

Role for NAD(P)H:quinone Oxidoreductase 1 and Manganese-Dependent Superoxide Dismutase in 17-(Allylamino)-17-demethoxygeldanamycin-Induced Heat Shock Protein 90 Inhibition in Pancreatic Cancer Cells[§]

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ABSTRACT

Previous work demonstrated that NAD(P)H:quinone oxidoreductase 1 (NQO1) metabolized the heat shock protein 90 (Hsp90) inhibitor 17-(allylamino)-17-demethoxygeldanamycin (17AAG) to the corresponding hydroquinone (17AAGH₂). The formation of 17AAGH₂ by NQO1 results in a molecule that binds with greater affinity to Hsp90 compared with the parent quinone. 17AAG induced substantial growth inhibition in human pancreatic cancer cell lines expressing NQO1. Growth inhibition induced by 17AAG could be reduced by pretreatment with 5-methoxy-1,2-dimethyl-3-[(4-nitrophenoxy)methyl]-indole-4,7-dione (ES936), a mechanism-based inhibitor of NQO1. After treatment with 17AAG, biomarkers of Hsp90 inhibition, including markers of cell-cycle arrest, were more pronounced in NQO1-expressing cells compared with NQO1-null cells. The intracellular concentrations of 17AAG and 17AAGH₂ were measured in human pancreatic cancer cells, and it was observed that larger amounts of 17AAG and 17AAGH₂

could be detected in cells with catalytically active NQO1 compared with cells lacking NQO1 activity or cells pretreated with ES936. These data demonstrate that, in addition to generating an inhibitor with greater affinity for Hsp90 (17AAGH₂), reduction of 17AAG to 17AAGH₂ by NQO1 leads to substantially greater intracellular concentrations of 17AAG and 17AAGH₂. In addition, oxidation of 17AAGH₂ could be prevented by superoxide dismutase (SOD), demonstrating that 17AAGH₂ was sensitive to oxidation by superoxide. Stable transfection of manganese-dependent SOD into MiaPaCa-2 cells resulted in a significantly greater intracellular concentration of 17AAGH₂ with a corresponding increase in growth inhibitory activity. These data confirm the role of NQO1 in sensitivity to 17AAG and demonstrate that SOD functions in conjunction with NQO1 to maintain intracellular levels of 17AAGH₂, the active Hsp90 inhibitor derived from 17AAG.

Introduction

Inhibition of heat shock protein 90 (Hsp90) in tumor cells has been exploited as a potential mechanism to target cancer. Hsp90 is an attractive target because this protein chaperone participates in the folding of many oncogenic proteins used by cancer cells. Hsp90 uses the hydrolysis of ATP to help fold nascent forms of client proteins into their active forms. Preventing Hsp90 from performing its chaperone function through the inhibition of ATP binding has been accomplished by a structur-

ally diverse group of compounds (Taldone et al., 2009). Of these compounds the benzoquinone ansamycins including geldanamycin, 17-(allylamino)-17-demethoxygeldanamycin (17AAG), and 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17DMAG), have emerged as potential candidates. However, because of liver toxicity in preclinical studies with geldanamycin (Supko et al., 1995), 17DMAG and 17AAG were selected as candidates for further development (Tian et al., 2004). Both 17DMAG and 17AAG have completed phase I clinical trials and are currently in phase II studies.

By virtue of their quinone moiety the benzoquinone ansamycin class of Hsp90 inhibitors can undergo bioreduction to semiquinone and hydroquinone forms. Hydroquinone forms of 17AAG and 17DMAG are relatively stable but have been shown to be sensitive to copper-mediated reoxidation (Guo et al., 2008). Hydroquinone forms of 17AAG and 17DMAG have

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ABBREVIATIONS: Hsp90, heat shock protein 90; 17AAG, 17-(allylamino)-17-demethoxygeldanamycin; 17AAGH₂, 17AAG hydroquinone; 17DMAG, 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin; NQO1, NAD(P)H:quinone oxidoreductase 1; rhNQO1, recombinant human NQO1; SOD, superoxide dismutase; MnSOD, manganese-dependent SOD; ES936, 5-methoxy-1,2-dimethyl-3-[(4-nitrophenoxy)methyl]-indole-4,7-dione; HPLC, high-performance liquid chromatography; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase; IPI-504, retaspimycin HCl; DCPIP, 2,6-dichlorophenol-indophenol; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide.

also been shown to be more potent inhibitors of Hsp90 in *in vitro* studies using purified Hsp90 compared with their parent quinones (Guo et al., 2006; Maroney et al., 2006). An important feature of the hydroquinone of 17AAG (17AAGH₂) is increased water solubility, and this feature has been exploited in an attempt to reduce vehicle-related toxicities associated with the administration of the more hydrophobic 17AAG. 17AAGH₂ (IPI-504, retaspimycin HCl) has been developed by Infinity Pharmaceuticals (Cambridge, MA) and is currently in phase II/III clinical trials (Hanson and Vesole, 2009). IPI-504 is administered as a hydroquinone, but it is believed that this compound undergoes oxidation back to 17AAG before entering cells (Sydor et al., 2006).

