The antiproliferative activity of the heat shock protein 90 inhibitor IPI-504 is not dependent on NAD(P)H:quinone oxidoreductase 1 activity in vivo

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Abstract

IPI-504, a water-soluble ansamycin analogue currently being investigated in clinical trials, is a potent inhibitor of the protein chaperone heat shock protein 90 (Hsp90). Inhibition of Hsp90 by IPI-504 triggers the degradation of important oncogenic client proteins. In cells, the free base of IPI-504 hydroquinone exists in a dynamic redox equilibrium with its corresponding quinone (17-AAG); the hydroquinone form binding 50 times more tightly to Hsp90. It has been proposed recently that the NAD(P)H:quinone oxidoreductase NQO1 can produce the active hydroquinone and could be essential for the activity of IPI-504. Here, we have devised a method to directly measure the intracellular ratio of hydroquinone to quinone (HQ/Q) and have applied this measurement to correlate NQO1 enzyme abundance with HQ/Q ratio and cellular activity of IPI-504 in 30 cancer cell lines. Interestingly, the intracellular HQ/Q ratio was correlated with NQO1 levels only in a subset of cell lines and overall was poorly correlated with the growth inhibitory activity of IPI-504. Although artificial overexpression of NQO1 is able to increase the level of hydroquinone and cell sensitivity to IPI-504, it has little effect on the activity of 17-amino-17-demethoxygeldanamycin, the major active metabolite of IPI-504. This finding could provide an explanation for the biological activity of IPI-504 in xenograft models of cell lines that are not sensitive to IPI-504 in vitro. Our results suggest that NQO1 activity is not a determinant of IPI-504 activity in vivo and, therefore, unlikely to become an important resistance mechanism to IPI-504 in the clinic. [Mol Cancer Ther 2009;8(12):3369–78]

Introduction

Heat shock protein 90 (Hsp90), an emerging target for the treatment of cancer (1–3), is a highly expressed protein chaperone that associates with ~200 client proteins to support their conformational maturation, stability, trafficking, and function. Numerous Hsp90 client proteins are kinases or transcription factors that are involved in cellular proliferation, angiogenesis, invasion, and metastasis. As a result, cancer cells seem to be more dependent on Hsp90 function than normal cells. Indeed, Hsp90 inhibitors show a remarkable selectivity for cancer cells over normal cells in vitro (4).

Inhibition of Hsp90 leads to rapid degradation of client proteins through the ubiquitin-proteasome pathway (5). A large number of studies have shown the activity of Hsp90 inhibitors in multiple models of solid (e.g., lung, breast, prostate, pancreatic, melanoma) and hematologic (e.g., chronic myelogenous leukemia, multiple myeloma) cancers (6–12). Two Hsp90 inhibitors, IPI-504 (retaspimycin hydrochloride; refs. 13, 14) and 17-allylamino-demethoxygeldanamycin (tanespimycin, 17-AAG), are derivatives of the natural product geldanamycin that interconvert in vivo to a redox equilibrium between the hydroquinone and quinone forms (14, 15). Both compounds are currently in clinical trials and have shown biological activity in the treatment of gastrointestinal stromal tumors, lung, melanoma, and breast cancer (16–20).

However, several questions related to the mechanisms underlying the cell growth inhibitory activity of ansamycins remain unresolved. For example, ansamycins exhibit low nanomolar cellular activity, but submicromolar in vitro Hsp90 binding affinities have been reported (21). To explain this discrepancy, it has been proposed that the hydroquinone form of ansamycins—which is not present under in vitro binding assay conditions without the addition of reducing agents—binds more tightly to Hsp90 compared with the quinone (13, 15, 21, 22).

