The anti-proliferative activity of the Hsp90 inhibitor IPI-504 is not dependent on NQO1 oxidoreductase activity in vivo.

 # 4691

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Abstract

IPI-504 is a water soluble ansamycin analog currently being investigated in a phase III registration trial. IPI-504 inhibits heat shock protein 90 (Hsp90) chaperoning activity, triggering the degradation of important oncogenic proteins. In the cell, IPI-504 interconverts between a quinone and a hydroquinone form, with the latter binding more tightly to Hsp90. Hence, the cytotoxic activity of IPI-504 might depend on its hydroquinone to quinone (HQ/Q) intracellular ratio, and it has been recently proposed that an NADH:quinone oxidoreductase, NQO1, could increase this ratio.

Here, the role of NQO1 in cancer cell sensitivity to IPI-504 was investigated in a panel of 30 cell lines and it was found that NQO1 intracellular level barely correlates with IPI-504 growth inhibitory activity. However, high over-expression of NQO1 in MDA-MB-231 cells increased the HQ/Q ratio and IPI-504 sensitivity. Importantly, NQO1 over-expression had no effect on the *in vitro* growth inhibitory activity of the major metabolite of IPI-504, 17-amino-17-demethoxy-geldanamycin (17-AG). In MDA-MB-231 wild type xenografts, IPI-504 potently inhibited tumor growth, despite poor cytotoxic activity with these cells in culture. This unexpected strong *in vivo* anti-tumor activity of IPI-504 might be due to 17-AG, whose activity does not depend on cellular oxidoreductases.

These results suggest that the NQO1 level may determine *in vitro* cell sensitivity to IPI-504 in some cases. However, the antiproliferative activity of IPI-504 *in vivo* does not appear to depend on NQO1 status of the cell, possibly because the biological activity of its primary metabolite 17-AG is unaffected by NQO1.

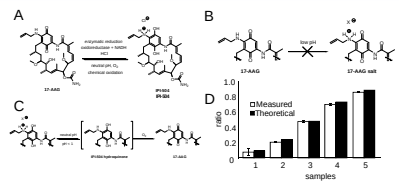
Objectives

- To determine the relationship between NQO1 protein expression/activity and cancer cell sensitivity to IPI-504 *in vitro* using a panel of 30 cancer cell lines
- To determine if NQO1 activity correlates with the hydroquinone : quinone ratio
- To determine if NQO1 activity influences the anti-tumor activity of IPI-504 and the IPI-504 major metabolite, 17-AG, in human NQO1-null breast cancer xenograft models

Assay to detect cytoplasmic HQ:Q ratio

Sample preparation for HPLC

Cells were incubated with 5 µM 17-AAG, an interconversion product of IPI-504, for 3 hr at 37°C, lysed (0.1 M HCl) and snap-frozen at – 80°C. The interconversion between hydroquinone and quinone chemotypes is ceased when cells are lysed in the presence of strong acid; at pH1, reducing enzymes are denatured which result in unchanged 17-AAG quinone levels. In addition, the acidic buffer stabilizes the hydroquinone as the protonated aniline moiety. For HPLC analysis, samples were thawed, mixed with a deuterated standard of 17-AAG, and split into 2 sets of aliquots. To convert the hydroquinone to quinone moiety, one set of aliquots was neutralized with 0.5 mL saturated $NaHCO_3$ for 30 min. The second set was left acidified to stabilize the hydroquinone as the protonated species. Dichloromethane was added to each sample, vortexed for 30 minutes, and centrifuged for 40 min. A 1 mL aliquot of the CH_2Cl_2 layer was removed and concentrated to dryness. The samples were reconstituted in 100 µL of HPLC diluent (80:20:0.1 DMSO:Acetonitrile:TFA) and analyzed immediately.



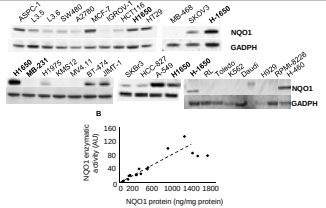
A - Dynamic redox equilibrium exists between 17-AAG and its hydroquinone (i.e. the freebase of IPI-504) in cells. The hydroquinone is the enzymatic reduction product of 17-AAG. The hydroquinone will readily oxidize to the quinone 17-AAG at physiological pH in aerobic conditions.

B - 17-AAG is not protonated at low pH. The 17-nitrogen of 17-AAG is a vinylogous amide and resonance delocalization through the quinone decreases the basicity of the nitrogen lone pair of electrons.

C - The hydroquinone is a pH sensitive molecule. The differential extraction method capitalizes on the pH sensitivity of the hydroquinone. At low pH, the basic aniline 17-nitrogen is protonated and soluble in the aqueous media. Basification of the aqueous media results in the deprotonation of the 17-nitrogen to form the hydroquinone which is prone to oxidize to 17-AAG under physiological conditions.

