19-Substituted benzoquinone ansamycin Hsp90 inhibitors. Biological activity and decreased off-target toxicity

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Introduction and Summary

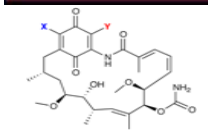
Benzoquinone ansamycins (BQAs) were the first class of Hsp90 inhibitors developed, but members of this class, particularly geldanamycin demonstrated hepatic toxicity.[1] 17-AAG and 17-DMAG are the BQA derivatives that have progressed clinically.[2,3] We have developed a new class of Hsp90 inhibitors with decreased off-target toxicity, while retaining Hsp90 inhibitory capacity in both neural and human cancer cells. The off-target toxicity of quinones is a function of their ability to arylate cellular nucleophiles and their ability to redox cycle.[4] To decrease the toxicity of the benzoquinone ansamycin (BQA) Hsp90 inhibitors such as geldanamycin, we designed novel 19-substituted BQAs (19BQAs), the 19-substituent preventing attack by cellular nucleophiles. Thus 19-methyl and -phenyl BQAs did not react with thiols at the 19-position, while marked reactivity was observed using parent BQAs. 19-substitution did not affect the ability of the BQAs to redox cycle. Importantly, while parent BQAs induced lethality in primary mouse hepatocytes, 19BQAs were essentially non-toxic. This data validated the overall approach and suggested that arylation reactions were primarily responsible for hepatotoxicity.

19BQAs inhibited purified Hsp90 in an NQO1-dependent manner demonstrating increased inhibitory efficacy of the hydroquinone ansamycin relative to its parent quinone.[5] Cellular assays confirmed the molecular signature of Hsp90 inhibition with decreases in client protein levels and compensatory induction of other Hsps in breast and pancreatic cancer cells. 19-Phenyl derivatives showed superior growth inhibitory activity relative to 19-methyl BQAs and notably, 19-phenyl-17-DMAG was of similar or greater potency to 17-DMAG. Finally, 19BQAs disrupted the interaction between p23 and Hsp90, but only 19-phenyl, and not 19-methyl BQAs disrupted the Hsp90-cdc37 interaction, resulting in differential Hsp90 client protein degradation. Cdc37 is an Hsp90 co-chaperone that can deliver client proteins, including kinases to Hsp90.[6,7] Hsp90 inhibitors have been proposed for use in neurodegenerative as well as anticancer applications and since the 19-methyl BQAs exert little cellular toxicity but induce Hsp90 inhibition and compensatory Hsp induction, they may be useful in preventing deregulated protein folding in neurodegenerative diseases. Additionally, the growth inhibitory 19-phenyl BQAs may have utility as anticancer agents. (Supported by CA51210)

Materials and Methods

The human breast cancer cell lines used included MDA468 and BT474 and we also used the human MiaPaCa-2 pancreatic cell line. The NQO1 stably transfected cell line MDA468/NQ16 has been described previously.[3] ATPase assay, molecular modeling, and X-ray crystallography were employed to show 19-substituted BQAs were Hsp90 inhibitors and bound to the *N*-terminal ATPase active site of Hsp90. Toxicity in freshly isolated primary mouse hepatocytes was assayed using MTT, Glutathione conjugation and oxygen consumption were measured using HPLC and polarographically, respectively. Growth inhibition in breast and pancreatic cells was assayed using the MTT assay. The effect of 19-substituted BQAs on client proteins was analyzed by western blot. For NQO1-dependent cellular effects of 19-subsituted BQAs, we compared NQO1-null MDA468 and NQO1-overexpressing MDA468/NQ16 cell lines. The interaction between Hsp90 and either p23 or cdc37 was investigated by co-immunoprecipitation.

Results



Cmpd	X	Y
17-DMAG	$NHCH_2CH_2N(CH_3)_2$	H
19Ph-17-DMAG	$NHCH_2CH_2N(CH_3)_2$	phenyl
19Me-17-DMAG	$NHCH_2CH_2N(CH_3)_2$	methyl

Figure 1. Chemical structure of BQAs and 19-phenyl BQAs.

19-Substituted BQAs inhibit purified yeast Hsp90 ATPase activity in the presence of NQO1

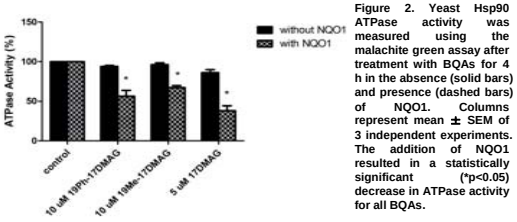


Figure 2. Yeast Hsp90 ATPase activity was measured using the malachite green assay after treatment with BQAs for 4 h in the absence (solid bars) and presence (dashed bars) of NQO1. Columns represent mean ± SEM of 3 independent experiments. The addition of NQO1 resulted in a statistically significant (*p<0.05) decrease in ATPase activity for all BQAs.