If the hydroquinone form of ansamycins is the more active Hsp90 inhibitor, the ability of cells to reduce the quinone to the hydroquinone form could play a role in the cellular activity of ansamycin derivatives. 17-AAG is a relatively weak substrate of the NADH:quinone oxidoreductase 1 (NQO1; DT-diaphorase, EC 1.6.99.2), which converts benzoquinone into hydroquinone (23). However, it is unclear whether NQO1 is the sole enzyme that can catalyze this reduction or whether other cellular factors influence this redox equilibrium. If NQO1 were the only enzyme...
to determine the amount of highly active hydroquinone in ansamycin-treated cells, NQO1 activity would presumably predict sensitivity of cancer cells to ansamycin derivatives and loss of enzyme activity could represent a possible resistance mechanism (24). Although it has been previously shown that overexpression of NQO1 in cells that do not express this enzyme can increase the hydroquinone to quinone (HQ/Q) ratio and sensitize the cells to ansamycins (15), the role of NQO1 on ansamycin-induced cytotoxic activity in cells that endogenously express the enzyme is debated (23, 25). In addition, NQO1 activity has been shown not to predict sensitivity to 17-AAG in the NCI 60 cell line panel (23).

The purpose of this study was to investigate the role of NQO1 in determining the levels of hydroquinone produced within cells and to correlate this HQ/Q ratio with the growth inhibitory activity of the ansamycin derivative IPI-504. To this end, a novel assay was developed to monitor the HQ/Q ratio in cell extracts. The hydroquinone is prone to chemical oxidation to the quinone derivative, whereas the quinone will only undergo enzymatic reduction to the hydroquinone. Upon cell lysis with an acidic buffer, the quinone derivative remains unchanged due to enzyme denaturation and the hydroquinone is stabilized as the protonated aniline derivative. We also studied the effect of overexpression of NQO1 on the HQ/Q ratio in cells and on the growth inhibitory effect of different geldanamycin derivatives in vitro and in vivo. Surprisingly, we found IPI-504 to be biologically active in xenograft models of tumor cell lines that show little sensitivity to the cytotoxic action of IPI-504 in tissue culture. We propose that this activity is due to the potent, redox-independent activity of 17-AG, the major active metabolite of IPI-504 in vivo.

Materials and Methods

Reagents and Cell Culture

17-AAG, IPI-504, and 17-AG were synthesized as previously reported (13, 26). H-460, HCC-4006, A-549, A2780, IGR-OV1, SKOV3, HCT-116, SW480, HT29, AsPC-1, L3.6p1, L3.5s1, MCF-7, MDA-MB-468, BT474, JIMT-1, K-562, Daudi, RL, and Toledo cell lines (all from American Type Culture Collection) were grown in RPMI 1640 containing 15% fetal bovine serum and HCC-827 in RPMI 1640 containing 10% fetal bovine serum, 1 μg/mL streptomycin, and 1 μg/mL penicillin. H-1975, H-1650 cells (American Type Culture Collection) were grown in RPMI 1640 containing 10% fetal bovine serum, 1 μg/mL streptomycin, and 1 μg/mL penicillin. H-1975, H-1650 cells (American Type Culture Collection) were cultured in RPMI 1640 containing 15% fetal bovine serum and HCC-827 in RPMI 1640 containing 5% fetal bovine serum. All the cell lines were tested for Mycoplasma and were maintained at 37°C in a humidified 5% CO2 atmosphere.

NQO1 Enzymatic Assay

NQO1 activity in cell lysates or from the human recombinant NQO1 enzyme (Sigma) was assayed in a Tris-HCl 25 mmol/L buffer (containing 0.3 mg/mL bovine serum albumin, 5 μmol/L FAD, and 0.05% Triton X-100) by adding 600 μmol/L NADH and 125 μmol/L of the substrate menadione (Sigma). NADH consumption due to NQO1 activity was monitored spectrophotometrically at 340 nm using a Spectramax (Molecular Devices). The same incubation without NADH or with 50 μmol/L of the NQO1 inhibitor dicoumarol was used as control. Human recombinant NQO1 enzyme was used as a standard.

