 17-DMAG

Primary rat hepatocytes were incubated for 30 min with CDCFH₂, 0.1 or 5 μM drug, and the fluorescence of DCF was measured. Results are presented as ratios and were compared using a Student's t test. *, p < 0.01 vs. Control; †, p < 0.05 vs. DMAG 5 μM.

Figure 6. Cytotoxicity of GM, 17-AAG, and 17-DMAG toward primary rat hepatocytes
 Cells were incubated with 0.1 or 250 μ M drug for 4 h at 37°C and assayed for viability using the MTT assay. Results are presented as ratios and were compared using a Student's t test. *, $p < 0.05$ vs. Control; †, $p < 0.05$ vs. GM 250 μ M; ††, $p < 0.01$ vs. GM 250 μ M; #, $p < 0.05$ vs. 17AAG 250 μ M.

Table 1

The half-wave potentials for the first and second reduction of the drugs in DMSO (V vs. Ag/AgCl)

Drug	$E_{1/2}(\text{I}) = (E_{\text{Ia}} + E_{\text{Ic}})/2$	$E_{1/2}(\text{II}) = (E_{\text{IIa}} + E_{\text{IIc}})/2$	$(E_{1/2}(\text{I}) + E_{1/2}(\text{II}))/2$
	$\text{Q} + \text{e}^- \rightleftharpoons \text{Q}^-$	$\text{Q}^- + \text{e}^- \rightleftharpoons \text{Q}^{2-}$	$\text{Q} + 2\text{e}^- \rightleftharpoons \text{Q}^{2-}$
GM	-0.37	-0.60	-0.49
17-AAG	-0.13	-0.87	-0.50
17-DMAG	-0.015	-0.26	-0.14