Computational-based molecular docking of the quinone and hydroquinone forms of 19-phenyl substituted 17-DMAG into the ATP binding domain of human Hsp90

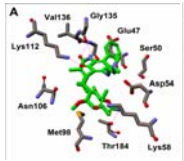


Figure 3. [A] quinone (green sticks) and [B] hydroquinone (cyan sticks) forms of 19-phenyl substituted 17-DMAG into the ATP binding domain of human Hsp90 (PDB ID: 2K5B; gray sticks). Dashed magenta lines represent predicted hydrogen bond interactions. Total binding energies (kcal/mol) were 19-phenyl substituted 17-DMAG (-18.9 kcal/mol), hydroquinone (-30.2 kcal/mol)

The data show more favorable binding energies (more negative) for 19-substituted hydroquinones than their parent benzoquinones in the active site of human Hsp90

19-Substituted 17-DMAG did not induce toxicity in primary mouse hepatocytes

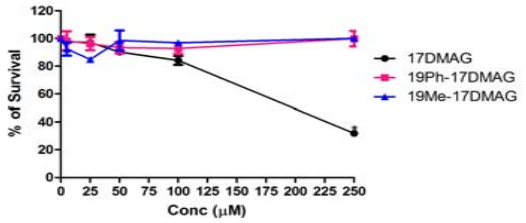


Figure 4., Toxicity to freshly isolated mouse hepatocytes was estimated using the MTT assay following incubation with the drug for 4 h. Only parental 17-DMAG caused diminished cell survival while both 19-phenyl and 19-methyl 17-DMAG were non toxic. Similar data was obtained in the 17-AAG and geldanamycin series.

19-Substituted BQAs do not react with GSH and 19-substitution did not affect the ability of BQAs to undergo redox cycling

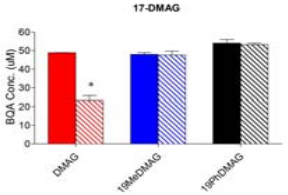


Figure 5A. 19-Substituted BQAs do not react with glutathione. Reactions were performed in 50 mM potassium phosphate buffer, pH 7.4 containing 50 μM BQA in the absence (solid bars) and presence (hatched bars) of 5 mM GSH. At the indicated times (DMAG series, 3h) the reactions were stopped and BQA concentrations were determined by HPLC. Data represents mean ± SD of three independent determinations (*p< 0.05)

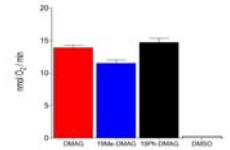


Figure 5B. 19-substitution does not affect ability to redox cycle Oxygen consumption was measured using a Clark electrode following the addition of BQAs to NADPH supplemented human liver microsomes. Reactions were performed in 50 mM potassium phosphate buffer, pH 7.4 containing 0.5 mM NADPH containing 0.2 mg of human microsomes at 37 °C. Reactions were started by the addition of 50 μM BQAs and oxygen consumption was measured over 5 min. Results are expressed as the mean, ±SD, n=3.

Table 1. Growth Inhibition induced by BQAs

Compound	IC_{50} (μM)		
	17-DMAG	19Ph-17-DMAG	19Me-17-DMAG
MDA468/NQ16	0.22 ± 0.05	0.1 ± 0.00	Non-toxic (highest dose 10 μM)
MDA468	2.06 ± 0.25	> 10	Non-toxic (highest dose 10 μM)
BT474	1.47 ± 0.42	14.5 ± 1.43	Non-toxic (highest dose 100 μM)
MiaPaCa-2	0.13 ± 0.01	2.99 ± 0.62	Non-toxic (highest dose 25 μM)

Interestingly, 19Ph-17-DMAG is more potent than 17-DMAG. Data shows a potentiation of growth inhibitory activity in high NQO1 (MDA468/NQ16 cells relative to NQO1-null (MDA468) cells.

19-Phenyl but not 19-methyl 17-DMAG inhibits human breast and pancreatic cancer cell growth

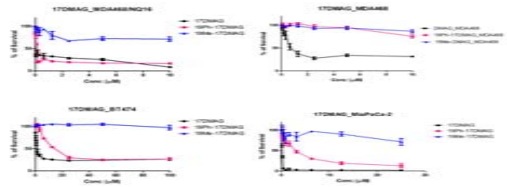


Figure 6. Growth inhibition in human breast and pancreatic cancer cells induced by BQAs in the 17-DMAG series. Cells were treated with BQAs for 4h and then allowed to grow for an additional 72 h. The MDA 468 (null NQO1)/MDA468-NQ16 comparison shows greater growth inhibitory effects in cells with elevated NQO1.