HQ/Q Ratio Method

Analytic liquid chromatography (high performance liquid chromatography) was done on an Agilent 1100 high performance liquid chromatography system equipped with a Waters C18-Symmetry reverse-phase column (4.6 × 150 mm, 5 μm). The mobile phases were acetonitrile (0.1% trifluoroacetic acid) and water (0.1% trifluoroacetic acid). The compounds were eluted with a step gradient of 10% acetonitrile for the first minute, 10% to 55% acetonitrile for 8 min, 55% to 90% acetonitrile for 9 to 11 min, followed by 90% acetonitrile for 3 min, at a flow rate of 0.4 mL/min. All flow was directed into a Thermo Scientific LCQdeca on which mass spectroscopy was done. The mass spectroscopy settings included a capillary temperature of 250°C, spray voltage of 4,500 V, capillary voltage of 4 V, tube lens offset of 15 V. Mass spectra was continuously recorded from 300 to 800 amu during the chromatographic analysis. All cell lines tested were incubated with 5 μmol/L of 17-AAG for 3 h at 37°C, lysed with an aqueous solution of HCl (0.1 mol/L), and snap frozen at ~80°C. Upon analysis, samples were thawed, mixed with a deuterated standard of 17-AAG, and split into two sets of 1-mL aliquots. To measure total ansamycin present (hydroquinone and quinone), the first set of 1-mL aliquots was neutralized with 0.5 mL saturated NaHCO3 to convert any hydroquinone to the quinone derivative. In the second set, samples were processed without a neutralization step to measure 17-AAG content. Dichloromethane (CH2Cl2, 2 mL) was added to each sample and a 1-mL aliquot of the CH2Cl2 layer was removed and concentrated to dryness. The samples were reconstituted in 100 μL of high performance liquid chromatography diluent (80:20:0.1 DMSO/Acetonitrile/trifluoroacetic acid) and analyzed immediately by liquid chromatography-mass spectrometry7.

Binding Assay

Hsp90 protein (SPP-770) was from Assay Designs, and [11,2H]-17-AAG and [11,3H]-17-AG were from Ambios Labs. The binding affinities (Ki) of 17-AAG and 17-AG for Hsp90 were determined using a spin column assayA series of [3H]-17-AAG and [3H]-17-AG (5 × 106 cpm/nmol) dilutions were prepared in Hsp90 binding buffer [20 mmol/L HEPES buffer (pH 7.3), 1.0 mmol/L EDTA, 100 mmol/L KCl, 5.0 mmol/L MgCl2, 0.5 mg/mL bovine γ globulin, 0.01% NP40 ± 2.0 mmol/L Tris (2-carboxyethyl)-phosphinohydroxide (TCEP)] containing a cocktail of protease inhibitors. Binding curves were obtained by incubating 100 nmol/L Hsp90 with a [3H]-17-AAG or [3H]-17-AG dilution series in Hsp90 binding buffer at 37°C for 30 min. Each sample was then passed twice through a Zeta spin plate (Pierce Biotechnology) to remove unbound ligand, and the final eluate was counted using a Microβ2 microplate scintillation counter (Perkin-Elmer).

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Immunoblotting
Western analyses were done using primary anti-NQO1 antibody (sc-16464) from Santa Cruz Biotechnologies according to previously reported protocols (27). Image analysis and band quantization were done with the Bio-Rad Versa Doc system. The expression of glyceraldehyde-3-phosphate dehydrogenase was used as a control for protein loading.

Cell Growth Inhibition Studies
Cells were incubated in 96-well plates (200 μL) ± compounds for 3 d with increasing concentrations of IPI-504 or 17-AG, and viability studies were done using the vital mitochondrial function stain Cell Titer Glow (Promega) according to the manufacturer instructions. The ratios obtained from drug-treated cells versus vehicle-treated cells were quantified and plotted against drug concentration to produce growth inhibition 50 (GI50) values.

NQO1 Expression in MDA-MB-231
An NQO1 ultimate open reading frame clone was purchased from Invitrogen and recombined using a Gateway LR clonase reaction into pLenti6/R4R2/DEST along with a cytomegalovirus promoter vector according to the manufacturer’s instructions. The resulting vector was used to generate recombinant lentiviral particles using Virapower kit (Invitrogen). MDA-MB-231 cells were infected with the NQO1-expressing lentiviral particles and infected cells were selected using Blasticidin.