D - The HQ:Q analytical method provides robust recovery of hydroquinone in cellular media. Different HQ:Q ratios were spiked into blank cellular lysis (ranging from 9-89% hydroquinone) and the actual % hydroquinone measured .

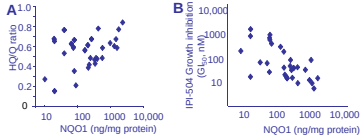
Cancer cell lines contain a broad range of NQO1protein levels



A . NQO1 protein expression level is determined by immunoblotting followed by densitometry as a ratio of NQO1 and GADPH bands

B. Densitometry values are plotted against NQO1 enzymatic activity assessed in cell lysates.

NQO1 expression level correlates with HQ/Q ratio in a subset of cell lines, but does not correlate with IPI-504 cytotoxic activity

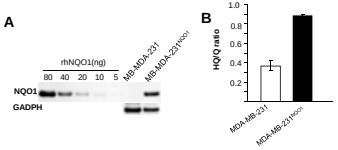


For each cell line, NQO1 protein levels are plotted against cytoplasmic HQ/Q ratio (A) or IPI-504 growth inhibitory activity (GI_{50}, nM) (B).

A . Overall there is not a good correlation between NQO1 levels and HQ/Q ratio. However 2 subsets of cells may exist. One where NQO1 levels correlate with HQ/Q ratio, another where HQ/Q ratio is independent of NQO1 expression levels.

B. There is not a good correlation between IPI-504 cytotoxic activity and NQO1 levels in the cell panel as a whole.

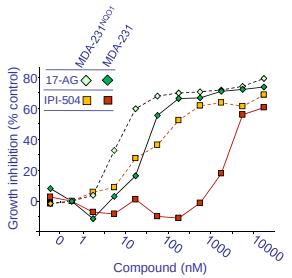
NQO1 overexpression in MBA-MD-231 cells increases the HQ/Q ratio



A. MBA-MD-231 NQO1 null cells infected with NQO1 expression vector, showed a very high expression level (5800 ngNQO1/mg protein).

B. The HQ/Q ratio was determined in both the parental and engineered cell lines

Growth inhibitory activity of IPI-504 increases with NQO1 protein expression, whereas 17-AG is largely unaffected by NQO1 expression



MBA-MD-231 NQO1 null and NQO1 overexpressing cells were incubated for 3 days with increasing concentrations of IPI-504 or 17-AG.

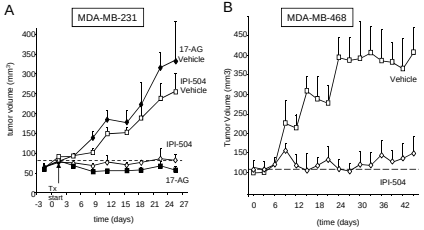
Hsp90 binding affinities for IPI-504 and 17-AG under reducing and non-reducing conditions

Assay conditions	K_i(nM) Reducing (+ TCEP)	K_i(nM) Non-reducing (- TCEP)
IPI-504	9 ± 5	530 ± 170
17-AG	3 ± 2	20 ± 7

17-AG and IPI-504 binding affinities were analyzed in a reducing and non-reducing environment to determine both their quinone and hydroquinone affinities for Hsp90.

- 17-AG has a high affinity for Hsp90 in reducing or non-reducing environments
- PI-504 binding affinity for Hsp90 is substantially weaker in a non-reducing environment

IPI-504 is active in xenograft models of tumor cells that do not express NQO1 and that are not sensitive to IPI-504 in vitro



1.10^7 MDA-MB-231 cells were injected into the flanks of 40 mice, and treatment began when tumors reached an average size of ~100 mm^3. IPI-504 75 mg/kg intraperitoneal (IP) and 17-AG 130 mg/kg oral (PO) or the vehicle equivalents were administered twice per week (Monday, Thursday).

1.10^7 MDA-MB-468 cells were injected into the flanks of 40 mice, IPI-504 100 mg/kg intraperitoneal (IP) was administered twice per week (Monday, Thursday).

Conclusions

- In a panel of 30 cancer cell lines, there is not a good correlation between NQO1 levels and HQ/Q ratio. However, two cell populations may exist: one where NQO1 determines the HQ/Q ratio and another where the ratio is independent of NQO1 abundance.
- NQO1 levels do not consistently correlate with IPI-504 cytotoxicity.
- In MBA-MD-231 NQO1-null cells, overexpression of NQO1 markedly increases the HQ/Q ratio and the cytotoxic activity of IPI-504. In contrast, 17-AG, the major metabolite of IPI-504, is largely unaffected by NQO1 overexpression.
- IPI-504 binding affinity to Hsp90 is dependent on the redox environment; whereas 17-AG binding affinity to Hsp90 is largely unaffected by the redox environment.
- In two different xenograft models that are insensitive to IPI-504 *in vitro*, IPI-504 significantly inhibits tumor growth *in vivo*; possibly because of the activity of its major metabolite 17-AG, which does not require conversion to the hydroquinone form for potent inhibition of Hsp90.