Previous studies have demonstrated that many of the benzoquinone ansamycins, including 17AAG, can undergo direct two-electron reduction by NAD(P)H:quinone oxidoreductase 1 (NQO1) to their corresponding hydroquinone ansamycins (Kelland et al., 1999; Guo et al., 2005, 2006). NQO1 is an FAD-dependent direct two-electron reductase that can use either NADH or NADPH as reducing cofactor and reduces quinones directly to hydroquinones. Relatively high levels of NQO1 protein and activity have been detected in many human solid tumors, including lung, breast, colon, ovary, and pancreas (Schlager and Powis, 1990; Siegel and Ross, 2000; Lewis et al., 2005). We have demonstrated that NQO1 protein levels in both normal and tumor tissues are influenced by a single-nucleotide polymorphism in the NQO1 gene. This polymorphism has been characterized as a C-to-T base-pair substitution at position 609 of the human NQO1 cDNA, which results in a proline-to-serine amino acid substitution at position 187 in the NQO1 protein (Traver et al., 1992). As a consequence of this proline-to-serine substitution the resultant mutant NQO1*2 protein has a substantially shorter half-life caused by rapid polyubiquitination and proteasomal degradation, and individuals genotyped as homozygous for the NQO1*2 polymorphism have been shown to be essentially devoid of NQO1 protein and activity (Siegel et al., 1999, 2001). In this study, we examined the role of NQO1 and the NQO1*2 polymorphism on the bioreduction of 17AAG to the more potent Hsp90 inhibitor 17AAGH₂ in human pancreatic cancer cells. Because 17AAGH₂ has been shown previously to be sensitive to oxidation (Guo et al., 2008) we examined the influence of superoxide on the oxidation of 17AAGH₂ and whether expression of superoxide dismutase (SOD) would lead to higher levels of 17AAGH₂.

Materials and Methods

Reagents. 17AAG was purchased from LC Laboratories (Woburn, MA). Dimethyl sulfoxide, radicicol, 2,6-dichlorophenol-indophenol (DCPIP), bovine serum albumin, NADH, FAD, dicumarol, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Triton X-100, 4,6-diamidino-2-phenylindole, and bovine erythrocyte Cu/Zn SOD were obtained from Sigma-Aldrich (St. Louis, MO). 5-Methoxy-1,2-dimethyl-3-[(4-nitrophenoxy)methyl]-indole-4,7-dione (ES936) was synthesized by Dr. Christopher J. Moody (School of Chemistry, University of Nottingham, Nottingham, U.K.).

Antibodies. Mouse anti-NQO1 monoclonal antibody (clone A180) was produced in our laboratory and is commercially available. Of the mouse monoclonal antibodies anti- β -actin (clone AC-15) was purchased from Sigma-Aldrich, and anti-Hsp70 (clone C92F3A-5) was purchased from Assay Designs (Ann Arbor, MI). The rabbit monoclonal antibodies anti-ERK1/2 (clone 137F5), antiphospho ERK1/2 (clone 197G2), antiphospho MEK (clone 41G9), and anti-cdc25 (clone 5H9) and the rabbit polyclonal antibodies anti-c-Raf and rabbit anti-

cdc2 were purchased from Cell Signaling Technology (Danvers, MA). Rabbit polyclonal anti-MnSOD (SOD-110) antibody was purchased from Assay Designs. Horseradish peroxidase-conjugated goat anti-mouse IgG and horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). All antibodies were diluted in 10 mM Tris-HCl, pH 8 containing 150 mM NaCl and 0.2% (v/v) Tween 20.

Cell Lines. Human pancreatic cancer cell lines Panc-1, MiaPaCa-2, and BxPC-3 were obtained from the American Type Culture Collection (Manassas, VA). Panc-1 cells were grown as attached monolayers in Dulbecco's modified Eagle's medium (American Type Culture Collection) supplemented with 10% (v/v) fetal bovine serum, penicillin (50 IU/ml), and streptomycin (50 μ g/ml). BxPC-3 cells were grown as attached monolayers in RPMI medium 1640 (Mediatech, Manassas, VA) supplemented with 10% (v/v) fetal bovine serum, penicillin (50 IU/ml), and streptomycin (50 μ g/ml). MiaPaCa-2 cells were grown as attached monolayers in Dulbecco's modified Eagle's medium (American Type Culture Collection) supplemented with 10% (v/v) fetal bovine serum, 2.5% (v/v) horse serum, penicillin (50 IU/ml), and streptomycin (50 μ g/ml). All cell lines were grown at 37°C in a humidified incubator with 5% carbon dioxide. Panc-1 cells were genotyped as homozygous for the NQO1*2 polymorphism by polymerase chain reaction-restriction fragment length polymorphism as described previously (Traver et al., 1997). The stably transfected Panc-1/C5 cell line expressing NQO1 was generated from Panc-1 cells by electroporation with an elongation factor 1 α -driven internal ribosome entry site expression vector (a generous gift from Dr. A. V. Patterson, University of Auckland, Auckland, New Zealand) containing full-length human NQO1*1 cDNA. Clones expressing NQO1 protein were selected by growth in complete medium containing 3 μ g/ml puromycin. The stably transfected MiaPaCa-2/SODII cell line was generated from parental MiaPaCa-2 cells by electroporation with an elongation factor 1 α -driven internal ribosome entry site expression vector containing full-length human MnSOD cDNA (Open Biosystems, Huntsville, AL). Clones expressing MnSOD protein were selected by growth in complete medium containing 3 μ g/ml puromycin.

Inhibition of NQO1 by ES936 and the NQO1 Activity Assays. The inhibition of NQO1 activity in pancreatic cancer cell lines by ES936 was determined in cell lines grown to 95% confluence in 100-mm tissue culture plates. Cells were treated with 100 nM ES936 in complete growth medium (10 ml), and after 30 min the medium was removed and cells were washed twice with phosphate-buffered saline. Cells were then scrapped into 1 ml of ice-cold 25 mM Tris-HCl, pH 7.4 containing 250 mM sucrose and 5 μ M FAD. The cell suspension was collected and then sonicated on ice for 5 s. The protein concentrations of sonicates were determined using the method of Lowry et al. (1951). NQO1 activity was measured in sonicates using the method of Ernster (1967) as modified by Benson et al. (1980). In brief, reactions (1 ml) contained 25 mM Tris-HCl, pH 7.4, 0.7 mg/ml bovine serum albumin, 200 μ M NADH, and 2 to 5 μ l of sonicate. Reactions were started by the addition of 40 μ M DCPIP, and the decrease in absorbance at 600 nm was measured for 1 min at 27°C using a HP8452 diode array spectrophotometer (Hewlett Packard, Palo Alto, CA). Reactions were performed in the absence and presence of 20 μ M dicumarol, and NQO1 activity was calculated using the dicumarol-inhibitable rate of DCPIP reduction.