In vivo Studies
Mice were maintained and treated in accordance with the Institutional Animal Care and Use Committee guidelines.

Six- to eight-week-old male NCr nude athymic (nu−/nu−) mice were purchased from Taconic Farms. MDA-MB-231 and MDA-MB-468 cells (1 × 10⁷) were injected into the flanks of 40 mice, and treatment began when tumors reached an average size of ∼100 mm³, which usually occurred ∼14 d after injection. IPI-504 (75 mg/kg) i.p. and 17-AG (130 mg/kg) oral or the vehicle equivalents were administered twice per week (Monday, Thursday). Tumor xenografts were measured with calipers twice per week. Ten mice were used for each arm. Results are presented as means and SEM.

Results
Determination of NQO1 Protein Levels and Enzymatic Activity in a Panel of Cancer Cell Lines
To investigate the potential role of NQO1 in the growth inhibitory activity of IPI-504 and in the setting of the HQ/Q ratio, NQO1 enzymatic activity and protein levels were determined in 30 cancer cell lines from nine different cancer types, and then correlated with the HQ/Q ratio in cell lysates and IPI-504 growth inhibitory activity in vitro. Among the cell lines tested, a broad range of NQO1 protein levels were observed, ranging from 20 to 2410 ng equivalent NQO1 per milligram of total protein (ng NQO1/mg; Fig. 1A). NQO1 was not detected in the acute myelogenous leukemia cell line MV4:11 or in the non–small cell lung cancer cell line HCC-4006. Among the cell lines tested, a broad range of NQO1 protein levels were observed, ranging from 20 to 2410 ng equivalent NQO1 per milligram of total protein (ng NQO1/mg; Fig. 1A). NQO1 was not detected in the acute myelogenous leukemia cell line MV4:11 or in the non–small cell lung cancer cell line HCC-4006. In contrast, the non–small cell lung cancer cell lines H-1650 and H-460 showed the highest level

Figure 1. NQO1 protein expression and enzymatic activity in cancer cell lines. A, the level of NQO1 protein was determined by immunoblotting followed by densitometry as a ratio of the NQO1 and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) band using recombinant NQO1 as standard. B, densitometry values were then plotted against enzymatic activity measured by monitoring the consumption of NADH using menadione as a substrate. Human recombinant NQO1 was used as a standard and inhibition by dicoumarol 10 μmol/L as a specificity control.
of NQO1 at 1,000 and 2,410 ng NQO1/mg, respectively. The level of NQO1 enzymatic activity correlated well with the amount of expressed protein in any given tested cell line ($R^2 = 0.9$, Fig. 1B). In addition, the relative NQO1 protein levels described here were in good agreement with those previously estimated in the NCI-60 cell lines panel (28).

**Development of an Analytic Method to Determine the HQ/Q Ratio in Cells**

To properly assess the level of ansamycin hydroquinone and quinone in cell extracts requires an analytic method that irreversibly and instantly locks the redox equilibrium, and that reproducibly avoids the conversion of the hydroquinone to the quinone form. Such a method was developed using acidic conditions to freeze the ratio and differential extraction to quantify each form.

Under normal intracellular conditions, the hydroquinone and quinone chemotypes exist in a dynamic equilibrium (Fig. 2A). The interconversion between hydroquinone and quinone chemotypes ceases when cells are lysed in the presence of strong acid, because reducing enzymes are denatured at pH 1.0 and the hydroquinone is stabilized because the protonated aniline moiety has a decreased ability to react with oxygen due to decreased electron donation into the aromatic ring. To quantify the HQ/Q ratio, a differential extraction method was developed that capitalizes on the basicity differences of 17-AAG quinone and hydroquinone. The 17-nitrogen of 17-AAG has decreased basicity compared with the 17-aniline nitrogen of the hydroquinone and is not protonated under the acidic conditions (Fig. 2B). Upon addition of an organic solvent, the protonated hydroquinone stays in the acidic aqueous media, whereas the unprotonated quinone is extracted into the organic layer and quantified in an analytic assay. The total amount of ansamycin (hydroquinone and quinone) is assessed by raising the pH of the cell lysate to pH 9.0, resulting in the deprotonation of the 17-nitrogen of 17-AAG to form the hydroquinone, which is prone to oxidize to 17-AAG under physiologic conditions.

