19-Substituted benzoquinone ansamycins. Decreasing the toxicity of the benzoquinone ansamycin class of Hsp90 inhibitors

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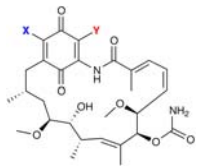
Introduction and Summary

Benzoquinone ansamycins (BQAs) were the first class of Hsp90 inhibitors developed but members of this class particularly geldanamycin demonstrated hepatic toxicity. One major mechanism of toxicity of the BQA class is manifested via the electrophilic properties of the quinone and alkylation of cellular nucleophiles at the 19-position of the quinone. We have developed novel 19-substituted BQAs in the geldanamycin, 17-AAG and 17-DMAG series as a means to prevent arylation of cellular nucleophiles and have validated this approach using model thiols including N-acetylcysteine and glutathione. 19-substituted BQAs did not react with thiols at the 19-position while marked reactivity could be detected using their parent quinones. 19-substituted BQAs were tested for their ability to inhibit recombinant yeast Hsp90 and 19-substitution did not block the capacity of these novel molecules to inhibit the ATPase functionality of the Hsp90 chaperone. This result was confirmed by molecular modeling of 19-substituted derivatives in the active site of human Hsp90 demonstrating that 19-substitutions did not block entry of the molecule into the active site. The addition of NAD(P)H:quinone oxidoreductase 1 (NQO1) potentiated inhibition of recombinant yeast Hsp90 by 19-substituted BQAs confirming our previous data demonstrating increased inhibitory efficacy of the hydroquinone ansamycin relative to its parent quinone. Cellular effects of 19-substituted BQAs were examined in NQO1 null MDA468 breast cancer cells and the isogenic MDA468/NQ16 cell line which over-expresses NQO1. Growth inhibitory effects were observed using 19-substituted BQAs and were potentiated by the presence of NQO1 in the MDA468/NQ16 line. Hsp90 inhibition in MDA468 and MDA468/NQ16 cells was confirmed using decreases in the Hsp90 client protein Raf1 and a compensatory increase in Hsp70 as biomarkers. In summary, these data demonstrate that 19-substituted BQAs do not react with thiols at the 19-position but retain their Hsp90 inhibitory capacity using purified enzyme and in cells suggesting that they should undergo further translational evaluation as therapeutic candidates (Supported by CA51210)

Results



Cmpd	X	Y
17AAG	$NHCH_2CHCH_2$	H
19Ph-17AAG	$NHCH_2CHCH_2$	phenyl
GA	OCH_3	H
19Ph-GA	OCH_3	phenyl
17DMAG	$NHCH_2CH_2N(CH_3)_2$	H
19Ph-17DMAG	$NHCH_2CH_2N(CH_3)_2$	phenyl

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

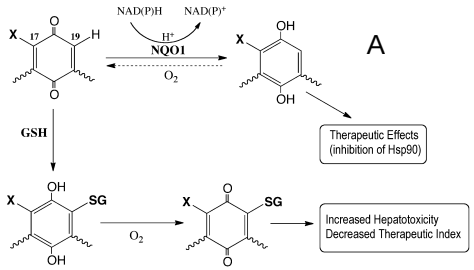


Figure 2A. In this work we synthesized 19-substituted BQAs in order to remove the potential for alkylation of cellular thiols and proteins. We have initially used 19-phenyl BQAs as proof of principle for this class of agents. A proposed model of BQA-induced hepatotoxicity caused by the glutathione conjugation.

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

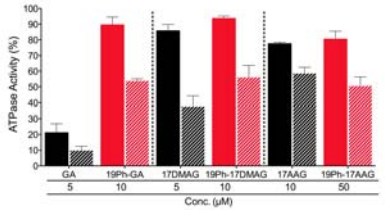


Figure 3. Yeast Hsp90 ATPase activity was measured using the malachite green assay after treatment with BQAs for 4h in the absence (solid bars) and presence (dashed bars) of NQO1. Columns represent mean ± SEM of 3 independent experiments. The addition of NQO1 resulted in a statistically significant ($p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

19-Substituted BQAs inhibit Hsp90 in human breast cancer cells

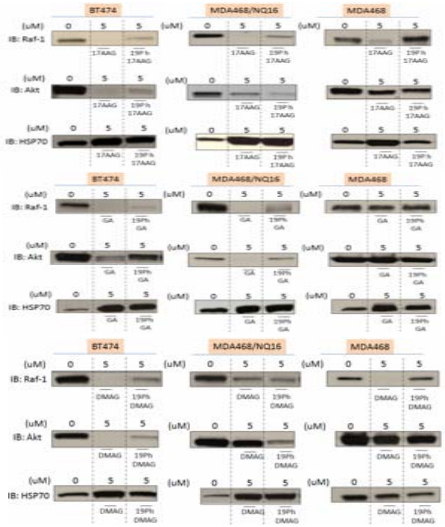


Figure 4. Immunoblot analysis of biomarkers of Hsp90 inhibition in BT474, MDA468, and MDA468/NQ16 cells treated with BQAs for 8h (Raf-1, Hsp70) or 48h (Akt). Results are typical of triplicate experiments.