Growth Inhibition Assays. Growth inhibition was measured in pancreatic cell lines using the MTT assay. Cells were seeded at 2×10^3 cells per well (96-well plate) in complete medium overnight. The next morning cells were pretreated with 100 nM ES936 or an equal amount of dimethyl sulfoxide. After 30 min the medium was replaced with fresh medium containing either 17AAG/dimethyl sulfoxide or 17AAG/ES936 (100 nM) for 4 h, after which cells were washed free of drug and incubated in fresh growth medium for an additional 72 h. For growth inhibition studies with radicicol cells were treated for 4 h, after which cells were washed free of drug and incubated in fresh growth medium for an additional 72 h. Cell viability was measured after treatment with MTT for 4 h as described previously (Mosmann, 1983).

17AAG Metabolism in Pancreatic Cell Lines. Intracellular metabolism of 17AAG by pancreatic cancer cells was performed under the following conditions. For each experiment four identical plates of cells (100 mm) were allowed to grow to 95% confluence in complete growth medium. Cells were then pretreated with 100 nM ES936 or an equal amount of dimethyl sulfoxide. After 30 min the medium was removed and replaced with fresh medium containing 25 to 50 μ M 17AAG and 100 nM ES936 (or dimethyl sulfoxide). At the indicated times drug-containing medium was removed and cells were washed twice (10 ml) with phosphate-buffered saline containing 1% (w/v) bovine serum albumin. The cell number was determined on one plate of cells using a Countess automated cell counter (Invitrogen, Carlsbad, CA). The remaining three plates of cells were then analyzed for intracellular concentrations of 17AAG and 17AAGH₂. For these studies 200 μ l of ice-cold stop buffer [0.1% (v/v) trifluoroacetic acid in 80% (v/v) acetonitrile/H₂O] was added to each plate on ice. Cell lysates were quickly harvested using a cell scraper and centrifuged at 13,000 rpm for 1 min at 4°C, and the supernatant (150 μ l) was removed for analysis of 17AAG and 17AAGH₂ by high-performance liquid chromatography (HPLC). For HPLC analysis of 17AAG and 17AAGH₂, 50 μ l of supernatant was separated on a Luna column (C₁₈-2; 250 \times 4.6 mm, 5 μ m; Phenomenex, Torrance, CA) using a flow rate of 1 ml/min with a detection wavelength of 270 nm. HPLC conditions were: buffer A, 0.1% (v/v) trifluoroacetic acid in water; buffer B, 100% acetonitrile. Peaks were eluted by increasing the concentration of buffer B from 20 to 80% over 20 min. The peak corresponding to 17AAG coeluted with authentic 17AAG and the peak corresponding to 17AAGH₂ coeluted with 17AAGH₂ prepared by the enzymatic reduction of 17AAG by purified recombinant human NQO1 (rhNQO1). Intracellular concentrations of 17AAG and 17AAGH₂ were quantified using standard curves generated with authentic 17AAG and 17AAGH₂.

Immunoblot Analysis. Cells were grown in 100-mm plates in complete growth medium. Cells were washed twice in phosphate-buffered saline and then scrapped into (200–400 μ l) of ice-cold 1 \times radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Waltham, MA) containing protease inhibitors (Halt Protease Inhibitor Cocktail; Thermo Fisher Scientific) and phosphatase inhibitors (Phosphatase Arrest I; G-Biosciences, Maryland Heights, MO). Samples were sonicated on ice (5 s) and then centrifuged at 13,000 rpm for 5 min at 4°C. The supernatant was collected and the protein concentrations were determined using the method of Lowry et al. (1951). For SDS-polyacrylamide gel electrophoresis samples were diluted in 2.5 \times Laemmli SDS sample buffer, heated to 70°C for 5 min, placed on ice for 5 min, and then centrifuged at 13,000 rpm for 5 min. SDS-polyacrylamide gel electrophoresis was performed using 12% precast minigels (Bio-Rad Laboratories, Hercules, CA). Proteins were transferred to 0.4- μ m polyvinylidene difluoride membranes (Immobilon; Millipore Corporation, Billerica, MA) in 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol. Membranes were blocked for 1 h at 27°C in 10 mM Tris-HCl, pH 8 containing 150 mM NaCl, 0.2% (v/v) Tween 20, and 5% (w/v) nonfat dry milk. All primary antibodies were added for 1 h at 27°C except antiphospho ERK1/2 and antiphospho MEK antibodies, which were added overnight at 4°C. Secondary antibodies were added for 30 min at 27°C. Proteins were visualized using enhanced chemiluminescence detection (Western Lighting Plus; PerkinElmer Life and Analytical Sciences, Waltham, MA).

Cell-Cycle Analysis. For cell-cycle analysis Panc-1 and Panc-1/C5 cells were grown to 50 to 60% confluence on 100-mm plates and treated with either dimethyl sulfoxide or 17AAG (0.5, 1 μ M) in complete growth medium. After 28 h cells were harvested by trypsinization, washed in phosphate buffer saline, and resuspended at 1 \times 10⁶ cells/ml in saponin stain [Dulbecco's phosphate-buffered saline containing 0.3% (w/v) saponin, 50 μ g/ml propidium iodide, 0.1 mM EDTA, and 125 U/ml RNase] and then analyzed for cell-cycle distribution by flow cytometry.