19-Substituted BQAs inhibit Hsp90 in human breast and pancreatic cancer cells

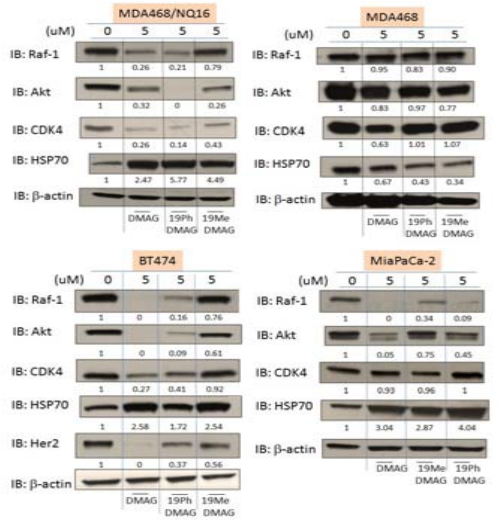


Figure 7. Immunoblot analysis of biomarkers of Hsp90 inhibition in BT474, MDA468, MDA468/NQ16, and MiaPaCa-2 cells treated with BQAs for 8 h (Raf-1, Hsp70) or 48h (Akt, CDK4, and Her2). Results are typical of triplicate experiments.

Only 19-phenyl 17-DMAG disrupts the Hsp90-cdc37 complex

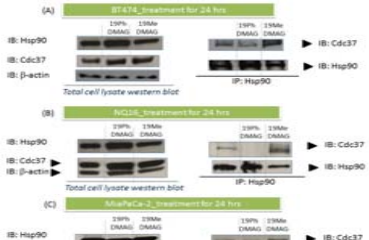


Figure 9. Only 19-phenyl 17-DMAG disrupts the Hsp90-Cdc37 interaction in both breast and pancreatic cancer cells. 19-phenyl 17-DMAG decreases the amount of Cdc37 proteins associated with Hsp90. (A) BT474, (B) MDA468/NQ16, and (C) MiaPaCa-2 cells were treated with 5 uM 19-phenyl or 19-methyl 17-DMAG or 17-DMAG. Cell lysate was immunoprecipitated with Hsp-90 antibody. Immunoblot anaysis blot was performed for detection of Cdc37.

Both 19-phenyl and 19-methyl 17-DMAG disrupt the Hsp90-p23 complex

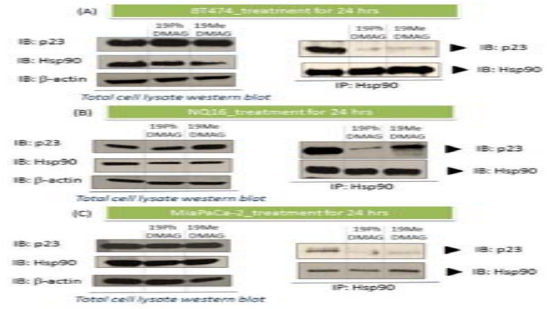


Figure 8. (A) BT474, (B) MDA468/NQ16 and (C) MiaPaCa-2 cells treated with 5 uM 19-substituted 17-DMAG or 17-DMAG for 24 h. The cell lysate was immunoprecipitated with either Hsp90 antibody and analyzed for p23 or immunoprecipitated with p23 antibody, and immunoblot blot was performed for detection of Hsp90.

Conclusions

- **19BQAs did not react with thiols at the 19-position and 19-substitution did not affect the ability of BQAs to redox cycle.**
- **A key finding was that 19BQAs were non toxic relative to parent quinones in primary mouse hepatocytes validating the overall rationale and suggesting arylation reactions were critical for hepatotoxicity.**
- **19BQAs retained their ability to inhibit Hsp90 using both recombinant protein (ATPase assay) and human breast and pancreatic cancer cells (client proteins and compensatory Hsp response).**
- **19-Methyl derivatives exhibited little growth inhibitory activity, while 19-phenyl derivatives demonstrated marked activity. 19-Phenyl 17-DMAG had similar or greater growth inhibitory activity to 17-DMAG.**
- **Both 19-methyl BQAs and 19-phenyl BQAs disrupted the p23/Hsp90 interaction, but only 19-phenyl BQAs disrupted the cdc37/Hsp90 interaction. Cdc37 is an Hsp90 co-chaperone which delivers kinases important for cell growth to Hsp90 and differential effects on these downstream clients were also observed.**
- **Hsp90 inhibitors have been proposed for use in neurodegenerative applications. Since the 19-methyl BQAs exert little toxicity but induce Hsp90 inhibition and compensatory Hsp induction, they may be useful in preventing deregulated protein folding in neurodegenerative diseases.**
- **The lack of toxicity of 19BQAs and their ability to induce a compensatory Hsp response in dopaminergic neural cells is shown on a companion poster (Kitson *et. al.*).**
- **19-Phenyl BQAs in the DMAG series show greater growth inhibitory potency than their parent quinones and may have utility as anticancer agents.**

References

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