![Figure 2](image-url)
the hydroquinone and hence a more electron rich aromatic ring that is prone to chemical oxidation to the quinone derivative (Fig. 2C). The quinone is extracted and analyzed as described above. The level of hydroquinone is determined by subtracting the amount of quinone established in the acidic extraction from the total amount detected in the basic extraction. This method has shown to accurately detect different HQ/Q ratios spiked into blank cell lysate (Fig. 2D).

**NQO1 Protein Levels and HQ/Q Ratios**

To use this method to correlate cellular NQO1 level with the HQ/Q ratio of 17-AAG, each cell line in the panel was incubated with 5 μmol/L of 17-AAG for 3 hours and lysed with an aqueous solution of HCl (0.1 mol/L). The HQ/Q ratio was determined and compared with the level of NQO1 measured (Table 1).

Overall, there is a poor correlation between NQO1 protein levels and the HQ/Q ratios (R² = 0.23; Fig. 3A). However, closer inspection of Fig. 3A reveals that there may exist two populations of cells: one, where the HQ/Q ratio is high, independent of the abundance of NQO1, and another where the HQ/Q ratio increases with increased enzyme abundance.

**NQO1 Protein Levels and HQ/Q Ratios Correlate Poorly with IPI-504 Growth Inhibitory Activity**

To investigate the correlation between NQO1 protein levels and the growth inhibitory activity of IPI-504, cell growth was assessed in all cell lines after 72 hours of incubation with increasing concentrations of IPI-504 (Table 1). Overall, the correlation between NQO1 expression level and IPI-504 cytotoxic activity is poor (R² = 0.37; Fig. 3B), suggesting that IPI-504 activity depends on other parameters. Furthermore, we also showed that the HQ/Q ratio itself does not correlate well with IPI-504 cytotoxic activities (Fig. 3C) because sensitive cell lines (GI₅₀ < 100 nmol/L) exhibited either a low (e.g., H-929) or a high HQ/Q ratio (e.g., H-460). Taken together, these data suggest that both NQO1 expression level and NQO1 activity (i.e., the production of hydroquinone and the increase of the HQ/Q ratio) do not influence IPI-504 cytotoxic activity in vitro.

### Table 1. NQO1 expression level, HQ/Q ratio, and IPI-504 GI₅₀

<table>
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<th>Cell line</th>
<th>Cancer type</th>
<th>IPI-504 GI₅₀ (nmol/L)</th>
<th>HQ/Q (ratio)</th>
<th>NQO1 (ng/mg prot)</th>
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NOTE: Cells were incubated with increasing concentrations of IPI-504 for 3 d, and cell growth was evaluated. NQO1 intracellular levels were quantified by immunoblot followed by densitometry analysis using glyceraldehyde-3-phosphate dehydrogenase as a loading control and human recombinant NQO1 as a standard. HQ/Q ratio was determined using the method described in Fig. 2.

Abbreviations: NSCLC, non–small cell lung cancer cell line; CRC, colorectal cancer; PaCa, pancreatic cancer; AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia.
Overexpression of NQO1 Increases the HQ/Q Ratio and the Growth Inhibitory Activity of IPI-504, but Has Little Effect on 17-AG Activity