Oxygen Consumption. Oxygen consumption was monitored during the reduction of 17AAG to 17AAGH₂ by rhNQO1 using a Clark electrode with a stirred 3-ml chamber at 37°C (YSI Inc., Yellow

Springs, OH). The experiments were performed in 50 mM potassium phosphate buffer, pH 7.4 containing 1 mg/ml bovine serum albumin, 500 μ M NADH, 20 μ g of rhNQO1, and 50 μ M 17AAG. Reactions were performed in the absence and presence of 50 μ g of purified bovine erythrocyte Cu/ZnSOD. Oxygen consumption was adjusted for barometric pressure and altitude.

Confocal Imaging. Confocal imaging was used to confirm mitochondrial localization of the overexpression of human MnSOD in MiaPaCa-2/MnSODII cells (Supplemental Fig. 1). In brief, MiaPaCa-2 and MiaPaCa-2/MnSODII cells were grown on glass coverslips in complete medium, fixed in 3.7% (v/v) formaldehyde in phosphate-buffered saline, pH 7.4, for 10 min, and then permeabilized in 0.15% (v/v) Triton-X100 in phosphate-buffered saline, pH 7.4, for 10 min. Cells were then blocked for 1 h in complete medium diluted 1:1 with phosphate-buffered saline, pH 7.4. Primary antibody was diluted in blocking buffer and added for 1 h. Dylight (594 nm, red)-conjugated goat anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories) diluted in blocking buffer containing 1 μ g/ml 4,6-diamidino-2-phenylindole was added for 30 min. All steps were performed at 27°C. Coverslips were mounted onto glass slides and visualized using a Nikon (Tokyo, Japan) C-1 confocal microscope.

Statistical Analysis. Statistical significance was determined using unpaired, one-tailed *t* test with Prism software (GraphPad Software Inc., San Diego, CA).

Results

To examine the role of NQO1 and the NQO1*2 polymorphism on 17AAG cytotoxicity in human pancreatic cancer we used the Panc-1, BxPC-3, and MiaPaCa-2 cell lines. The Panc-1 cell line was selected because it had no detectable NQO1 catalytic activity or protein (Table 1 and Fig. 1) caused by homozygous expression of the NQO1*2 polymorphism. We stably transfected the Panc-1 cell line with a plasmid containing the human NQO1 coding region to generate an isogenic cell line (Panc-1/C5) expressing moderate levels of NQO1 protein and activity (Table 1 and Fig. 1). In these studies we included the BxPC-3 and MiaPaCa-2 cell lines that also express moderate levels of NQO1 protein and activity (Table 1 and Fig. 1). To further elucidate the role of NQO1 in 17AAG-induced growth suppression in pancreatic cancer cell lines we used ES936, a potent mechanism-based inhibitor of NQO1 (Winski et al., 2001). Treatment with

TABLE 1
NQO1 activity and growth inhibition induced by Hsp90 inhibitors in pancreatic cancer cell lines
Results are the mean \pm standard deviation of three separate determinations.

Cell Line	NQO1 Activity ^a	17AAG IC ₅₀ ^b	Radicicol IC ₅₀ ^b
		μ M	
MiaPaCa-2	787 \pm 6	0.38 \pm 0.04	
MiaPaCa-2 + ES936	N.D. ^c	0.76 \pm 0.07 ^{d,e}	
BxPC-3	376 \pm 7	1.17 \pm 0.10	
BxPC-3 + ES936	N.D. ^c	24.90 \pm 0.14 ^{d,e}	
Panc-1	N.D.	51.10 \pm 3.90	4.72 \pm 0.07
Panc-1 + ES936	N.D. ^c	52.90 \pm 5.87 ^d	
Panc-1/C5	388 \pm 7	5.90 \pm 1.03	5.35 \pm 0.19
Panc-1/C5 + ES936	N.D. ^c	28.22 \pm 3.6 ^{d,e}	

N.D., nondetectable (< 5 nmol DCPIP/min/mg protein).

^a DCPIP reduction assay (nmol DCPIP/min/mg protein).

^b 17AAG and radicicol growth inhibition (4-h exposure) was determined using the MTT assay as described under *Materials and Methods*.

^c ES936 (100 nmol/l) was added to cells for 30 min.

^d Pancreatic cell lines were pretreated with ES936 (100 nmol/l) for 30 min and ES936 (100 nmol/l) was present during 17AAG treatment.

^e Pretreatment with ES936 resulted in a statistically significant (*P* < 0.05) increase in the IC₅₀ for 17AAG.

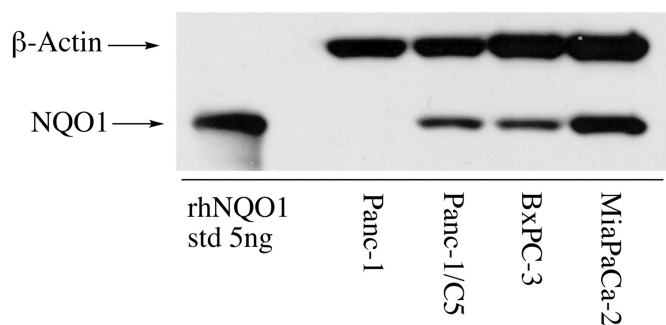


Fig. 1. Immunoblot analysis of NQO1 protein expression in human pancreatic cancer cell lines. Whole-cell sonicates (20 μ g) of pancreatic cancer cell lines Panc-1 (NQO1 null), Panc-1/C5 (NQO1 overexpressing), BxPC-3, and MiaPaCa-2 were assayed by immunoblotting for NQO1 protein expression. Purified rhNQO1 (5 ng) was included as a positive control, and β -actin was included as a loading control.

ES936 resulted in essentially complete inhibition of NQO1 activity in these cell lines (Table 1).