19-Substituted BQAs do not react with GSH

B

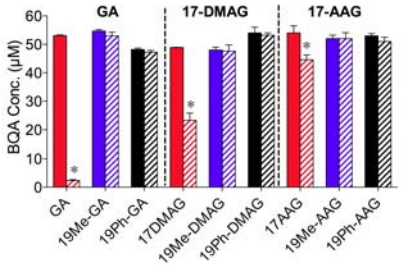


Figure 2B. 19-Substituted BQAs do not react with glutathione. Reactions were performed in 50mM potassium phosphate buffer, pH 7.4 containing 50μM BQA in the absence (solid bars) and presence (hatched bars) of 5mM GSH. At the indicated times (GA series, 15min; DMAG series, 3hr; AAG series, 16hr) the reactions were stopped and BQA concentrations were determined by HPLC. Data represents mean ± SD of three independent determinations (*p< 0.05)

19-Substituted BQAs inhibit human breast cancer cell growth

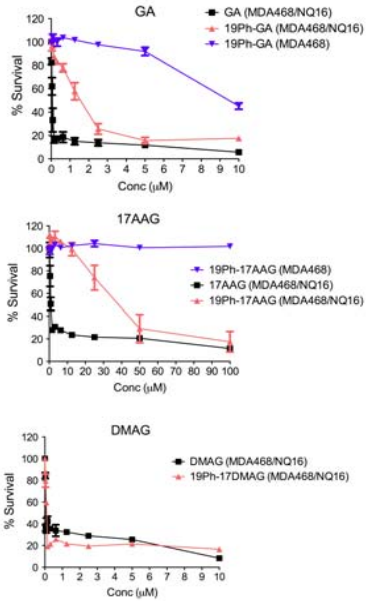


Figure 5. A comparison of growth inhibition of human breast cancer cells induced by 19-substituted BQAs and their corresponding 19-unsubstituted parent quinones. Cells were treated with BQAs for 4h and then allowed to grow for an additional 72h.

Table 1 Growth Inhibition induced by BQAs

compound	IC_{50} (μM)			IC50-fold difference between MDA468/NQ16 and MDA468	IC50-fold difference between with or without ES936
	MDA468	NQ16	NQ16+ES936		
17AAG	10.05 ± 1.07	0.86 ± 0.16	7.67 ± 1.36	11.7	8.9
19Ph-17AAG	88.37 ± 2.46	38.79 ± 10.9	83.87 ± 2.02	2.28	2.16
GA	0.063 ± 0. 011	0.023 ± 0.0003	0.057 ± 0.01	2.78	2.47
19Ph-GA	9.43 ± 0.53	1.52 ± 0.41	1.93 ± 0.44	6.2	1.27
DMAG	2.06 ± 0. 25	0.22 ± 0.05	1.81 ± 0.2	9.4	4
19Ph-17DMAG	*****	0.1 ± 0.00	0.19 ± 0.08	*****	1.90

Interestingly, 19Ph-17DMAG is more potent than 17DMAG which is in agreement with client protein data (see Fig 4).

19-Substituted BQAs induce apoptosis in human breast cancer cells

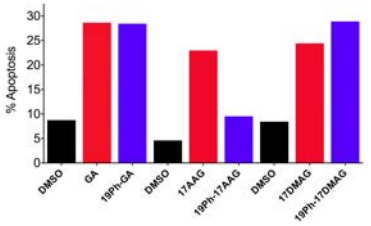


Figure 6. Induction of apoptosis by 19-phenyl substituted BQAs. Apoptosis was measured by annexin V-FITC/ PI dual staining in MDA468/NQ16 cells following treatment with BQAs (1μM for GA and 5μM for all other BQAs) for 48h. Data represents mean of 2 independent determinations.

19-Substituted BQAs prevent cell cycle progression in human breast cancer cells

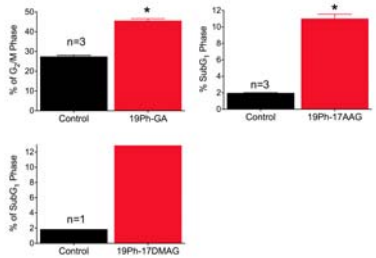


Figure 7. Effect of 19-phenyl BQAs on cell cycle progression. Cell-cycle analysis was performed by flow cytometry on MDA468/NQ16 cells after treatment with 19-phenyl BQAs for 48h (19Ph-GA, 0.5μM; 19Ph-17AAG, 2.5μM; 19Ph-17DMAD, 1μM). The bar chart shows the percentage of cells in sub-G1 and the percentage of cells in G2/M. Data represents mean ± SD of three independent determinations (*p< 0.05 significantly different from untreated MDA468/NQ16 cells).

Conclusions

- 19-phenyl substitution of benzoquinone ansamycins (BQAs) prevented glutathione conjugation and reaction with thiols.
- 19-phenyl substituted BQAs inhibited the ATPase activity of recombinant yeast Hsp90 and inhibition was potentiated by generation of the hydroquinone ansamycin by NQO1.
- 19-phenyl BQAs demonstrated growth inhibition in human breast cancer cells and of the 19-substituted derivatives, 19Ph-17-DMAG was the most potent. MDA468/NQ16 cells containing high NQO1 activity were more sensitive to 19-substituted BQAs when compared with NQO1-null parental MDA468 cells indicating the importance of the hydroquinone ansamycin.
- 19-substituted BQAs induced degradation of the Hsp90 client proteins Raf-1 and Akt and a compensatory induction in Hsp70 in breast cancer cells. This data indicated that 19-substituted BQAs inhibited cellular Hsp90.
- 19-Phenyl BQAs induced apoptosis in MDA468/NQ16 cells.
- Differential effects of 19-substituted BQAs were observed on cell cycle progression. Both 19Ph-17AAG and 19Ph-17DMAG induced MDA468/NQ16 cells to go into sub-G1 while 19Ph-GA caused G2 arrest.
- In summary, these data demonstrated that 19-substituted BQA's do not react with thiols at the 19-position but retain their Hsp90 inhibitory capacity using purified enzyme and cells suggesting that they should undergo further translational evaluation as therapeutic candidates.

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