Our data show a lack of correlation between NQO1 levels and the growth inhibitory activity of IPI-504. However, it has been reported in the literature that overexpression of NQO1 in NQO1-null cell lines (e.g., MDA-MB-468 or BE cells; refs. 15, 23) led to a higher sensitivity to 17-AAG in vitro. To extend these findings, we investigated the effect of NQO1 overexpression on the HQ/Q ratio and cellular activity of ansamycin derivatives in a cell line that is devoid of NQO1 activity and protein (MDA-MB-231; see Figs. 1A and 4A). MDA-MB-231 cells were infected with a viral NQO1 expression vector and a robust overexpression of NQO1 was achieved (5,830 ng NQO1/mg; Table 1; Fig. 4A). In the MDA-MB-231 parental cell line, the HQ/Q ratio was low (0.36), which was significantly increased to 0.88 with NQO1 overexpression (Fig. 4B). In a 3-day growth inhibition assay, the engineered cell line MDA-MB-231NQO1 was 60 times more sensitive to IPI-504 than the parental cell line (GI50 = 31 and 1,253 nmol/L, respectively; Fig. 4C). However, both the parent and engineered cells exhibit a similarly high sensitivity toward 17-AG, the major metabolite of IPI-504 (GI50 = 20 nmol/L in MDA-MB-231 and 7 nmol/L in MDA-MB-231NQO1; Fig. 4C).

To understand the molecular basis for such a striking difference between IPI-504 and 17-AG, the hydroquinone and quinone forms of 17-AAG and 17-AG were tested for their ability to bind to purified human Hsp90 in a direct binding assay using radioactively labeled 17-AAG and 17-AG. This assay was run under either reducing (+2 mmol/L TCEP) or nonreducing (no TCEP) conditions, which permitted the complete conversion to either the hydroquinone or quinone forms, respectively. Using this method, binding affinities (K_i) of 21 ± 8 nmol/L and 3.0 ± 1.8 nmol/L were determined for the 17-AG quinone and hydroquinone, respectively (Fig. 2D). The quinone form of 17-AAG, however, exhibited ∼50-fold weaker K_i compared with the hydroquinone form (530 ± 170 and 9.0 ± 4.7 nmol/L, respectively; Table 2). These results indicate that, unlike 17-AAG, 17-AG in both the quinone and hydroquinone form binds to Hsp90 potently, and reduction results in only modest increase in potency. Interestingly, the difference in binding between the quinone and the hydroquinone form of 17-AAG was similar to the difference in growth inhibitory activity of IPI-504 (50 and 60 times, respectively).

In vivo Tumor Growth Inhibition by IPI-504 in Xenografts of Cells Lacking NQO1

To study whether the striking difference in activity of IPI-504 and 17-AG on MDA-MB-231 cells was maintained in vivo, nude mice bearing subcutaneous MDA-MB-231 xenografts were treated with IPI-504 (75 mg/kg i.p.) and 17-AG (130 mg/kg p.o.). The IPI-504 dose administered was reduced compared with 17-AG to account for the systemic bioavailability of the orally administered 17-AG. Both compounds showed comparable strong growth inhibition for >3 weeks using a twice per week dosing schedule, with no significant difference observed between the two compounds.
To confirm these findings, the in vivo efficacy of IPI-504 was tested on an additional NQO1-null xenograft model, MDA-MB-468. Nude mice bearing subcutaneous MDA-MB-468 xenografts were treated with IPI-504 (100 mg/kg i.v.) twice weekly. Again, in this cell line, lacking NQO1 and insensitive to IPI-504 in tissue culture (GI50 = 950 nmol/L; Table 1; ref. 15), IPI-504 exhibits a strong tumor growth inhibition after 3 weeks of treatment (Fig. 5B).

Discussion

The interest in IPI-504 as a clinical candidate warrants investigation of how NQO1 contributes to the activity of IPI-504 and whether lack of NQO1 represents a possible clinical mechanism for resistance. A possible role of NQO1 in the mechanism of action of benzoquinone ansamycins has been proposed based on previous work that showed that NQO1 is involved in the quinone-hydroquinone conversion of other small molecules including RH1 (29), streptonigrin (30), or mitomycin C (31). In the case of 17-AAG, it has been shown that the hydroquinone form binds 50 times more tightly to Hsp90 than the quinone derivative, presumably because of increased hydrogen bonding of the inhibitor to its protein target (15). To understand whether NQO1 plays an important role in the cellular activity of ansamycins, we have extended this analysis by directly measuring the product/substrate ratio of the enzymatic activity of NQO1 in a broad panel of cancer cell lines. Within our panel, 11 of the 30 cell lines had barely detectable NQO1 protein and, accordingly, exhibited almost no NQO1 enzymatic activity. In some cells, this can be explained by a mutation in NQO1 (P187S; refs. 32, 33) that leads to the rapid proteasomal degradation of NQO1 (e.g., MDA-MB-468 and MDA-MB-231; ref. 34). Almost all cell lines derived from hematologic cancers (multiple myeloma, acute myelogenous leukemia, chronic myelogenous leukemia, and lymphoma) were devoid of NQO1 protein expression (Fig. 1A), but no other correlation was found between tumor type and levels of NQO1. Others have noted this discrepancy in NQO1 expression in solid versus hematologic tumors (35).