A role for NQO1 in the toxicity of 17AAG was examined using the MTT growth inhibition assay in pancreatic cancer cell lines. A comparison of the relative growth inhibition of 17AAG between the isogenic cell lines Panc-1 and Panc-1/C5 cell lines demonstrated that 17AAG was approximately 10 times more potent at inducing growth inhibition in NQO1 expressing Panc-1/C5 cells compared with the NQO1-null parental Panc-1 cells (Table 1). In addition, pretreatment of Panc-1/C5 cells with ES936 decreased the growth inhibitory effect of 17AAG substantially (Table 1). Likewise, growth inhibition induced by 17AAG could also be attenuated in BxPC-3 and MiaPaCa-2 cells by pretreatment with ES936 (Table 1). These data confirm a role for NQO1 in the growth inhibitory activity of 17AAG in pancreatic cancer cell lines.

The role of NQO1 on growth inhibition induced by the nonquinone Hsp90 inhibitor radicicol was also examined. In these studies the growth inhibitory activity of radicicol was slightly greater in the NQO1-null Panc-1 cell line compared with the Panc-1/C5 cell line (Table 1), suggesting that NQO1 expression by itself does not lead to increased sensitivity to nonquinone Hsp90 inhibitors.

To confirm that NQO1 expression resulted in more pronounced Hsp90 inhibition after 17AAG treatment we examined downstream biomarkers of Hsp90 inhibition by immunoblot analysis using the isogenic Panc-1 and Panc-1/C5 cell lines. Results from these studies clearly demonstrated greater compensatory Hsp70 induction and increased degradation of cRaf in the NQO1-expressing Panc-1/C5 cells compared with the NQO1-null Panc-1 cells (Fig. 2, A and B). Correspondingly, markers of downstream signaling pathways of cRaf including MEK and ERK1/2 phosphorylation were also modulated to a greater degree in Panc-1/C5 cells compared with Panc-1 cells (Fig. 2, C and D). The effect of 17AAG treatment on cell-cycle progression was also examined in Panc-1 and Panc-1/C5 cells. In these studies G₂M arrest and inhibited progression to S phase was detected in Panc-1/C5 cells but not Panc-1 cells (Fig. 3). Proteins associated with cell-cycle progression were also analyzed in Panc-1 and Panc-1/C5 cells after treatment with 17AAG, and in these studies substantially lower levels of CDC2 were detected at all time points in Panc-1/C5 cells compared with Panc-1 cells. Protein levels of CDC25 were also lower in Panc-1/C5 cells compared with Panc-1 after treatment with 17AAG. However, for both cell lines after 24-h treatment with

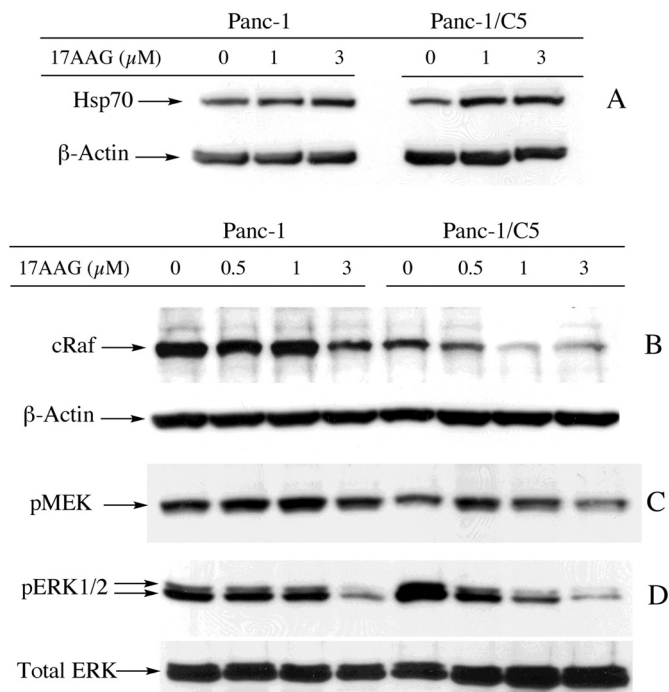


Fig. 2. Immunoblot analysis of biomarkers for Hsp90 inhibition in Panc-1 and Panc-1/C5 cells treated with 17AAG. Whole-cell sonicates (20 μ g) of Panc-1 and Panc-1/C5 cells were assayed by immunoblot analysis for Hsp70 (A), cRaf (B), pMEK (C), and pERK1/2 (D) after treatment with 17AAG (0–3 μ M) for 8 h. β -Actin was used as a loading control for cRaf and pMEK, and total ERK was used as a loading control for pERK1/2. Results are representative of duplicate experiments.

17AAG the protein levels of CDC25 rebounded to those observed in nontreated cells (Fig. 4). These data confirm that 17AAG is more effective at inducing G₂M arrest in NQO1-expressing Panc-1/C5 cells compared with NQO1-null Panc-1 cells.

To account for the increased growth suppression and greater Hsp90 inhibition in human pancreatic cancer cell lines with catalytically active NQO1 we measured the intracellular concentrations of 17AAG and 17AAGH₂. For these studies, pancreatic cancer cell lines were treated with 17AAG, and at the indicated times cells were collected and intracellular concentrations of 17AAG and 17AAGH₂ were determined by HPLC. In experiments using the isogenic Panc-1 and Panc-1/C5 cell lines, much higher concentrations of 17AAG and 17AAGH₂ could be detected at all time points in the NQO1-expressing Panc-1/C5 cell line compared with the NQO1-null Panc-1 cell line (Fig. 5A). In experiments with BxPC-3 and MiaPaCa-2 cells inhibition of NQO1 by ES936 resulted in much lower intracellular concentrations of 17AAG and 17AAGH₂ (Fig. 5B). Taken together, these data clearly demonstrate that metabolism of 17AAG to 17AAGH₂ by NQO1 and subsequent partial reoxidation of 17AAGH₂ back to 17AAG results in higher intracellular concentrations of both 17AAG and 17AAGH₂.