To understand what role the oxidoreductase NQO1 may play in determining the redox equilibrium of IPI-504, we developed a method that can accurately determine how much of the intracellular concentration of 17-AAG exists in the reduced form (HQ) compared with the oxidized form (Q). This HQ/Q ratio was determined for every cell line in our panel and correlated with the abundance of the NQO1 protein in that cell line (Fig. 3A). Although there is a poor overall correlation (R² = 0.23), our analysis suggests that there may exist two cell populations, one where NQO1 influences the redox equilibrium and another one where hydroquinone is produced in the absence of NQO1, presumably through the action of other cellular oxidoreductases. There are multiple enzymes (e.g., Xanthine dehydrogenase, NADPH:cytochrome P450 reductase, b6 Reductase, NQO2, b5 reductase, etc.).
and xanthine oxidase) that have been shown to participate in the intracellular reduction of quinones (29). We have looked at the expression of three of these (NQO2, b5 reductase, and xanthin oxidase) in five cell lines with low NQO1 levels and could not detect significant expression levels (data not shown). If one accepts the interpretation of Fig. 3A as two groups of cells in one of which NQO1 is obligatory for HQ generation, then these data also imply that nonenzymatic mechanisms are not operative (at least in the cells with low NQO1). This notion is consistent with a very strong reducing potential of 17-AAG (36) and our own experiments that show that neither mercapo-ethanol nor reduced glutathione were able to significantly reduce 17-AAG in physiologic buffer (data not shown).

Knowing that there was not a strong correlation found between the HQ/Q ratio and NQO1 expression levels, we next compared the cellular growth inhibitory activity of IPI-504 to the levels of NQO1. The overall correlation between IPI-504 GI50 values and corresponding NQO1 protein levels was weak ($R^2 = 0.37$), although a trend is apparent. The data included several cell lines that are devoid of NQO1 protein expression, but are extremely sensitive to IPI-504 (e.g., MV4.11 or K562), clearly indicating that a low intracellular level of this enzyme does not always lead to a low activity of IPI-504. A possible explanation for the poor correlation between IPI-504 activity and intracellular NQO1 protein expression could come from the fact that in different cell lines, the degradation of different Hsp90 client proteins is thought to be relevant for cell growth inhibition after Hsp90 inhibition. For example, MV4.11 or K-562 cells are very sensitive to IPI-504, presumably because these cells are driven by the oncogenes Flt3-TR and Bcr-Abl, both of which are very sensitive client proteins of Hsp90 (10, 37, 38). In these cells with a low HQ/Q ratio, even a relatively low concentration of the active hydroquinone could be sufficient to destabilize these key oncogenic client proteins and induce apoptosis.

Because a good correlation was not found between HQ/Q ratios and NQO1 abundance in our cell line panel, we asked whether NQO1 was capable of modulating intracellular levels of the IPI-504 hydroquinone. For this purpose, NQO1 protein was overexpressed in a cell line that is devoid of endogenous NQO1 activity. As expected from previous reports in the literature with 17-AAG (15, 23), overexpression of NQO1 increased the HQ/Q ratio in cells after treatment with IPI-504 and enhanced the cell growth inhibitory activity of IPI-504 (Fig. 4C). However, 17-AG, the major metabolite of IPI-504 that can undergo a similar redox equilibrium, exhibited potent activity against both the wild-type cell line and the NQO1-overexpressing cells.