Because the stability of 17AAGH₂ in solution has been shown previously to be sensitive to oxidation catalyzed by redox active metals, including copper (Cu²⁺; Guo et al., 2008), we examined whether superoxide was responsible for catalyzing the oxidation of 17AAGH₂. Initially, we measured oxygen consumption during NQO1-mediated reduction of 17AAG in a cell-free system. The polarographic data shown in Fig. 6, A and B clearly

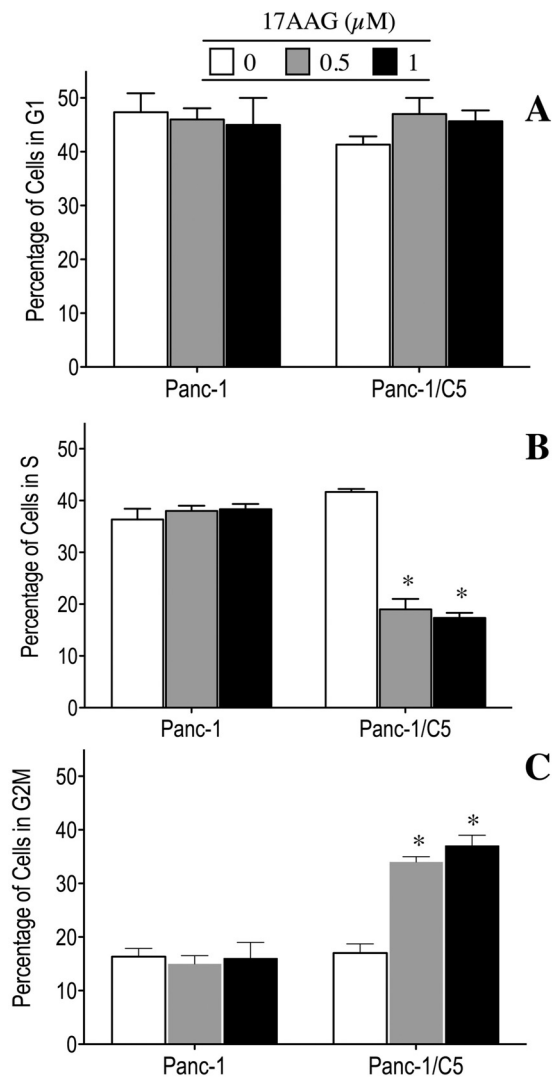


Fig. 3. Cell-cycle analysis of Panc-1 and Panc-1/C5 cells treated with 17AAG. Cell-cycle analysis was performed by flow cytometry on Panc-1 and Panc-1/C5 cells after treatment with 17AAG (0.5 and 1 μ M) for 28 h. A, percentage of cells in G₁. B, percentage of cells in S phase. C, percentage of cells in G₂M. *, $p < 0.05$ significantly different from untreated Panc-1/C5 cells.

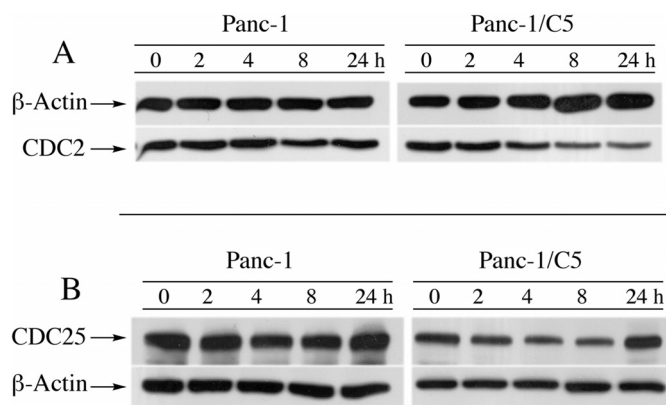


Fig. 4. Immunoblot analysis of CDC2 and CDC25 protein expression in Panc-1 and Panc-1/C5 cells treated with 17AAG. Whole-cell sonicates (20 μ g) of Panc-1 and Panc-1/C5 cells were assayed by immunoblot analysis for CDC2 (A) and CDC25 (B) protein expression after treatment with 17AAG (0.5 μ M) for the indicated times. β -Actin was included as a loading control. Results are representative of duplicate experiments.

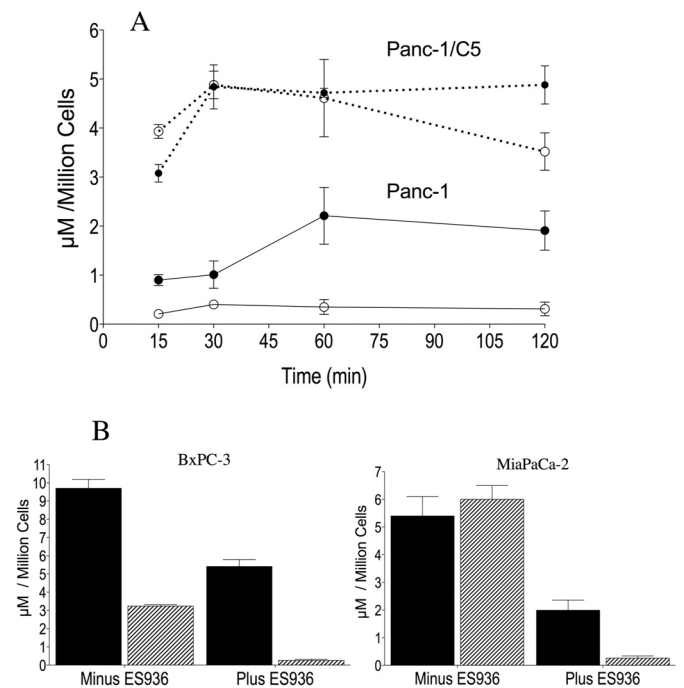


Fig. 5. Effect of NQO1 on the accumulation of 17AAG and 17AAGH₂ in pancreatic cancer cell lines. A, time course of 17AAG and 17AAGH₂ accumulation in Panc-1 and Panc-1/C5 cells. Intracellular concentrations of 17AAG and 17AAGH₂ were measured by HPLC after treatment with 17AAG for the indicated times. Solid lines, Panc-1 cells; dashed lines, Panc-1/C5 cells. ●, 17AAG; ○, 17AAGH₂. Results are the mean \pm standard deviation of three separate determinations. B, effect of NQO1 inhibition on the accumulation of 17AAG and 17AAGH₂ in BxPC-3 and MiaPaCa-2 cells. Intracellular concentrations of 17AAG and 17AAGH₂ were measured by HPLC after treatment with 17AAG for 1 h in the presence and absence of the NQO1 inhibitor ES936. Cells were pretreated with ES936 or dimethyl sulfoxide for 30 min, and ES936 was included with 17AAG for 1 h. Solid bars, 17AAG; hatched bars, 17AAGH₂. Results are the mean \pm standard deviation of three separate determinations.

show oxygen consumption during the metabolism of 17AAG by rhNQO1 caused by oxidation of 17AAGH₂. The addition, however, of purified SOD to the reaction reduced oxygen consumption substantially, suggesting that 17AAGH₂ is susceptible to superoxide-mediated oxidation (Fig. 6, A and B). To examine the ability of SOD to prevent 17AAGH₂ oxidation in cells we generated a stable clone of the MiaPaCa-2 cell line overexpressing human MnSOD (MiaPaCa-2/SODII; Fig. 6C and Supplemental Fig. 1). After treatment with 17AAG higher levels of 17AAGH₂ were detected in MiaPaCa-2/SODII cells compared with parental MiaPaCa-2 cells (Fig. 6D). In addition, overexpression of MnSOD in MiaPaCa-2 cells led to a significant increase in growth inhibition induced by 17AAG as measured using the MTT assay (Fig. 6E).

Discussion

In these studies we have confirmed the role of NQO1 in the sensitivity of human pancreatic cancer cells to 17AAG. Using MiaPaCa-2 and BxPC-3 pancreatic cancer cells that express moderate levels of NQO1 in combination with ES936, a mechanism-based inhibitor of NQO1, we have shown that sensitivity to 17AAG could be decreased several-fold by inhibiting the catalytic activity of NQO1. Furthermore, studies using the Panc-1 (NQO1-null) and the Panc-1/C5 (NQO1-expressing) isogenic cell lines confirmed that expression of NQO1 increased sensitivity to 17AAG. A role for NQO1 in

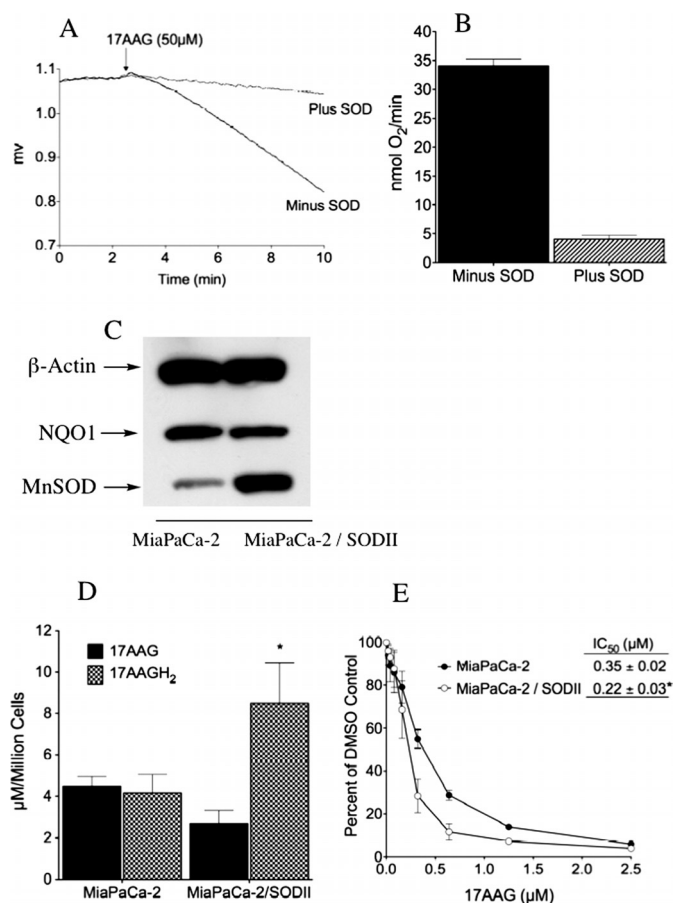


Fig. 6. Effect of superoxide dismutase on inhibiting the oxidation of 17AAGH₂. A, polarographic analysis of the inhibition of oxygen consumption by Cu/ZnSOD during the reduction of 17AAG by rhNQO1. Reaction conditions are described under *Materials and Methods*. B, quantification of A. Results are the mean \pm standard deviation of five separate determinations. C, immunoblot analysis demonstrating overexpression of MnSOD in MiaPaCa-2/SOD II cells. D, intracellular levels of 17AAG and 17AAGH₂ in MiaPaCa-2 and MiaPaCa-2/SODII cells after treatment with 17AAG (25 μ M) for 30 min. Results are the mean \pm standard deviation of three separate determinations. *, $p < 0.05$ significantly different from 17AAGH₂ in parental MiaPaCa-2. E, effect of MnSOD overexpression on the growth inhibition induced by 17AAG in MiaPaCa-2 and MiaPaCa-2/SODII cells. Growth inhibition was determined using the MTT assay after a 4-h 17AAG treatment. Results are the mean \pm standard deviation of three separate determinations. *, $p < 0.05$ significantly different from parental MiaPaCa-2.

generating substantially greater intracellular concentrations of both 17AAG and 17AAGH₂ was also demonstrated. The increased intracellular concentrations of 17AAG and 17AAGH₂, in combination with the more efficient inhibition of Hsp90 by 17AAGH₂ (Guo et al., 2005, 2006; Maroney et al., 2006), are most likely the mechanisms whereby NQO1 contributes to the increased cytotoxicity of 17AAG. The NQO1 activity in the MiaPaCa-2 cell line was approximately 2-fold greater than the BxPC-3 and Panc-1/C5 cell lines; however, there was a more than 15-fold difference in the IC₅₀ values between MiaPaCa-2 cells and Panc-1/C5 cells, suggesting that the sensitivity to 17AAG in pancreatic cancer cell lines is not linearly related to NQO1 and other susceptibility factors in addition to NQO1 are involved.