One possible explanation for the striking difference between IPI-504 and 17-AG activity in this isogenic pair of cell lines are the different Hsp90 binding parameters of the two

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<th>Table 2. Binding affinities of 17-AG and 17-AAG to purified Hsp90 protein in reducing and nonreducing conditions</th>
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Figure 5. In vivo tumor growth inhibition by IPI-504 in both MDA-MB-231 and MDA-MB-468 xenograft models. A, $1 \times 10^7$ MDA-MB-231 cells were injected into the flanks of 40 mice, and treatment began when tumors reached an average size of 100 mm³. IPI-504 75 mg/kg i.p. and 17-AG 130 mg/kg oral or the vehicle equivalents were administered twice per week (Monday, Thursday). B, $1 \times 10^7$ MDA-MB-468 cells were injected into the flanks of 40 mice; IPI-504 100 mg/kg was administered twice per week i.v. (Monday, Thursday).
molecules. In the case of 17-AG, both the hydroquinone and the quinone bind strongly to Hsp90 (Table 2), indicating that a shift in the HQ/Q ratio should not significantly affect its binding and its activity. In contrast, the 17-AAG quinone binds Hsp90 50 times weaker than the hydroquinone form, suggesting that the HQ/Q ratio for IPI-504 is more important in determining the degree of Hsp90 inhibition and, thus, the subsequent cellular activity of IPI-504. The biochemical potency of 17-AG and 17-AAG hydroquinone is comparable with recently reported nonansamycin Hsp90 inhibitors (23, 39, 40). Thus, if enough of the redox-independent metabolite 17-AG is produced in vivo, the need for NQO1 or any other oxidoreductase for the biological activity of IPI-504 would be eliminated. In a phase 1 trial of 17-AAG, 17-AG has been found to account for half of the total area under the curve (41, 42).

To test this hypothesis, a xenograft experiment was undertaken examining the effects of both IPI-504 and 17-AG on a cell line that is devoid of NQO1 activity and resistant to the cytotoxic action of IPI-504 in tissue culture. In this model (MDA-MB-231), 17-AG and IPI-504 exhibit the same tumor growth inhibition, suggesting that enough of the redox-insensitive metabolite 17-AG is produced in vivo to overcome any effect that lack of NQO1 might have on IPI-504 activity. These findings were confirmed in another in vivo model (MDA-MB-468), a breast cancer line that also does not contain any active NQO1 and is insensitive to IPI-504 in vivo. In this model again, IPI-504 administration resulted in a strong tumor growth inhibition.

Beyond our experimental model, one would expect that in hypoxic solid tumors, the equilibrium between hydroquinone and quinone might be shifted toward the reduced hydroquinone. We expect this effect to be of limited importance, however, because our data imply that a large part of the in vivo activity of IPI-504 is due to the redox insensitive, active metabolite 17-AG.

In conclusion, we have shown that there is not a good correlation between the abundance of the enzyme NQO1 and the activity of IPI-504 in a panel of 30 cancer cell lines. Our analysis does point to the possible existence of two cell populations, one in which NQO1 is the predominant enzyme that can catalyze the reduction of ansamycin antibiotics to the active hydroquinone and another one where other enzymes are present that can fulfill this function. The possible existence of two populations of cells might explain why the role of NQO1 in the activity of ansamycin-derived Hsp90 inhibitors has remained controversial. Our analysis also reveals that experiments in tissue culture may underestimate the activity of IPI-504 in vivo (xenograft models and presumably patients) because the major active metabolite of IPI-504, 17-AG, does not depend on reduction to the hydroquinone for activity. These experiments also imply that NQO1 deficiency is less likely to evolve as a clinical resistance mechanism to ansamycin-based Hsp90 inhibitors.

Disclosure of Potential Conflicts of Interest
All authors: employees with stock ownership, Infinity Pharmaceuticals.

References


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The antiproliferative activity of the heat shock protein 90 inhibitor IPI-504 is not dependent on NAD(P)H:quinone oxidoreductase 1 activity in vivo


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