The increased potency of hydroquinone ansamycins relative to their parent quinones as Hsp90 inhibitors is caused by a more favorable interaction energy in the Hsp90 ATPase

active site (Guo et al., 2006; Maroney et al., 2006). Therefore, cells that generate and maintain higher intracellular concentrations of 17AAG and 17AAGH₂ would be predicted to sustain greater levels of Hsp90 inhibition. However, a study that examined IPI-504, the hydroquinone of 17AAG, in a panel of more than 20 human tumor cell lines questioned the role of NQO1 (IPI-504 is rapidly converted to 17AAG at physiological pH). Although no statistical methods were reported in that study the authors concluded that there were two groups of cell lines, one of which depended on NQO1 for IPI-504 activity and the other that was independent (Douglas et al., 2009). Nevertheless, the study using IPI-504 (Douglas et al., 2009) and our own work (Guo et al., 2005) clearly demonstrated that NQO1 activity is only one of the molecular correlates predicting anti-tumor response to 17AAG in a wide range of human tumors. The related quinone reductase NQO2 can also reduce 17AAG to 17AAGH₂ in cell-free studies using rhNQO2 and nicotinamide riboside (Siegel et al., 2007); however, at present there are no data in cellular systems demonstrating a role for NQO2 in the reduction of 17AAG. Other factors include the level of expression of drug transporters such as multi-drug resistance 1 and p-glycoprotein as well as the compensatory induction of heat shock proteins including Hsp27 and Hsp70, which all have been linked to resistance to 17AAG (McCullum et al., 2006, 2008; Zhang et al., 2010). The role of Hsp90 oncogenic client proteins in stimulating cell proliferation is also an important determinant of tumor sensitivity to Hsp90 inhibitors. For example, tumors driven by mutations in b-Raf and n-Raf may be more sensitive to Hsp90 inhibition compared with tumors lacking mutations in these oncogenes (da Rocha Dias et al., 2005; Banerji et al., 2008).

An alternative approach to examining correlations in multi-cell line panels has been to examine the sensitivity of 17AAG in isogenic cell lines that differ in the expression of a single protein such as NQO1. In experiments using isogenic pairs of cancer cell lines derived from the colon (Kelland et al., 1999) and breast (Guo et al., 2005) and in our work in this study using pancreatic cell lines the expression of NQO1 greatly enhanced the sensitivity to 17AAG compared with their corresponding NQO1-null daughter cell lines. Pretreatment with ES936, a mechanism-based inhibitor of NQO1 in either the isogenic models or in cell lines that normally express NQO1, decreased the sensitivity of cells to 17AAG, confirming the role of NQO1 in these studies (Guo et al., 2005). Similar data were demonstrated using IPI-504 in breast cancer cells transfected with NQO1 (Douglas et al., 2009). NQO1 expression has also been implicated as an important molecular determinant in the sensitivity of glioblastoma and melanoma cell lines to 17AAG. In these experiments cells were exposed to sublethal concentrations of 17AAG for 2 to 8 weeks after which cellular resistance to 17AAG developed in conjunction with extensive down-regulation of NQO1 protein expression (Gaspar et al., 2009). All of these studies implicate NQO1 as a susceptibility factor in the response to the benzoquinone ansamycin Hsp90 inhibitors but do not imply that NQO1 is the only protein responsible for sensitivity to 17AAG.

In this article we also demonstrate that 17AAGH₂ could be oxidized back to the parent quinone by superoxide and that by increasing intracellular levels of MnSOD higher concentrations of 17AAGH₂ could be maintained inside the cell, leading to increased growth inhibition. This discovery could

have therapeutic value because the protein levels of both NQO1 and MnSOD have been shown to be increased substantially in cells after radiation exposure (Boothman et al., 1993; Lin et al., 1993; Wong, 1995; Choi et al., 2007). A number of studies have shown that 17AAG synergizes with radiation to increase the anti-tumor response (Bisht et al., 2003; Russell et al., 2003; Machida et al., 2005). Although these studies did not examine intracellular levels of 17AAG and 17AAGH₂ directly they did demonstrate with combination therapy more pronounced alterations in Hsp90 client proteins, which may be explained by higher intracellular levels of 17AAGH₂. By increasing NQO1 protein expression greater intracellular levels of 17AAGH₂ could be generated and in combination with increased MnSOD expression higher concentrations of 17AAGH₂ could be maintained. It is noteworthy that in cells with very high levels of NQO1 both the reduction of 17AAG to 17AAGH₂ and protection against superoxide-induced hydroquinone oxidation could be performed by NQO1 because NQO1 has also been shown to scavenge superoxide (Siegel et al., 2004).

In summary, these data demonstrate that NQO1 increased the sensitivity of human pancreatic cell lines to 17AAG via the reduction of 17AAG to 17AAGH₂. The oxidation of 17AAGH₂ could be inhibited by the overexpression of MnSOD, implicating superoxide as an important oxidant in limiting the accumulation of 17AAGH₂.

Authorship Contributions

Participated in research design: Siegel, Shieh, Yan, and Ross.

Conducted experiments: Siegel, Shieh, and Yan.

Contributed new reagents or analytic tools: Kupa.

Performed data analysis: Siegel, Shieh, and Yan.

Wrote or contributed to the writing of the manuscript: Siegel and Ross.

Other: Siegel and Ross acquired funding for the research.

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